

Optimization of aptamer microarray technology for multiple protein targets

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Abstract

Aptamer-based microarrays for the quantitation of multiple protein analytes have been developed. A multiplex aptamer microarray was generated by printing two RNA aptamers (anti-lysozyme and anti-ricin) and two DNA aptamers (anti-IgE and anti-thrombin) on to either streptavidin (SA) or neutravidin (NA)-coated glass slides. However, substantial optimization was required in order to ensure the simultaneous function of the aptamer:analyte pairs. The effects of protein labeling, assay buffer, surface coating, and immobilization chemistry and orientation were investigated. A single buffer (PBS buffer containing 5 mM MgCl₂ and 0.1% Tween 20) was found to work well with all the aptamers, even though this was not the buffer originally used in their selection, while neutravidin-coated slides yielded a lower detection limit, wider detection range, and more uniform background than streptavidin-coated slides. Incubation with Cy3-labeled proteins yielded sensitive, target-specific, and dose-dependent responses to each protein. Target protein concentrations as low as 72 pg/mL (5 pM, lysozyme), 15 ng/mL (0.5 nM, ricin), 1.9 ng/mL (0.01 nM, IgE), and 170 ng/mL (5 nM, thrombin) could be detected. These results show that aptamer arrays can potentially be used with numerous proteins in parallel, furthering the notion that aptamer arrays may be useful in proteomics.

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1. Introduction

The development of sensitive and selective high-throughput assays for multiplexed protein detection remains an extremely important goal in drug discovery and clinical diagnosis [1,2]. In conjunction with DNA microarray techniques [3–5], a wide variety of protein-detecting microarrays have been developed by immobilizing capture reagents onto glass slides. Microarray technology based on sensitive fluorescence scanning provides a means of high throughput, parallel, and rapid detection of analytes. In addition, the miniaturized array format has the advantages of automation and economy of sample and reagents.

In developing multiplexed protein detection microarrays, the critical parameters are the sensitivity, specificity, reproducibility, and signal-to-noise (S/N) ratio. All of these will become more difficult to achieve as arrays becoming larger and the analyte mixtures increasingly complex. Most reports to date have utilized antibodies [2,6,7] as protein capture agents and many

studies have been conducted within the past 2–3 years to improve the efficiency of protein detection associated with antibody-based microarrays, including optimizing surfaces, immobilization, assay and detection protocols, and the affinities, specificities, and stabilities of the antibodies themselves [8,9]. Despite these extraordinary efforts, antibody cross-reactivity remains a significant problem and continues to limit the application of antibody-based microarrays to proteomics [10–12]. In addition, the cumbersome *in vivo* immunization procedure by which most antibodies are still produced is far from high throughput.

Aptamers are functional nucleic acids selected from combinatorial oligonucleotide libraries by *in vitro* selection [13,14] against a variety of targets, such as small organics, peptides, proteins, and even whole cells [15,16]. Numerous aptamers against a wide range of protein targets have already been selected and have affinities in the pM to low nM range. Aptamers have shown increasing utility as affinity reagents and can successfully compete with antibodies in a number of analytical applications [17–25], and more generally as biosensors, including in microarrays [26–35]. In addition to having selectivities comparable to those of monoclonal antibodies [36,37], some advantages of aptamers over traditional antibody-based reagents are that they can be stabilized against degradation by the introduction

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of modified nucleotides, can be stabilized against denaturation by manipulating their secondary structures, and can even be refolded following denaturation [38]. Because aptamers can be synthesized chemically, batch-to-batch variability can be greatly reduced, and labels for detection and linkers for conjugation can be site specifically introduced. Most importantly, the *in vitro* selection process allows aptamers to be acquired against otherwise toxic targets, and its automation should allow the rapid, parallel production of multiple aptamers against complex target sets such as proteomes [39]. Because of these advantages it would be extremely useful to expand the high-density DNA microarray technology currently used to quantitate mRNA expression to aptamer arrays that could be used to recognize and quantitate protein expression.

There have been several proofs-of-principle for aptamer microarray development. Stanton and researchers at the company Archemix have generated a small aptamer array that relies on scanning fluorescence anisotropy measurements and demonstrated the performance in detecting three cancer-associated proteins [32]. Somalogic, Inc. selected aptamers that contained photoreactive 5-bromodeoxyuridine (BrdU) and that could covalently cross-link bound protein targets [40] and developed arrays with these photoaptamers [41]. Finally, we have previously developed aptamer arrays by immobilizing biotinylated RNA aptamers onto streptavidin-coated microbeads and demonstrated that the biothreat protein, ricin, could be selectively quantitated in real time [42]. We also developed an anti-lysozyme aptamer microarray and showed that this protein could be detected over 7 orders of magnitude with a lower limit of detection of 1 pg/mL [43].

Although aptamers mimic the protein recognition properties of antibodies, aptamer research is still in its infancy compared with antibody technology. The same problems that have troubled researchers developing antibody microarrays must also be solved for aptamer microarrays. Unfortunately, since nucleic acids have very different physicochemical properties than proteins, the lessons learned with antibodies cannot be immediately applied to aptamers. Therefore, as a step towards developing aptamer microarrays for numerous protein targets, we have developed microarray procedures for four aptamers and their cognate proteins, and have optimized a variety of parameters to improve the sensitivity and specificity of simultaneous protein detection. As a result, we could determine target protein

concentrations as low as 72 pg/mL (5 pM, lysozyme), 15 ng/mL (0.5 nM, ricin), 1.9 ng/mL (0.01 nM, IgE), and 170 ng/mL (5 nM, thrombin), respectively.

