Signaling Aptamer

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Self-Assembled Signaling Aptamer DNA Arrays for Protein Detection**

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Herein we report a DNA-tile directed self-assembly of signaling aptamers into high-density nanoarrays for detection of protein molecules. A signaling aptamer for human α-thrombin was created by replacing one nucleotide that is located close to the protein binding site in the aptamer sequence with a fluorescent nucleotide analogue, which generates a significant increase in fluorescence upon binding to thrombin. The signaling aptamer was linked to a DNA tile designed to self-assemble into 2D DNA arrays with ≈27-nm periodic spacing between neighboring aptamers. The high local density of the signaling aptamers displayed on the self-assembled DNA arrays allowed us to detect low concentrations of thrombin protein bound to the arrays by using confocal fluorescence microscope imaging.

This water-soluble, self-assembled signaling aptamer aggregate provides a novel strategy for developing programmable sensors. An important design concept in this work is that the molecular recognition process (protein find-and-bind aptamers) is in solution, but the physical measurement of the recognition is on the surface. The water-soluble signaling aptamer assemblies can lead to more-efficient molecular recognition compared to binding on solid surfaces. After being deposited on solid substrates, each signaling aptamer aggregate collectively gives strong-enough signals that allow the binding events to be read by a fluorescence microscope, which resembles the readout method in microarray technology. Overall, the method described herein involves no surface-chemistry steps, which are common in solid surface-based microarrays, and reveals no reduction in the aptamer-binding efficiency as compared with the unmodified aptamer. Thus, it has the advantage of microarray technology, but avoids its disadvantages.

In recent years, there has been a substantial advancement in the use of DNA as a "smart" material to construct

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structures with periodical patterns on the nanometer scale.[1–9] Structure formation by using DNA tiles begins with the design of the sequence and chemical synthesis of single-stranded DNA oligonucleotides. When the DNA oligonucleotides are properly annealed together, they self-assemble into DNA branched-junction building blocks or DNA tiles through Watson–Crick base pairing. DNA tiles can carry sticky ends (single-stranded overhangs) that preferentially hybridize to the sticky ends of other DNA tiles, thereby facilitating further assembly into ordered periodic lattices that display designed patterns. Micrometer to millimeter sized 2D DNA-tile lattices containing millions to billions of tiles have been constructed.[10,11] Self-assembled DNA arrays might ultimately offer great opportunities for biomedical applications. For example, self-assembling DNA arrays may be used as templates for the assembly of biomimetic materials. Multiplex biosensing systems could also be built by encoding variable molecular recognition events in a multitile DNA array.

Besides serving as nanosized building blocks, another nonclassical application for nucleic acids has been the discovery of aptamers. Aptamers are short DNA or RNA oligonucleotides that are selected from pools of random sequences based on their ability to bind to other molecules. By using in vitro selection techniques, aptamers that can bind to other nucleic acids, proteins, small organic compounds, and even entire organisms have been identified.[12,13] Some of them exhibit subnanomolar affinities for a variety of protein targets.[14,15] Selection and synthesis in vitro, low molecular weight, and the highly specific molecular recognition of ligands make aptamers appealing for sensing and diagnostic applications as a substitute for antibodies.[16]

Signaling aptamers are aptamer probes that couple target binding to fluorescent-signal generation. This is generally done by introducing a fluorophore in a region of the aptamer that is known to undergo environmental change upon target binding, such as conformational or polarity change, so the molecular-recognition event can be transduced to detectable optical signals. Ellington and co-workers showed that by modifying an ATP binding aptamer with a single fluorescent dye (fluorescein, rhodamine green, or cascade blue), a fluorescence signal increase up to 220% upon the addition of ATP was observed.[17,18] Similarly, Merino and Weeks converted three aptamers into sensor devices by tethering an environmentally sensitive fluorescent group (Bodipy) at an appropriate 2′-ribose position.[19] A variety of other approaches to create signaling aptamers were devised over the years. For example, Stojanovic and Kolpashchikov developed a modular allosteric sensor that combined two aptamers with a connecting stem in which the recognition event in one aptamer was transduced into fluorescence change of a noncovalently attached fluorophore in the other aptamer.[20] Li and co-workers designed "structure-switching" signaling aptamers that showed target binding by the fluorophore-labeled aptamer switching binding partner between quencher-labeled DNA and the target.[21–23] A wide range of fluorescence enhancements of up to 50-fold between the bound and free states had been reported, but very often, depending on the design strategy, the binding affinities for the targets decreased several or even 60-fold when compared with the unmodified aptamers.[17–23]

