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High-Density Fiber-Optic Genosensor Microsphere Array Capable of Zeptomole Detection Limits

Jason R. Epstein, Myoyong Lee, and David R. Walt*

The Max Tishler Laboratory for Organic Chemistry, Department of Chemistry, Tufts University, Medford, Massachusetts 02155

The detection limit of a fiber-optic microsensor array was investigated for simultaneous detection of multiple DNA sequences. A random array composed of oligonucleotide-functionalized 3.1-μm-diameter microspheres on the distal face of a 500-μm etched imaging fiber was monitored for binding to fluorescently labeled complementary DNA sequences. Inherent sensor redundancy in the microarray allows the use of multiple microspheres to increase the signal-to-noise ratio, further enhancing the detection capabilities. Specific hybridization was observed for each of three sequences in an array yielding a detection limit of 10^{-21} mol (∼600 DNA molecules).

New methods for high-throughput DNA detection and analysis have transformed the biological sciences.[1,2] High-density DNA microarray sensors are being developed to exploit the potential of the Human Genome Project.[3−8] Standard DNA biochip array technology suffers from a rigid fabrication protocol and relatively large feature sizes,[9,10] and because low detection limits are uncommon for most DNA microarrays, amplification is usually necessary for detection.[10] The fiber-optic array platform developed in our laboratory provides smaller feature sizes and flexibility in design.[11−13] In this report, we describe how these arrays can achieve unprecedented detection limits, 2 orders of magnitude lower than previously reported.[12] A random microsphere array situated on the distal face of a 500-μm etched imaging fiber was measured for binding to three different fluorescently labeled complementary DNA sequences.[12] Inherent redundancy in the microarray allows the use of multiple microspheres to increase the signal-to-noise ratio as the square root of the number of sensors examined.[14,15] Redundancy improved the specific detection for each of three DNA sequences in an array and afforded a detection limit of 10^{-21} mol (∼600 molecules).

Our approach to achieving low detection limits was based on two premises. First, a limited number of hybridized target molecules occupying an extremely small volume provide a high local concentration.[16] Second, examining multiple identical sensors simultaneously improves the signal-to-noise ratio by allowing incoherent noise to be signal averaged. These two strategies are at odds: for a limited number of target molecules, having fewer microsensors present will increase the signal since there will be more target molecules per microsphere; however, greater microsphere numbers present in the array have a positive impact on the signal-to-noise ratio. The signal-to-noise ratio increases with the square root of the number of microspheres observed; therefore, replicates in the array should provide an improved detection limit. For fixed microsphere numbers, as target concentrations were decreased, individual microspheres exhibited significant variability such that it was not possible to ascertain signal from noise, but averaged signals from identical microspheres provided detectable signals. The present array maximized the numerical advantage of target molecules to microspheres (i.e., concentration effect), while still maintaining a signal-averaging benefit (i.e., S/N effect).

EXPERIMENTAL PROTOCOL

Microsensor Generation. A 5-μL aliquot of 3.1-μm amine-functionalized microsphere stock suspension (Bang's Laboratories, Fishers, IN; 5 μL contains ∼1 × 10^6 microspheres) was treated for 1 h with 150 μL of 5% glutaraldehyde in 0.2 M phosphate buffer (PBS pH 7).[17] The microspheres were washed three times with PBS buffer and mixed for 1 hr with 150 μL of 5% polyethyleneimine (PEI) in PBS.[17] The microspheres were again washed three times with PBS buffer. All oligonucleotides were synthesized in the Tufts University Physiology Department (Boston, MA) using an ABI synthesizer, with DNA concentrations determined by measuring the solution's optical density at 265 nm. Oligonucleotide probes

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were made with a 5′-amino-C6 modifier (Glen Research, Sterling, VA). Activation of the 5′-amino terminal DNA probe sequences [20 nmol in 180 μL of 0.1 M sodium borate buffer (SBB, pH 8.3)] was performed with 1 μM cyanuric chloride (40 nmol in 40 μL of acetonitrile) for 1 h,[17] after which unreacted cyanuric chloride was separated by three cycles of centrifugal ultrafiltration (Microcon 3, Amicon). The PEI-coated microspheres were shaken overnight with the activated oligonucleotide solution and then washed three times with SBB buffer. Unreacted amine groups were capped with succinic anhydride (100 μL of 0.1 M in a 90:10 solution of DMSO and SBB). The microspheres were washed three times with SBB and three times with 6× saline sodium phosphate EDTA buffer containing 0.1% SDS (SSPE), in which they were stored at 4 °C. The use of detergent avoids microsphere aggregation, and buffers containing EDTA can inhibit nuclease activity. Target DNA were labeled with fluorescein and dissolved in SSPE buffer.