2. Experiments

2.1. Aptamers and proteins

Biotin-modified RNA aptamers were prepared as follows: (1) 5' biotin-modified RNA aptamers (anti-lysozyme and anti-ricin) were prepared by including biotin-GpG in the *in vitro* transcription reaction, as we [42] and others [44] have previously described; (2) 3' biotin-modified RNA aptamers were prepared by RNA oxidation and hydrazide coupling. In greater detail, 1 nmol of RNA aptamer was incubated with 100 nmol of NaIO₄ (Pierce Biotechnology, Rockford, IL) in 200 μ L of 100 mM NaOAc (pH 5.0) for 1.5 h at room temperature in the dark to promote 3'-end oxidation. An equal volume of 50% glycerol was added to the reaction mixture to stop the oxidation and the RNA samples were then ethanol precipitated. The RNA was re-suspended in 440 μ L NaOAc (100 mM, pH 5.0), freshly prepared biotin-LC-hydrazide (50 μ L; 40 μ M in DMSO, Pierce Biotechnology) was added, and the conjugation reaction was incubated for 3.5 h at room temperature. The 3' biotinylated RNAs were separated by size exclusion chromatography on a NAP 5 column (Amersham Bioscience, Piscataway, NJ), gel purified, and then ethanol precipitated. The degree of biotin labeling was determined via a streptavidin–biotin gel shift assay. The proportion of the RNA sample that was labeled proved to be 20–40% for 5'-end labeling and ~50% for 3'-end labeling. DNA aptamers containing biotin at either their 5'- or 3'-end (anti-IgE aptamer), or biotin-TEG-T₅ at either their 5'- or 3'-end (anti-thrombin aptamer) were purchased from Integrated DNA Technology, Inc. (Coralville, IA). The aptamer sequences and the composition of their original selection buffers are summarized in Table 1.

Lysozyme, thrombin (human), and ricin A chain were from Sigma (St. Louis, MO). Immunoglobulin E (IgE), purified from human polyclonal serum, was obtained from Fitzgerald Industries International, Inc. (Concord, MA). Most target proteins were labeled with a Cy3–NHS ester (Amersham Bioscience, Arlington Heights, IL) according to the manufacturer's protocols, except that ricin A chain was labeled with Cy3–maleimide

Table 1
Aptamer sequences, selection buffer compositions, and binding constants from the original *in vitro* selections

Target protein	Aptamer sequence	Selection buffer	K_d^a (nM)	Reference
Lysozyme	GGGAAUGGAUCCACAUCUACGAAUUCACAGGGCU-AAAGAGTGCAGAGUUACUUAGUUCACUGCAGACUUGACGAAGCUU	20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl ₂	31	[58]
Ricin (A chain)	GGGAAUGGAUCCACAUCUACGAAUUCAGGGGACGU-AGCAAUGACUGAGAUGCUGGUUCACUGCAGACUUGACGAAGCUU	PBS, 5 mM MgCl ₂	7.4 $\pm$ 1.1	[59]
IgE (human)	GGGGCACGTTTATCCGTCCTCCTAGTGGCGTGCCCC	PBS, 1 mM MgCl ₂	10	[60]
Thrombin (human)	AGTCCGTGGTAGGGCAGGTTGGGGTGACT	20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM MgCl ₂	0.5	[51]

^a K_d s were obtained using filter binding assays with unmodified aptamers and targets in solution.

(Amersham Bioscience, Arlington Heights, IL). In general, Cy3 dyes were dissolved in 20 μ L DMSO and added to 1 mL of protein solution (1 mg of protein in 0.1 M NaHCO_3 buffer, pH 9.2). After 30 min gentle rotation at room temperature in the dark, unincorporated Cy3 dye was removed from Cy3-labeled protein by using a NAP 10 desalting column (Amersham Bioscience, Arlington Heights, IL). Cy3-labeled proteins were further concentrated by using an Amicon ultra-4 centrifugal filter device (Millipore Co., Billerica, MA) with PBS buffer.

2.2. Fabrication of aptamer microarrays

Biotinylated aptamers were immobilized on the surfaces of modified avidin (streptavidin or neutravidin)-coated glass slides (Pierce Biotechnology) via avidin–biotin interactions. In greater detail, aliquots of aptamers (500 nM) in PBS (137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) were denatured at 70 °C for 3 min, cooled to room temperature, and then glycerol stock solution was added to a final concentration of 5%. The aptamer solutions were then printed on glass slides using a manual arrayer (V&P Scientific, Inc., San Diego, CA) at room temperature and a relative humidity of 70–90%. Pins on the manual arrayer deposited approximately 3 nL of aptamer solution (according to manufacturer) and the resulting spot sizes were around 500–700 μ m with a spot-to-spot distance of $\sim$ 1.5 mm. Three replicates of four 5' biotinylated aptamers were deposited, making four subarrays (Fig. 1A). To compare the effects of immobilization orientation, sets of 5' (solid-colored circle in Fig. 1B) and 3' (solid-colored diamond in Fig. 1B) biotin-labeled

aptamers against each target protein were printed in subarrays, respectively.

After printing, the slides were incubated within the humidity chamber for 0.5 h to allow the biotin moieties on the aptamers to be bound by the modified avidin molecules coating the slides. The printed area on each slide was then enclosed within a CoverWell incubation chamber (Schleicher and Schuell, Keene, NH) comprised of four, independent water-tight wells and a clear plastic cover (Fig. 1). To remove unbound aptamers, 1 mL of PBS containing 5 mM MgCl_2 and 0.1% Tween 20 (PBSMT) was passed through the chamber. The remaining PBSMT was left for 1 h to block the unbound modified avidin molecules on the glass slide, and then removed.