We have previously shown that by embedding a thrombin-binding-aptamer (TBA) sequence into a linear DNA tile, thrombin can be organized into periodical 1D linear arrays,[24] demonstrating that aptamers keep their molecular-recognition capability when they are introduced into DNA-tile arrays. Herein we demonstrate that signaling aptamers can be organized into micron sized 2D arrays for biosensing applications. Figure 1 schematically illustrates the design of the array based on a two-tile system (A and B tiles) in which the tiles are designed to associate with each other in a periodic fashion to form a 2D nanogrid.[25] In this work, a DNA hairpin–loop (colored green in Figure 1) containing the sequence of the thrombin-binding signaling aptamer is incorporated in the A tile (colored pink), which protrudes out of the tile plane. The periodical spacing between neighboring signaling aptamers is ≈27 nm in the self-assembled array (Figure 1b).

TBA is a well characterized 15-mer DNA aptamer with a consensus sequence of d(GGTTGGTGTGGTTGG) that folds into a unimolecular guanine quadruplex and displays

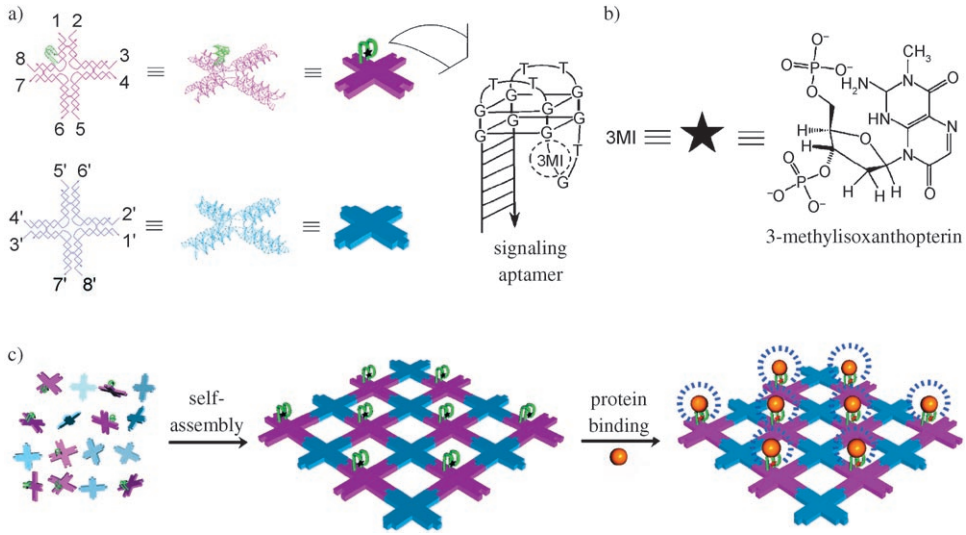

Figure 1. Schematic drawing of the design and operation of the signaling aptamer array created by DNA-tile self-assembly. a) The two tiles of the DNA nanogrid array. Complementary sticky ends are numbered as *n* and *n*′. The pink tile contains the thrombin aptamer sequence (a green loop) in a G-quadruplex structure. At the 7-position, the original dT is substituted by the fluorescent nucleic acid analogue 3 MI. b) The molecular structure of 3 MI. It is also labeled as a black star in the tile without protein. c) The self-assembly of the two-tile system into a 2D array that displays the thrombin-binding aptamer at every other DNA tile. Upon protein binding, the array containing the signaling aptamers "lights up" (labeled as red star).