Array Fabrication. The optical substrate used for the array was 500-μm-diameter imaging fiber bundles (Galileo Electro-Optics Corp., Sturbridge, MA), each containing ∼6000 individually cladded 3-μm optical fibers, which were sequentially polished on an automated fiber polisher (Ultra-tec Mfg Inc., Santa Ana, CA), using 30-, 12-, 9-, 3-, 1-, and 0.3-μm lapping films. The polished fibers were etched using a solution consisting of 0.2 g of NH_4F, 100 μL of 50% HF, and 600 μL of distilled water. Fibers were exposed to the solution for 75 s, rinsed in water for another 2.5 min, and sonicated for 15 s to remove salts remaining from the etching process. The different DNA-functionalized microspheres were mixed, and an aliquot of the suspension was added to the fiber's etched distal face.

Imaging System. The custom-built, modified epifluorescence imaging system includes a xenon arc lamp, excitation and emission filter wheels, microscope objectives, and an intensified charge-coupled device (ICCD) camera (Princeton Instruments, Trenton, NJ). The camera is fitted with a microchannel plate image intensifier coupled to a CCD array. Filter wheels and shutters were software controlled, and fluorescence images were processed using IPLab software (Scananalytics, Fairfax, VA). The system was assembled so the fiber containing the microsensors was fixed during all hybridization and rinsing processes. Fluorescent images were acquired with 2-s acquisition times while the tip was in buffer.

Positional Registration and Array Decoding. Although positional registration strategies for individual microsensor placement based on a multiple-dye bar code format have been developed,[11−13] the present array simplicity, i.e., fewer microsensors, enabled a less complex design. Positional microsphere registration was performed by exposing the array to complementary 1 μM target concentrations. Fluorescence changes from hybridizing 1 μM concentrations were visualized within seconds with the ICCD. After hybridization with the target solution, the fiber's distal tip containing the array was washed with warm buffer to remove nonspecific target binding. The microsphere positions in the array are not affected by washing and remain fixed unless subjected to a sonication bath. To ensure the microsphere arrangement remained constant, rapid decoding was performed throughout the experiment as needed.

Detection Limit Experiments. Experiments were carried out by exposing the array to solutions containing various labeled target concentrations. Since only the tip of the 500-μm-diameter fiber is exposed to the target solution, a minimum solution volume of 4 μL is required with this system. For all detection limit experiments, 10-μL volumes were used for easier handling and to avoid effects due to evaporation. These small volumes can evaporate over extended hybridization times, so miniature hybridization vessels were designed to counter this effect. These vessels were structured to allow the arrays access to the hybridization solution, while keeping the hybridization environment constant. Each target solution was allowed to hybridize to the array for 12 h. Microspheres were randomly selected and monitored from each array. Three background images were first obtained, followed by target hybridizations. Each hybridization exposed the array to a target solution complementary to one of the probe types. The array was rinsed with SSPE buffer, and three fluorescence images were collected. From these hybridization images, signal and background values were obtained by averaging the response of identical microspheres. The same images were also used to calculate the standard deviations associated with the average microsphere response. A positive hybridization signal was defined as a signal greater than 3 times its standard deviation ($>3\sigma$). At lower total target molecules numbers, elongating the hybridization time was necessary to achieve positive detection. Longer hybridization times increase both the specific and nonspecific hybridization signals. Hybridized arrays were rinsed until each nonspecific signal was below the criterion of a successful signal ($>3\sigma$), except for 10 aM target concentrations, where no assay gave acceptable signals. Experiments detecting unknown targets would require a control standard introduced into the hybridization solution.

Inhibition Experiments. A total of 100 mg of salmon sperm DNA (587−831 base pairs, Sigma) was incubated with nuclease S1 [3.96 units/mL (Gibco-BRL)] at 37 °C for 1 h in 30 mM sodium acetate buffer (pH 4.6) with 30 mM sodium chloride and 1 mM zinc acetate. After the reaction, the enzyme was extracted from the DNA preparation with phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated to pH 8.0). DNA between 10 and 125 base pairs was recovered by ultrafiltration with Microcon 3 (10-bp cutoff) and Microcon 50 (125-bp cutoff). The DNA was quantified with OligoGreen single-stranded DNA quantification reagent (Molecular Probes, Eugene, OR). Approximately 10 ng of salmon sperm was added for inhibition experiments with the hybridization solutions.