2.3. General assaying protocol

After blocking, 70 μ L of a target protein in PBSMT was introduced to each well and incubated for 5 min at room temperature in the dark. Each well was rinsed with 3 mL PBSMT followed by 2 mL PBSM (PBS containing 5 mM of MgCl_2). The incubation chamber was then peeled away from the surfaces of the slides and the slides were dried by spinning for 20 s at room temperature in a Slide Spinner minicentrifuge (Labnet International, Inc., Edison, NJ).

Protein analytes were also prepared in either TrisMT (50 mM Tris–Cl buffer at pH 7.4, 5 mM of MgCl_2 , 0.1 M NaCl, 0.1% Tween 20) or in HEPESMT (20 mM HEPES at pH 7.4, 1 mM of MgCl_2 , 5 mM KCl, 0.1% Tween 20). Proteins in TrisMT or HEPESMT were incubated with aptamers on the microar-

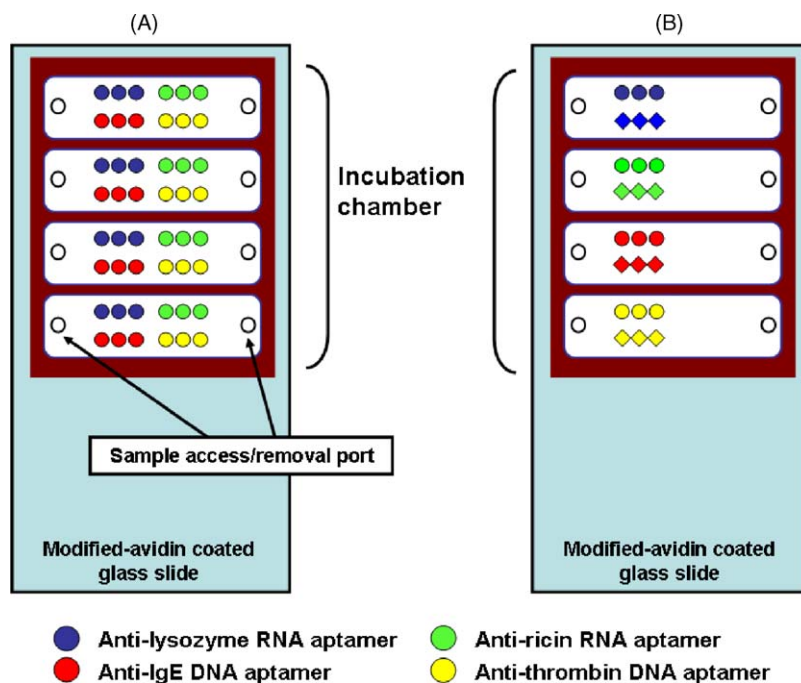


Fig. 1. Sample arrangement for the aptamer microarray. A CoverWell incubation chamber was aligned on the four aptamer microarrays. Target proteins and washing buffers were subsequently introduced through one of sample access/removal ports, and removed via the other sample access/removal port. (A) The arrangement of four 5' biotin-labeled aptamers printed in four microarrays. (B) The arrangement of four sets of 5' and 3' biotin-labeled aptamers printed in four microarrays. Each color corresponds to a different target protein. Circles represent 5' biotinylated aptamers and diamonds represent 3' biotinylated aptamers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rays, washed with 3 mL TrisMT or HEPESMT followed by 2 mL TrisM or HEPESM, and then dried as described above.

As a positive control, aptamer microarrays were stained with SYBR 555 oligonucleotide stain (Molecular Probes, Eugene, OR) for 5 min, washed according to the manufacturer's protocol, and dried as described above.

2.4. Array imaging and data analysis

Dried microarray slides were immediately scanned using a GenePix 4000B microarrayer (Axon Instruments, Union City, CA) and the resulting images were analyzed using the GenePix 4.1 software that accompanied the scanner. Spot intensity was determined as the local background-corrected mean fluorescence intensity of the pixels within the spot ellipse. Median background values could be used to determine local background because of the uniform distribution of fluorescence intensity throughout the background. In analyzing the spots themselves, however, mean values were used instead of median values because the images were not uniform [45]. Statistical analyses were performed using SigmaPlot 7.0 (SPSS, Chicago, IL).

3. Results and discussion

Determining whether common microarray assay conditions used for multiple targets and aptamers is a critical challenge because most aptamers have been generated under very specific buffer regimes. Some of the key parameters to be considered in deriving common assay conditions and optimizing aptamer microarray performance include: the derivatization chemistry for protein analytes; buffers for printing, blocking, and assaying; the chemistry for aptamer immobilization; the protocol for the washing and drying process.

3.1. Target protein labeling

In most antibody-based immunoassays, proteins have been detected either via direct assay [46] or by a sandwich assay [47,48]. While sandwich methods yield highly sensitive detection (down to the fM range) of low abundance proteins [49], this method also of necessity requires two specific receptors for each protein target, and thus drastically increases the problems associated with generating large numbers of specific receptors. Furthermore, sandwich methods may not prove suitable for aptamer microarrays since aptamers selected against protein targets often 'home' to a single dominant epitope [50]. Aptamers that bind distinct epitopes can be identified, but require a series of selection experiments [51].

In principle, a direct assay approach utilizing fluorophore-labeled proteins has the potential to capture thousands of proteins using high-density microarray technology. The degree of protein labeling (the number of molecules of dye conjugated to each molecule of protein), however, plays an important role; low labeling may yield weak signals on the microarray, while high labeling may destroy protein epitopes needed for binding or rapidly saturate the signal.