an approximate 10 nM apparent-dissociation constant to human α-thrombin.[26,27] Herein we used TBA modified with 3-methylisoxanthopterin (3 MI) at position 7 (Figure 1b). The fluorescence quantum yield of 3 MI, a fluorescent guanosine (G) analogue, is highly sensitive to changes in the local environment, in particular to the extent of base-stacking interactions.[28] Crystal-structure analysis[29] of TBA bound to thrombin suggests that dT_7 undergoes significant unstacking from the neighboring bases upon binding to the protein. Therefore, the 3 MI-modified TBA showed a large fluorescence-intensity change upon binding with thrombin (Katilius et al.[34]). 3 MI has a relatively large Stoke shift with excitation and emission maxima at 350 nm and 430 nm, respectively, suitable for fluorescence imaging with a confocal fluorescence microscope. In comparison with other reported signaling aptamers, 3 MI-modified aptamers have the advantage of a decent fluorescence-signal increase upon protein binding, no decrease in binding affinity, and importantly, high resistance to photobleaching.[28]

Fluorescence spectra were measured at two different DNA array concentrations suspended in buffer solution to investigate 3 MI fluorescence-intensity changes as a function of human α-thrombin concentration (Figure 2). With a constant concentration of the DNA arrays equivalent to 1 μM TBA, the thrombin concentration in solution increased from 0 to 1.6 μM, and a twofold increase in the 3 MI fluorescence intensity was observed. The emission peak is also red shifted ≈4 nm from 413 nm to 417 nm. A fit to the "Langmuir model" for the fluorescence-response curve gives an apparent dissociation constant of $\approx 4 \pm 2$ nM. This is obtained by taking into account the depletion in the bulk concentration of protein owing to binding to the aptamer, and with the assumptions of 1) a linear fluorescence response with the concentration of the bound protein, 2) a single binding site for a 1:1 ratio of protein and aptamer, and 3) no interactions between individual binding sites. This dissociation constant shows an ≈2.5-fold increase when compared with the published effective dissociation constant values for TBA, ≈10 nM.[30,31] The detection limit of protein was estimated to be ≈20 nM based on the signal-to-noise (S/N) level.

When the concentration of the DNA array is lowered to 10 nM, as shown in Figure 2c, the addition of human α-thrombin causes ≈60% fluorescence increase at a saturation concentration of ≈30 nM. The detection limit was estimated to be ≈5 nM. The better sensitivity for the lower DNA