RESULTS

Imaging fibers were selectively etched using the difference in etching rates between the fiber cores and clad to form a high-density array of microwells on the fiber's distal end.[18] The different DNA-functionalized microsphere types were prepared prior to addition to the fiber-optic array and combined to form a multiplexed sensor stock suspension. An aliquot of the microsensor stock suspension was added to the distal face. Upon solvent evaporation, the complementary-sized microspheres localize into the etched wells of the fiber,[11] creating an array of optically connected, individually addressable DNA sensors.[12] The DNA sequences used for this study, shown in Table 1, were parts of the sequences of interleuken 2 (IL2), interleuken 6 (IL6), and the F508C mutation in the cystic fibrosis gene. Detection capabilities are influenced by sequence length, base composition, and experi-

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Table 1. Probe and Target Sequences Used for DNA Detection Limit Experiments[a]

probe name	sequence
IL2 (interleuken-2)	5′-TA-CAA-GAA-TCC-CAA-ACT-CAC-CAG-3′
IL6 (interleuken-6)	5′-GT-TGG-GTC-AGG-GGT-GGT-TAT-T-3′
F508C	5′-TAG-GAA-ACA-CCA-CAG-ATG-ATA-3′
target name	**sequence**
IL2 (interleuken-2)	5′-CT-GGT-GAG-TTT-GGG-ATT-CTT-GTA-3′
IL6 (interleuken-6)	5′-AA-TAA-CCA-CCC-CTG-ACC-CAA-C-3′
F508C	5′-TA-TCA-TCT-GTG-GTG-TTT-CCT-A-3′

[a] Sequences were chosen because of their importance in either the immune system or recognized disease states.

Table 2. Required Hybridization Times for 10-μL Sample Volumes To Obtain a Signal Greater than Three Times the Standard Deviation of the Background ($>3\sigma$)

concn	total target molecule nos.	hybridization time (min)
1 pM	$\sim 6 \times 10^6$	10
100 fM	$\sim 6 \times 10^5$	20
10 fM	$\sim 6 \times 10^4$	30
1 fM	$\sim 6 \times 10^3$	<60

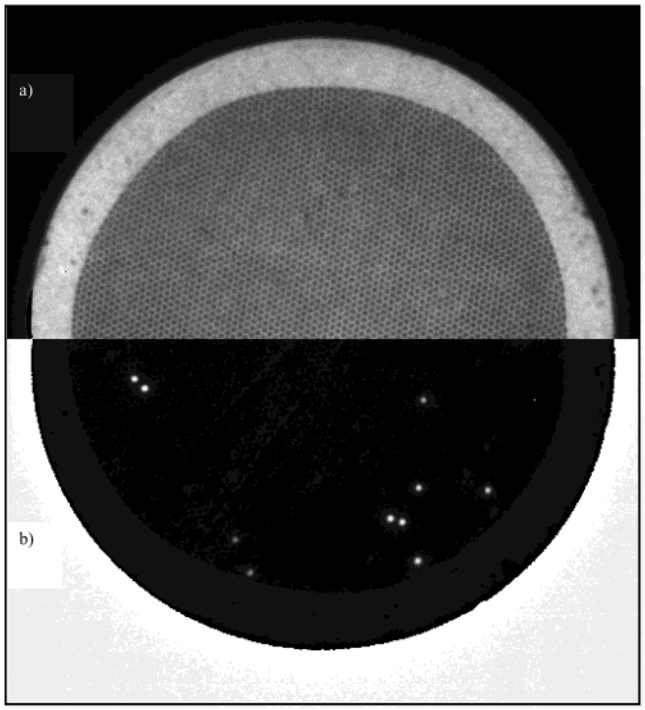

Figure 1. Overlayed images of the fiber-optic, microsphere-based array. The fiber bundle is 500 μm in diameter and contains ~6000 individually addressable 3-μm etched wells capable of housing complementary oligonucleotide-functionalized microspheres. (a) A white light image of the array before hybridization. (b) Posthybridization (10 μL of 1 μM target concentration) with 10 complementary microspheres in the array.

mental protocols. These three sequences are similar in length and base composition, and hybridizations were performed under identical conditions.

To evaluate the array's detection capabilities, a CCD camera was used in conjunction with an epifluorescence imaging system. By employing an intensified CCD camera, the exposure time required to analyze the lowest concentrations was significantly reduced relative to an unintensified camera, and any effects due to photobleaching were minimized. One-picomolar target concentrations (10 μL, $\sim 6 \times 10^6$ target molecules) were detected with 10-min hybridization times, and 1 fM concentrations ($\sim 6 \times 10^3$ target molecules) were distinguished within 1 h (Table 2). This array platform provides the shortest hybridization times of any documented array format for such low concentrations. For detection limit purposes, all hybridization times were extended to 12 h to more readily compare the results from different target concentration hybridizations.