Following the manufacturer's protocol that was designed to label 1 mg of IgG to a final dye–protein molar ratio of either between 4 and 12 (using Cy3 monofunctional NHS-ester) or between 0.5 and 3 (using Cy3 monofunctional maleimide), we initially achieved Cy3:protein ratios of: Cy3–lysozyme = 1.0, Cy3–ricin = 1.3, Cy3–IgE = 4.6, and Cy3–thrombin = 3.6. We were concerned that the higher dye–protein ratios might inhibit binding, and by decreasing the dye–protein ratio from 3.6 to 0.8 for thrombin we in fact increased the observed fluorescence signal by 30–50% and S/N ratio by 40–50% (data not shown). We did not attempt to further optimize IgE labeling because it was sensitively bound by the anti-IgE aptamer and showed low background adsorption to the chip.

3.2. Selection of a universal assay buffer

The strong influence of electrostatic interactions on the folding and tertiary structure of aptamers, and on their binding to protein targets, means that the binding performance of aptamers are usually highly dependent on the ionic composition and strength of the binding buffer, as previously investigated by us [52] and others [34]. We have recently reported that there are variety of productive buffer conditions under which in vitro selection experiments can be carried out [53]. However, the variety of observed and potential buffers is potentially a critical obstacle to expanding the applications of aptamer microarray to measurements involving thousands of different protein targets. While it is difficult to predict the general suitability of a given assay buffer to all possible aptamer:protein sets, we wished to at least determine whether a buffer could be chosen that would allow aptamers that had previously been selected under a variety of buffer regimes to retain their affinities and selectivities for protein targets.

As listed in Table 1, the four anti-protein aptamers employed in this study were originally selected in either PBS or Tris buffers that spanned a variety of salt compositions and concentrations. We therefore screened several common buffers (PBS, Tris–Cl, and HEPES at pH 7.4 supplemented MgCl₂, NaCl (or KCl), and Tween 20; PBSMT, TrisMT, and HEPESMT, respectively) as potential 'universal' assay buffers. It has been our experience that addition of Tween 20 to assay buffers greatly reduces non-specific absorption and yields a uniform background that is better than commercially available blocking buffers. The four individual protein analytes were prepared in the three assay buffers and their binding performance was examined with 5' biotin-labeled aptamers arrayed on SA slides.

Fig. 2 presents the S/N ratio for each aptamer incubated with its cognate target protein in the three buffer compositions. While the anti-lysozyme RNA aptamer shows the highest S/N ratio in its original selection buffer, TrisMT, the other three aptamers (anti-ricin RNA, anti-IgE DNA, and anti-thrombin DNA aptamers) show the highest S/N ratios in HEPESMT buffer. However, while anti-lysozyme and anti-ricin RNA aptamers show minimal buffer dependence in binding their targets, the anti-IgE and anti-thrombin DNA aptamers do not bind (or bind very weakly) to their target proteins in TrisMT. In addition, when HEPESMT was used as an assay buffer, the anti-

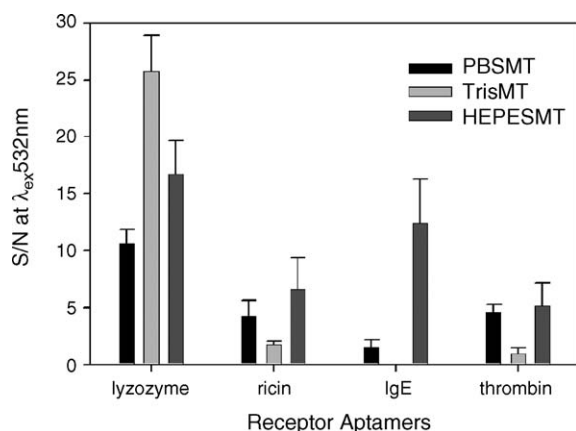


Fig. 2. Effect of assay buffer composition on the S/N ratio of protein binding. Data were derived from the fluorescence signals associated with aptamer spots arrayed on SA slides and probed with 1 nM Cy3–lysozyme, 10 nM Cy3–ricin, and 1 nM Cy3–IgE. Error bars represent the standard error in triplicate assays.

thrombin DNA aptamer exhibited a high degree of cross-binding to IgE (40% relative to the anti-IgE aptamer). Therefore, we chose to use PBSMT in all further experiments as a universal buffer.

3.3. Array measurements with multiple proteins

To demonstrate the utility of our multi-aptamer microarrays, it was of interest to first determine the dose-dependent array response for individual target protein analytes using the universal buffer. 5' biotin-labeled aptamers arrayed on SA slides were incubated with Cy3-labeled proteins diluted in PBSMT and washed with PBSMT/PBSM. Control arrays were stained with SYBR555. Fig. 3 represents typical fluorescence images of aptamer microarrays when they are incubated with a single individual analyte (Fig. 3A–D) or stained with SYBR555 (Fig. 3E). Each RNA and DNA aptamer yielded a highly specific response to its corresponding target protein and showed no cross-reactivity with other proteins. In Fig. 3E, all of the aptamer spots exhibit a bright signal, confirming the quality and consistency of surface immobilization.

The dose-dependence curves in Fig. 4 were generated by plotting the background corrected mean fluorescence intensity $\pm$ S.D. for three replicate experiments as a function of protein concentration. The dotted lines in each curve represent the best fits through the data points using 3 parameter sigmoidal functions (SigmaPlot, SPSS). The detection ranges for individual proteins were: lysozyme = 0.005–10 nM (72 pg/mL–143 ng/mL); ricin = 0.5–100 nM (15 ng/mL–3 μ g/mL); IgE = 0.01–2 nM (1.9–380 ng/mL); thrombin = 5–50 nM (170 ng/mL–1.7 μ g/mL).