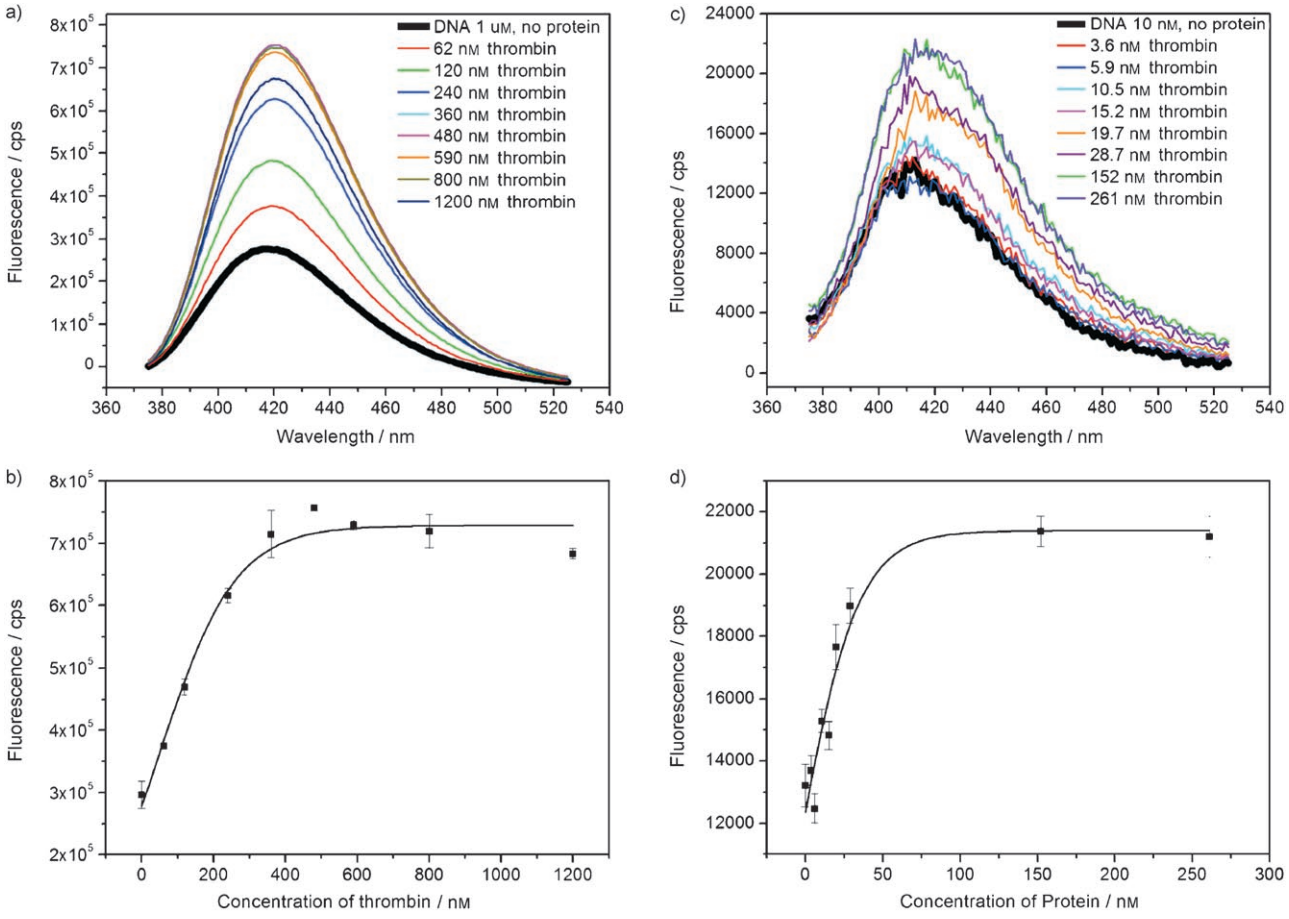

Figure 2. a, c) Fluorescence spectra for the signaling aptamer array with different amounts of thrombin. The aptamer array concentrations are 1 μM (a) and 10 nM (c). Increasing thrombin concentrations were added into the solution as labeled in the Figure legends. b, d) Plots of the fluorescence peak intensity as a function of the thrombin concentration, corresponding to the spectra in (a) and (c). The curves through the data points are fit to Langmuir model. The error bars are estimated from repeating the experiments three times. cps = counts per second.

nanoarray concentration is due to the lower background signal from the signaling aptamer alone. As the overall signal level decreases, the S/N ratio also decreases significantly. When the concentration of the DNA array is further lowered to 1 nM, the S/N ratio is so low that the signal generated from 6–18 nM thrombin, which should nearly saturate the binding sites, is just buried in the noise (data shown in the Supporting Information). Therefore, the detection limit is difficult to improve for analysis in bulk solutions.

Confocal fluorescence microscopy was used to directly visualize the DNA arrays before and after the addition of the protein (Figure 3). The top image in Figure 3a shows the arrays deposited on the glass slides before addition of the protein. Arrays were assembled and deposited at the effective concentration of 1 μM TBA, some conglomeration of arrays was observed. This is because no terminal tiles were included in the assembly of the arrays and as a result, the final arrays formed are all irregularly shaped with "sticky edges". Therefore, touching of the edges of nearby DNA arrays or even some overlapping or folding of DNA arrays upon binding to the surface is common.[10,11] This phenomenon can also be observed by AFM for the self-assembled signaling aptamer arrays before and after the addition of thrombin (see the Supporting Information). AFM images clearly show the formation of the signaling aptamer array and the protein array. However, scanning across the surface of the AFM tip can scratch some proteins off the array owing to the non-covalent interaction between the protein and aptamer. Therefore, the coverage of the protein on the signaling aptamer array does not reflect the binding efficiency of the protein to the array. It is also notable that smaller domains of the array come together to form larger aggregates which can lead to measurement of the array by fluorescence microscope imaging.