The number of probe molecules on the microsphere surface is important to the array's sensitivity. Microsensors with different amounts of probe molecules on their surface were hybridized to 1 fM target solutions in order to ascertain the probe density effect. Sensors made with 4 times diluted activated oligonucleotide did not generate a signal of $>3\sigma$ after 1 h, demonstrating that the amount of probe on the microsensor surface is crucial for detection. The probe coupling method used in this work provides each microsensor with a large excess of probe sites on its surface, such that virtually all the target molecules are bound to the microsphere.

Signal Averaging. To ascertain the advantage attributed to microsphere summing, the 1 fM target solution, containing ~6000 target DNA molecules, was examined with a separate array containing 500 identical microspheres. With this number of microspheres in the array, we would expect an average of 12 target molecules to be hybridized to each microsphere if hybridization goes to completion. Signals from the randomly positioned microspheres were averaged to produce a definitive hybridization signal. Although individual microspheres varied in their ability to define a positive signal, averaging 10 microspheres produced an acceptable signal with 7% coefficient of variation (CV), while 100 microspheres provided even higher precision with average 3% CV (data not shown). With 10 μL of a 1 fM target concentration, signal generation could be confidently affirmed with the average signal of at least 10 microspheres; therefore, this system is capable of detecting sufficient signal from 120 target molecules bound to the microspheres. In some cases, we were able to identify the correct sequence with as few as 5 identical microspheres; however, 10 microspheres provided a higher level of certainty.

Detection Limits. Once we determined that signal averaging with 10 microspheres provided a sufficient signal-to-noise ratio, the number of microsensors placed on the array was limited to ~10 per probe type (Figure 1). Table 3 shows data from a ~30-microsensor array (10 microsensors of each probe type) illustrating the specificity of each microsphere type with target concentrations of 100 aM. Since the detection limit assays were performed with arrays containing all three different probe types, each assay contained two internal controls to address specificity. Each assay was performed in triplicate, and the full data illustrating the specificity are included in the Supporting Information. The results presented were obtained with 10-μL target volumes and 12-h hybridization times. Each data set shows the averaged signals from three hybridization images and 10 microsensors, including their standard deviations. Experiments performed near the detec-

Table 3. Average Fluorescent Intensity and Background of 100 aM (10^{-16} M, ~600 Molecules) F508C, IL2, and IL6 Target Concentrations Hybridized to the Multiplexed Array[a]

probe	hybridization ± SD	mean background ± SD	signal ± SD
	Target: F508C		
F508C	560.79 ± 8.5	522.17 ± 2.2	38.62 ± 8.5
IL2	481.01 ± 12	454.20 ± 4.0	{26.81} ± 13
IL6	403.38 ± 6.3	382.27 ± 3.4	{21.11} ± 7.2
	Target: IL2		
F508C	341.42± 2.1	327.47 ± 4.5	{13.95} ± 5.0
IL2	378.20 ± 7.8	343.14 ± 6.6	35.06 ± 10
IL6	471.38 ± 13	466.21 ± 11	{5.17} ± 17
	Target: IL6		
F508C	262.40 ± 3.8	246.31 ± 3.9	{16.09} ± 5.4
IL2	242.34 ± 2.7	233.35 ± 4.9	{8.99} ± 5.6
IL6	351.25 ± 5.3	314.49 ± 1.3	36.76 ± 5.5

[a] Signals in broken brackets are less than 3 times the standard deviation of the signal and are below the acceptable signal-to-noise range. Each probe correctly identified its complementary target.

Table 4. Average Fluorescent Intensity and Background of 1 fM (~6000 Molecules) and 100 (~600 Molecules) and 10 aM (~60 Molecules) F508C Target Concentrations Hybridized to the Multiplexed Array[a]

probe	hybridization ± SD	mean background ± SD	signal ± SD
	Target: F508C; 1 fM (~6000 Target Molecules)		
F508C	543.02 ± 0.71	472.23 ± 5.0	70.79 ± 5.1
IL2	485.39 ± 2.3	476.96 ± 8.0	{8.43} ± 8.3
IL6	390.63 ± 0.87	375.91 ± 5.2	{14.72} ± 5.3
	Target: F508C; 100 aM (~600 Target Molecules)		
F508C	560.79 ± 8.5	522.17 ± 2.2	38.62 ± 8.5
IL2	481.01 ± 12	454.20 ± 4.0	{26.81} ± 13
IL6	403.38 ± 6.3	382.27 ± 3.4	{21.11} ± 7.2
	Target: F508C; 10 aM (~60 Target Molecules)		
F508C	540.21 ± 3.8	528.14 ± 2.6	{12.07} ± 4.6
IL2	462.29 ± 2.9	454.85 ± 0.72	{7.44} ± 3.0
IL6	394.15 ± 2.9	390.44 ± 2.2	{3.71} ± 3.6