For individual target proteins, the calculated binding constants (K_d s, summarized in Table 2) are somewhat higher (up to 5 times) or lower (down to 34 times) than the K_d s determined by filter binding assays (summarized in Table 1). This deviation provides a rough estimate of the range of perturbations to aptamer affinities that may be expected based on target modification, immobilization, and assay methods.

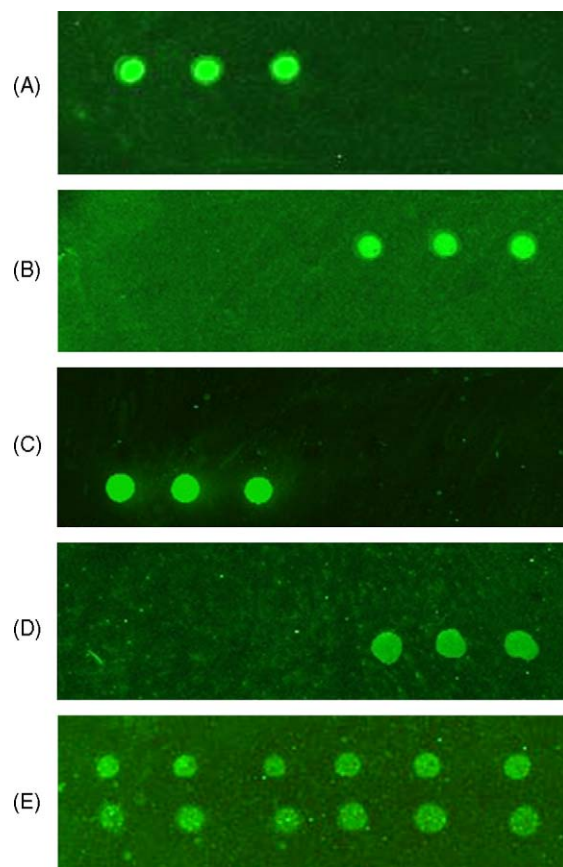


Fig. 3. Fluorescence images of aptamer microarrays. The aptamers were arrayed on SA slides for the detection of individual Cy3-labeled proteins: (A) 1 nM Cy3–lysozyme; (B) 10 nM Cy3–ricin; (C) 1 nM Cy3–IgE; (D) 10 nM Cy3–thrombin. The image in (E) is an aptamer microarray stained with SYBR555 as a positive control.

The limits of detection (LODs, the lowest concentration where the measured S/N is above 3) are also summarized in Table 2 along with the LODs for similar assay methods reported by others. It should be noted that differences between background and signal can be readily apprehended at even lower concentrations, but these differences become more difficult to quantitate when the S/N ratio is less than three. The binding affinities of different aptamers against the same target are known to vary; for example, 14 different anti-lysozyme aptamers showed a broad range of detection limits in a microarray format, spanning 5 orders of magnitude from 0.01 to 100 ng/mL [43].

For a more precise comparison with other methods, we limited references to those employing exactly the same aptamer sequences that were used in this work (except for the anti-lysozyme RNA aptamer, where a different sequence that arose from the same selection was used). Some of the difference between our and others' assay methods are: aptamer end modification (biotin or amine), detection platform (CCD camera or laser scanner), protein modification (the type of fluorophore and protein/fluorophore ratio), and detection strategy (direct versus indirect versus competitive detection, or label-free detection via fluorescence anisotropy).

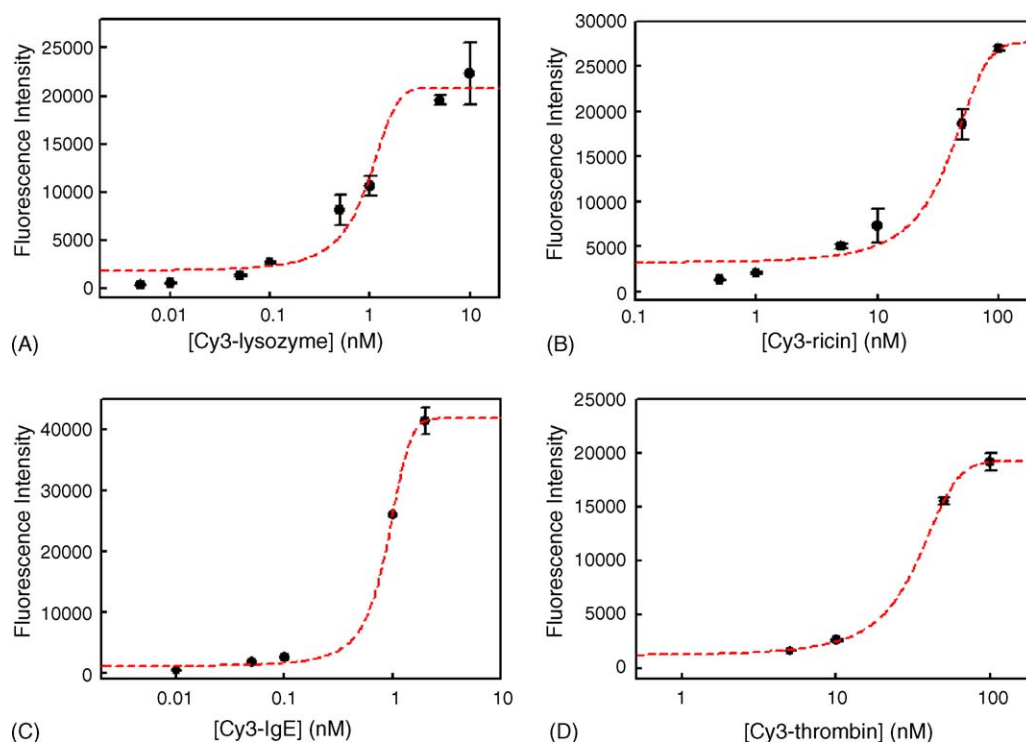


Fig. 4. Dose-dependent detection of proteins by aptamers. The aptamers were arrayed on SA slides: (A) Cy3-lysozyme; (B) Cy3-ricin; (C) Cy3-IgE; (D) Cy3-thrombin. Error bars represent the standard error in triplicate assays.