Control experiments were performed to test the specificity of the fluorescence response. First, the common serum protein, bovine-serum albumin (BSA), was added to the arrays instead of α-thrombin and no significant fluorescence-intensity change was observed (Figure 3a). Further addition of 1 μM human β-thrombin and human γ-thrombin to the arrays caused a small increase in the signal intensity, ≈15–20%, which was similar to the observations in solution (see the Supporting Information). Finally, when 1 μM α-thrombin was added, the arrays "light-up" as the fluorescence signal increases significantly (the bottom image in Figure 3a). Immunoglobulin E (IgE) was also used as control and showed no competition in binding to the aptamer (Figure 3b). These experiments show that the fluorescence-signal increase was highly specific to binding of the thrombin protein to the aptamer array.

To further confirm that the fluorescence change was caused by the specific binding of TBA to thrombin, the sequence d(TTTTTT(3 MI)TTTTTTTT) was incorporated into the array instead of the TBA sequence. In this case, the DNA-tile arrays could still self-assemble, but no fluorescence-signal changes were detected before or after addition of the α-thrombin to the solution (Figure 3c). Thus, the self-assembled signaling aptamer array specifically detects the presence of α-thrombin in solution.

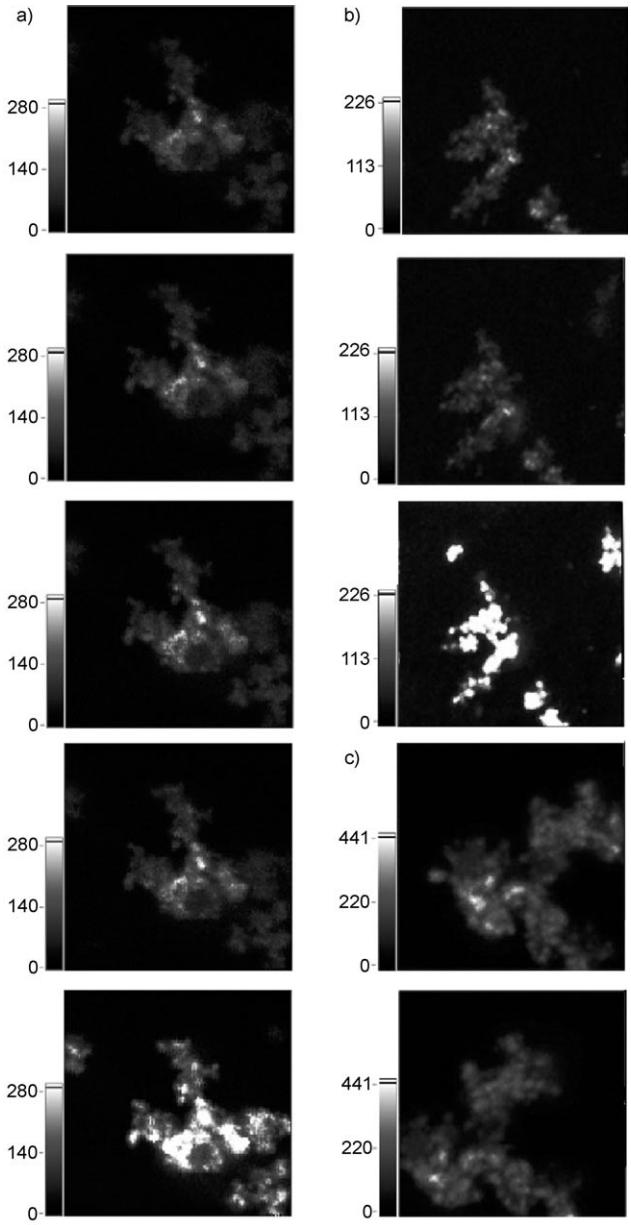

Figure 3. Images of DNA arrays. All images shown are samples containing 1 μM DNA-tile arrays (a–e) in 50×50 μm² scale. a) From top to bottom are the DNA-tile arrays containing thrombin binding aptamer with 3 MI, after addition of 1 μM BSA, after addition of 1 μM human β-thrombin, after the addition of 1 μM human γ-thrombin, and finally after addition of 1 μM human α-thrombin. b) From top to bottom are the DNA-tile arrays without protein, after the addition of 1 μM IgE, and after the addition of 1 μM human α-thrombin. c) Top, DNA-tile array containing a random sequence of DNA loop with 3 MI. Bottom, after the addition of 1 μM α-thrombin to the array.