tion limit will have relatively small signal increases compared to the background. The background intensity is a function of many parameters, including camera exposure time, dark current noise, and the substrate's intrinsic fluorescence. Table 4 demonstrates the array detection limits by presenting the specific hybridization of the F508C target at 1 fM, 100 aM, and 10 aM concentrations, with the multiplexed array. Similar experiments were performed with IL2 and IL6 targets, each done in triplicate, and are included in the Supporting Information. Using 10 microsensors in the array, sample detection was successfully performed on 10-μL sample volumes down to concentrations of 100 aM, which corresponds to 10^{-21} mol or ~600 molecules. For the 10 aM target concentration experiments, no assay gave acceptable signals.

Inhibition Studies. In addition to pure target solutions, studies were performed on samples containing noncomplementary single-stranded salmon sperm DNA (587−831 base pairs). When the same target concentrations were examined containing excess sperm DNA (10 ng, MW ~79 000, ~126 μM, roughly 10^9-fold higher concentration of irrelevant DNA), there was no observable inhibition of target hybridization. The same analyses were also performed with shorter length sperm DNA (10 ng, 10−125 base pairs, MW ~37 000, ~260 μM). In this case, hybridization to target concentrations of 10 fM and lower were inhibited. The same signals as in the pure target solutions could be observed (>3σ), however, by incubating for intervals one and a half times that of pure target solutions.

DISCUSSION

Detection limits can be defined by two criteria: sample volume and target concentration, or absolute number of target molecules. The ability of our platform to detect extremely small absolute numbers of molecules stems from the small sensor feature sizes. To illustrate this point, consider that 12 molecules confined to a well volume (bead and liquid) of ~30 fL provide a local concentration of 1 nM. Our fiber-optic array platform can readily detect nanomolar fluorophore concentrations with the ICCD imaging system.[12] Such low detection limits generally require lasers, confocal optics, and avalanche photodiodes,[19] while these experiments employ a standard white light source, CCD camera, and optics. As the volume decreases, the less a sample needs to be amplified for detection since the same absolute number of target molecules in a smaller volume generates a higher local concentration. Fewer sensor numbers also provide a numerical advantage because more target molecules hybridize per microsphere, resulting in a signal increase. By using a 10-microsensor array and assuming the hybridization goes to completion, the local target amount associated with 100 aM solutions averages 60 target molecules/microsphere. The same array exposed to 10 aM target concentrations provides 6 target molecules/microsphere and was below the limit of detection. While 1 fM (~6000 target molecules) detection results were reproducible 100% of the time, the detection experiments that were closer to our actual detection limit (100 aM, ~600 target molecules) had a success rate of 80%. Similarly, experiments performed with increased numbers of identical microspheres in the array and 100 aM target concentrations provided lower signal values, diluting the number of target molecules per microsphere, and definitive signals (>3σ) were not always obtainable.

This array design provides both lower detection limits and shorter analysis time than other documented arrays. The ability to monitor hybridization directly in the hybridization solution, along with the versatility of the array platform, establishes this detection process as a viable alternative to other current DNA detection protocols that typically require PCR amplification for analysis. Standard PCR starts with 10^2−10^5 copies of template and amplifies each target by 10^9 or higher. We demonstrated that the DNA microsensor array is capable of detecting as few as 600 target molecules (10^{-21} mol). Although this process exhibits low detection limits, it is still limited by the need to incorporate fluorescence onto the DNA targets. The three target sequences examined for this study were markedly different, and an array analyzing single base mismatches may necessitate more experimental stringency to achieve similar results. Longer strands of DNA similar to that present in template DNA did not affect the system's detection capabilities, but the presence of shorter noncomplementary DNA required extended hybridization times to acquire a signal of >3σ.

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Ongoing multiplexed experiments will address simultaneous detection of mixed target solutions. The uncertainty associated with such low absolute molecule numbers is countered by confining a small number of molecules to a small volume, providing a high local concentration. Bead replicates improve the confidence level even further by increasing the signal-to-noise ratio and prevent the occurrence of false positive and false negative signals.

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SUPPORTING INFORMATION AVAILABLE

Additional data as indicated in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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