For the selective quantitation of target proteins, it appears that the aptamer microarray technology is competitive with or even superior to antibody-based microarrays and to other aptamer-based detection methods, such as an electrochemical sensor array (LOD for thrombin = 10 nM or 374 ng/mL) [54] or a quartz crystal biosensor (LOD for IgE = 0.5 nM or 94 ng/mL) [26]. One exception is the determination of lysozyme, where the LOD was found to be approximately 70 times higher than our previous result using a similar aptamer-based microarray

technique [43]. It is likely that the difference between the anti-lysozyme clones and the change of assay buffer from the original selection buffer (Tris-Cl) to the universal buffer (PBS) caused the aptamer sensitivity change. The sensitivity of non-covalent capture of proteins on our aptamer microarray was also comparable to results with photoaptamer microarrays, in that we could detect IgE concentrations as low as 1.9 ng/mL (0.01 nM), while the photoaptamer array could detect IgE concentrations down to 2.1 ng/mL [41].

Table 2
Summary of individual target protein detection using aptamer microarrays on SA-coated slides

Protein	K_d (nM)	LOD ^a	LOD ^b (ng/mL)	Assay method ^c	Reference ^d
Lysozyme	0.9 ± 0.1	72 pg/mL (5 pM)	0.3 1×10^{-3}	ELISA <u>Aptamer</u> :F-protein	[61] [43]
Ricin (A chain)	36 ± 6.0	15 ng/mL (0.5 nM)	10 320	<u>Antibody</u> :protein:F-antibody <u>Aptamer</u> :protein:F-antibody	[62] [42]
IgE (human)	N/A ^e	1.9 ng/mL (0.01 nM)	2.4 2.1	<u>Aptamer</u> ^f :F-protein <u>Photoaptamer</u> :protein:antibody:F-antibody	[35] [41]
Thrombin (human)	N/A ^e	170 ng/mL (5 nM) 34 ng/mL ^g (1 nM) ^g	34 62	<u>F-aptamer</u> :protein <u>Aptamer</u> :F-protein/protein ^h	[30] [34]

^a The lowest concentrations where the measured $S/N > 3$ in experiments, represented in both ng/mL and nM units.

^b The lowest concentrations where detection could be demonstrated by other authors. Units are ng/mL.

^c Assay methods employed by other authors. The receptor reagents immobilized on surfaces are underlined. F denotes a fluorescent modification to either aptamers or target proteins.

^d References for LODs and assay methods reported by other authors.

^e Data not available.

^f The anti-IgE aptamer has four additional nucleotides at each end compared with the original sequence.

^g LOD obtained from NA-coated slide.

^h F-protein/protein represents competitive assay methods, in which the aptamer was incubated with a mixture of F-labeled protein and unlabeled protein.

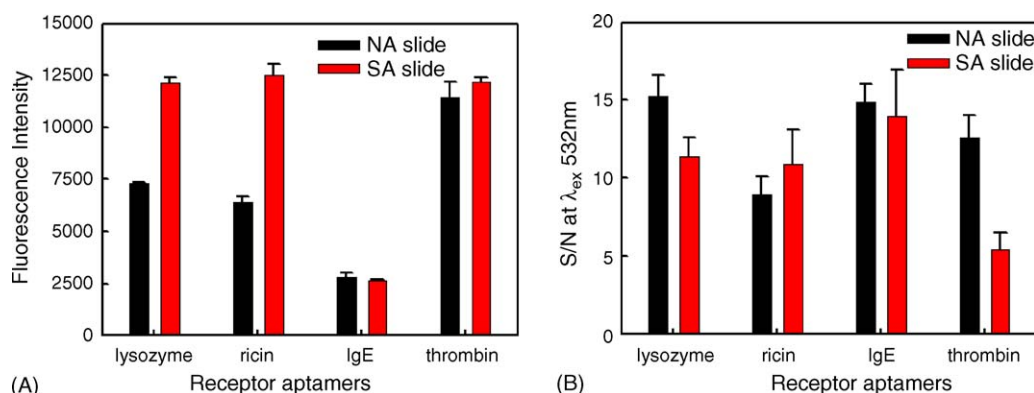


Fig. 5. Effect of surface coatings on signal. Both raw fluorescence intensities (A) and S/N ratios (B) are shown for the detection of 1 nM Cy3–lysozyme, 10 nM Cy3–ricin, 1 nM Cy3–IgE, and 10 nM Cy3–thrombin. Error bars represent the standard error in triplicate assays.

3.4. Surface coating effects

An important issue in the development of antibody microarrays is to determine the optimum surface coating that can maintain reagent affinity while minimizing non-specific absorption. This is a continuing challenge in the development of aptamer microarrays as well.