Fluorescence imaging was also performed on the DNA arrays with an effective concentration of the aptamer of 1 nM (Figure 4). Dilution of the arrays 100 times from 1 μM minimizes their conglomeration, allowing single arrays to be resolved. Imaging of arrays under these conditions showed that most of them exist in sizes ranging from 1–10 μm, as limited by the self-assembly process. An approximate 100% increase in the average fluorescence signal was obtained from

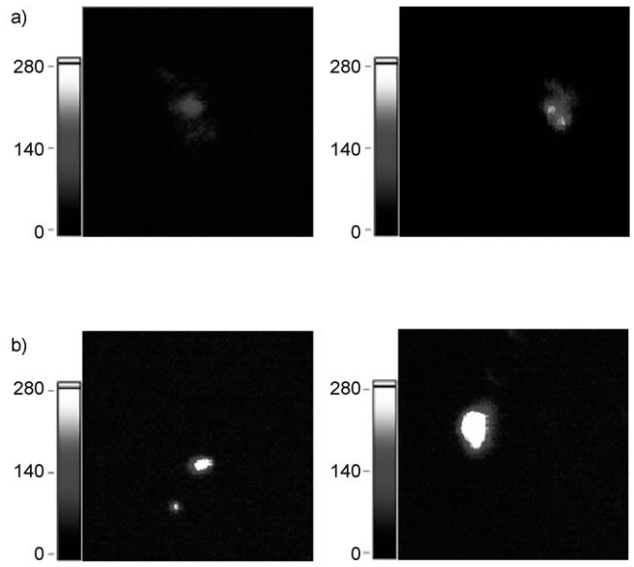

Figure 4. Images of DNA arrays deposited on the slides at low concentration. All images shown are samples containing 1 nM DNA-tile arrays in $25 \times 25\ \mu m^2$ scale. a) Representative samples of single DNA arrays. b) Representative samples of DNA arrays that were preincubated with 1 nM thrombin and then deposited on the slides. Notice a significantly higher fluorescent intensity of the array. The average intensity of arrays before the addition of protein range from 130–190 counts μm^{-2}, whereas the average intensity when 1 nM thrombin is added increases to 300–400 counts μm^{-2}.

images taken in the presence and absence of thrombin protein (Figure 4a and b).

These data may seem contrary to the fluorescene data obtained in solution. with the dissociation constant of $\approx 4 \pm 2$ nM at initial concentrations of the protein and aptamer of 1 nM, the percentage of aptamers binding with protein is expected to be lower than 20%. As a result of this, a maximum 20% increase in the signal is expected based on this calculation. Previous data have indicated the binding of TBA with thrombin maybe very complicated, 1:1, 1:2, 2:1, and 2:2 binding ratios are all possible.[24,32] Therefore, the dissociation constant of 4 nM obtained by fitting the data to the simple Langmuir model may not be accurate. Furthermore, when the aptamers are assembled into nanoarrays, the dissociation of bound protein from the aptamer array is different from that of the individual aptamer molecules in solution. Because of rebinding of the released protein by a nearby aptamer on the array, the dissociation constant may decrease an order of magnitude or more. The data shown in Figure 4 support this argument. This ligand rebinding phenomenon has been examined theoretically on cell-membrane surfaces and macromolecule systems.[33,34] It was pointed out that rebinding can play a major role in the performance of surface-based biosensors. These results show that by incorporating the 3 MI-modified signaling aptamer on DNA nanoarrays and imaging with confocal microscope, we can effectively detect nanomolar and subnanomolar concentration of a target protein in solution.

In summary, we have demonstrated that the DNA-tile directed self-assembly of a signaling aptamer into micron-sized DNA arrays can be used to detect proteins with high specificity and sensitivity at subnanomolar concentrations. However, the strength of the signals that were detected allow us to believe that even picomolar concentrations should be easily detectable if signaling aptamers with higher affinity are selected. This methodology could present future opportunities to construct water-soluble sensor arrays in a programmable fashion. Of course, the use of fluorescent nucleotides as fluorophores for signaling aptamers limits the possibility of multiplexing the assay. Alternatively, different signaling aptamers that are labeled with fluorophores that emit at different wavelengths can be incorporated into the same DNA array (e.g. a multitile system) for multicolor and multicomponent detection.

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