Avidin and modified avidin coatings are routinely used in the production of immunoassays because the biotin–avidin interaction is known to be extremely tight (with a dissociation constant 10^{-15} M [55]) and robust. We have previously shown and find again in this study that aptamers immobilized on streptavidin-coated agarose beads or glass slides maintain their selectivity and sensitivity for target proteins [42,43]. In addition, the four binding sites on the avidin tetramer promote the high-density deposition of attachment points compared with chemically derivatized glass slides. For example, we found that aptamers at a concentration of 500 nM could saturate the streptavidin surface following incubation for 10–30 min at room temperature, whereas it is likely that a ~ 10 μ M aptamer solution and up to overnight incubation would be required for chemical immobilization; for example, Stadtherr et al. [35] used an aptamer concentration of 100 μ M in an immobilization procedure, while photoaptamer arrays were made from aptamer solutions of 10 μ M [41]. The high reagent concentration and long incubation time required for chemical immobilization are especially daunting if RNA aptamers are to be used, given their susceptibility to nucleases and the cost of either chemical or enzymatic RNA production.

We wished to determine if further optimization of S/N ratios could be garnered by switching from streptavidin (SA) to neutravidin (NA), since NA (60 kDa) does not contain carbohydrate and thus eliminates potential binding to lectins. In addition, NA has a *pI* of 6.3, which is closer to neutrality than non-glycosylated SA (*pI* = ca. 5), and thus helps to eliminate electrostatic interactions that might contribute to non-specific binding. Rowe et al. [56] and others [57] have shown that NA coatings help reduce non-specific binding in antibody immunoassays compared to other avidin species.

5' biotin-labeled aptamer microarrays were prepared on both SA- and NA-coated slides and then assayed with 1 nM Cy3–lysozyme, 10 nM Cy3–ricin, 1 nM Cy3–IgE, and 10 nM

Cy3–thrombin. Fig. 5A shows that the absolute fluorescence intensity captured by the two RNA aptamers (anti-lysozyme and anti-ricin) spots is higher on the SA-coated slide, while the fluorescence intensity attributed to the two DNA aptamers (anti-IgE and anti-thrombin) was not altered by the avidin species. Anti-thrombin aptamers arrayed on the NA slide showed a significantly improved S/N ratio (130% relative to SA), whereas the other three aptamers showed either a slight increase (anti-lysozyme aptamer, 34% higher) or no effect (Fig. 5B). Moreover, more uniform background images were obtained with NA-coated slides for most aptamers in most experiments (Fig. 6A and B).

As a further optimization, the anti-thrombin aptamer was modified at its 5'-end with biotin-TEG, printed on SA and NA slides, and the slides were assayed with serial dilutions of Cy3–thrombin in PBSMT. The NA slides (Fig. 6A) again produced spot intensities with higher S/N ratios than the SA slides (Fig. 6B) and more uniform background images. Dose-dependent response curves from the two different avidin-coated slides are summarized in Fig. 6C. The fitted binding constant for NA-coated slides was 50 ± 2.9 nM (K_d), while the same parameters for SA-coated slides were 34 ± 2.5 nM (K_d). The fluorescence signal saturated on the SA-coated slide above thrombin concentrations of 50 nM, but was more proportional to thrombin concentrations up to 100 nM on the NA-coated slide. In addition, the lowest thrombin concentration for which detection was demonstrated ($S/N > 3$) was 34 ng/mL (1 nM) using NA-coated slides, a five-fold smaller LOD than for SA-coated slides. Again, these results confirm that neutravidin is a superior coating reagent not just for protein receptors, but for aptamers as well.

3.5. Immobilization orientation effects

The chemical modification of aptamers and interactions with the microarray surface may cause steric hindrance that influences nucleic acid folding and structure, and the ability bind to target proteins. The effect of immobilization orientation on aptamer binding has previously been examined for anti-thrombin DNA aptamers [34]. However, we have already seen that the performance of the DNA and RNA aptamers can differ

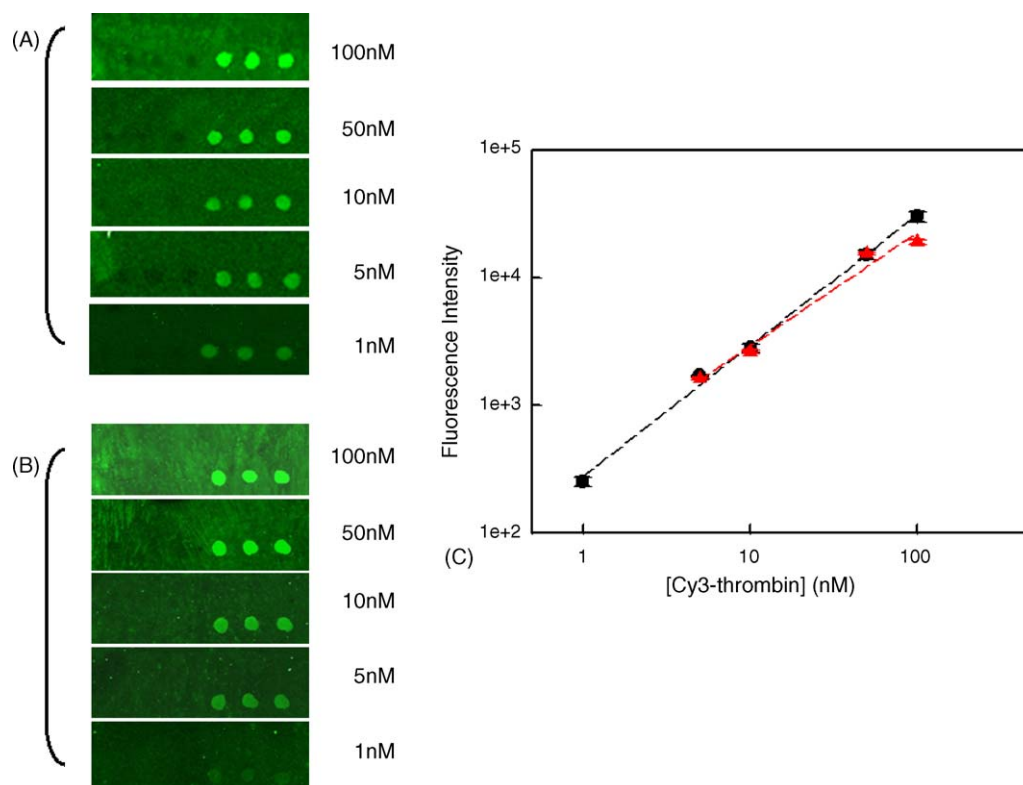


Fig. 6. Optimization of thrombin detection. The anti-thrombin aptamer modified at its 5'-end with biotin-TEG was arrayed on either NA-coated slides (A) or SA-coated slides (B). (C) Dose-dependent response in a log format. Dotted lines through the data points represent the linear regression ($\log F = K \log C + B$, where F is the fluorescence intensity and C is the protein concentration). Error bars represent the standard error in triplicate assays. Experimental data from: (black circle) NA-coated slides and (red triangle) SA-coated slides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in different buffers or on different surfaces, and thus we wished to examine the effect of orientation on RNA aptamers, as well.

To this end, we prepared both 5'- and 3'-end biotin-modified RNA aptamers (anti-lysozyme and anti-ricin) using biotin-GpG during in vitro transcription or via oxidation and hydrazide coupling, respectively. Biotin-modified aptamers were arrayed on NA slides alongside chemically synthesized 5'- or 3'-end biotin-modified DNA aptamers (anti-IgE and anti-thrombin) (Fig. 1B). The aptamers were probed with labeled target proteins (1 nM Cy3-lysozyme, 10 nM Cy3-ricin, 1 nM Cy3-IgE, and 10 nM Cy3-thrombin in PBSMT) and captured fluorescence intensity was compared as a function of immobilization.

As indicated in Fig. 7, the binding affinity changes based on immobilization direction was not significant. Anti-lysozyme and anti-IgE aptamers showed 14–30% higher sensitivity following 3'-end immobilization, whereas anti-ricin and anti-thrombin aptamers showed 26–28% higher sensitivity following 5'-end immobilization. These results were somewhat surprising for the anti-IgE aptamer, as this was a minimal aptamer structure that did not contain any linker regions (unlike both the RNA aptamers (anti-lysozyme and anti-ricin), that contained constant regions, and the anti-thrombin aptamer, which contained an introduced spacer sequence). Nonetheless, even direct attachment of aptamers to the microarray surface allowed the close approach and binding of cognate proteins.

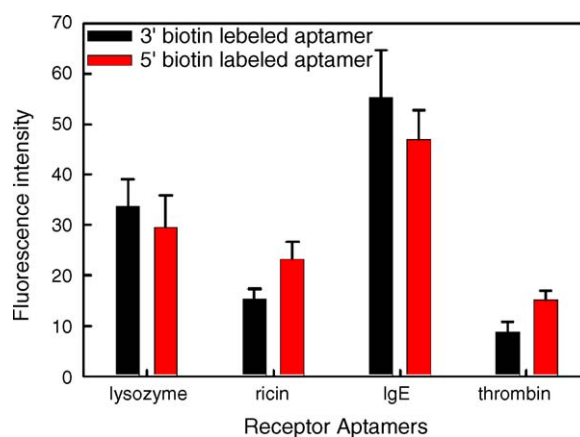


Fig. 7. Effects of immobilization orientation on signal. The target concentrations used were 1 nM Cy3-lysozyme, 10 nM Cy3-ricin, 1 nM Cy3-IgE, and 10 nM Cy3-thrombin. Error bars represent the standard in triplicate assays.

4. Conclusions

A multiplex aptamer microarray was generated by printing two RNA aptamers (anti-lysozyme and anti-ricin) and two DNA aptamers (anti-IgE and anti-thrombin) on to either streptavidin- or neutravidin-coated glass slides. However, substantial optimization was required in order to ensure the simultaneous func-

tion of the aptamer:analyte pairs. The effects of protein labeling, assay buffer, surface coating, and immobilization chemistry and orientation were investigated. Although the aptamers were originally selected under diverse buffer conditions, a single buffer (PBS buffer containing 5 mM MgCl₂ and 0.1% Tween 20) was found to work well with all the aptamers. Neutravidin-coated slides yielded a lower detection limit, wider detection range, and more uniform background than SA-coated slides. However, neither aptamer modification nor immobilization appeared to significantly influence affinity for target proteins, indicating that aptamers are robust to surface immobilization. Incubation with Cy3-labeled proteins yielded sensitive, target-specific, and dose-dependent responses to each protein. Target protein concentrations as low as 72 pg/mL (5 pM, lysozyme), 15 ng/mL (0.5 nM, ricin), 1.9 ng/mL (0.01 nM, IgE), and 170 ng/mL (5 nM, thrombin) could be detected. This performance was more sensitive than comparable antibody-based microarrays or any previous aptamer-based detection strategies, and showed none of the non-specific binding problems typically associated with antibody microarrays. Because the aptamer microarray strategy presented here is simple, sensitive, fast (total time from array printing to scanning was about 2 h), and readily applicable to diverse aptamers, this study should have broad implications for the future development of aptamer microarrays for proteomics.

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