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A microfluidic platform for electrical detection of DNA hybridization

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Abstract

Current methods used for detection of DNA hybridization involve the use of DNA microarrays which require overnight incubation times along with bulky and expensive fluorescent scanners. Here, we demonstrate electrical detection of DNA hybridization in an oligonucleotide functionalized microfluidic channel. We use microchannels functionalized with DNA probes integrated with electrodes for measuring conductance across the channel. As beads conjugated with the target DNA passing through the channel are captured on the surface, we are able to electrically detect changes in resistance due to bead capture. Our assay can be completed in less than an hour using less than a microliter of reagent, and has the potential for extensive multiplexing. Such a device can be useful as a handheld platform in a clinical setting where one would need to rapidly genotype a small number of genes rapidly.

Keywords

Microfluidics; Biosensors; DNA hybridization; Genotyping; Electrical detection

1. Introduction

A rapid and inexpensive methodology for detecting the hybridization of two DNA strands can be useful in detecting the presence of certain genes in a patient's DNA. By detecting such gene sequences it is possible to determine whether a patient has a predisposition to a certain type of disease allowing him to get treatment to prevent the disease. Common methods for genotyping include the use of DNA microarrays [1] and also real-time PCR [2]. Such techniques are expensive given that they require the use of fluorescent labels which result in high reagent costs. The other major cost comes from the use of expensive and bulky optical scanners required for measuring fluorescence. DNA hybridization also requires overnight incubation given that thousands of molecules must hybridize in order to produce enough optical signal to be readable by the fluorescent scanner. Electrical detection allows for the potential of developing a hand held platform capable of multiplexed detection due to ease of integration with CMOS [3,4]. Here, we demonstrate the ability of our microfluidic biochip to electrically detect the hybridization of two complementary DNA strands within seconds, without the need of any fluorescent labels. Previously we demonstrated the ability of this technique to detect target cells [5], protein-protein interactions [6], and protein biomarkers [7].

Although our technique is capable of multiplexed detection, we still will not be able to reach the order of multiplexing achievable with DNA microarrays. This level of multiplexing is

necessary in research settings where access to core facilities with DNA microarray scanners is more readily available. However, in clinical settings, particularly in poor areas, access to DNA microarray scanners which cost hundreds of thousands of dollars is quite rare. In this type of setting a common application would be biomarker detection in a patient's DNA, where it is not necessary to detect on the order of hundreds of thousands of target DNA strands (which DNA microarrays are capable of doing). In this type of setting, a portable scanner capable of detecting 10 or even 100 genes may be sufficient. So in this case, our electrical detection approach would be quite suitable. The use of microfluidic devices for genotyping is currently under exploration due to their ability to improve detection sensitivity, their decreased usage of reagent consumption, and the resulting decrease in assay time [8]. Most of the current successful microfluidic approaches for genotyping involve the use of fluorescent detection, which requires expensive equipment for scanning, making portability difficult. A detection platform based on electrical detection can overcome this barrier.

In the microchannel gating technique for DNA biomarker detection, DNA probe molecules are immobilized on the surface of the microchannel. Target DNA molecules are immobilized on the surface of micron sized beads. The beads are then injected into the microchannel (Fig. 1A) partially clogging the channel resulting in an instantaneous increase in the baseline resistance (Fig. 1C). The requirement for successful detection of the DNA hybridization (Fig. 1B) is that the surfaces of the microspheres contain target DNAs which are specific and complementary to the probe DNAs immobilized on the active area of the sensor. In order to be able to detect the hybridization resulting in the capture of a single bead, it is also necessary that the microspheres used be comparable in size to that of the channel geometry.

Alternatively it is possible to run this assay without immobilizing the target DNA on the beads. In this assay, the test sample containing the target DNA, functionalized with a linker molecule such as biotin at the 5' end, is injected into the channel pre-functionalized with DNA probe molecules, in which the 5' end is attached to the surface. After hybridization occurs, the 5' end of the target DNA will be exposed to the solution. Thus if a bead coated with a molecule such as streptavidin passes through, it can bind to the biotin on the target DNA. This format is more suitable for multiplexing since it would not be necessary to separately functionalize each bead with a different target DNA.

2. Experimental procedures

In this section we describe the procedure required to fabricate the microsensors, and also the procedure to functionalize the beads and the channel surface with the target DNA and the probe DNA molecules respectively.

2.1. Fabrication

A schematic of the sensor is shown in Fig. 2. The microsensor consists of gold electrodes on a glass substrate covered with a PDMS slab embedded with a microfluidic channel. Au/Cr electrodes (with a thickness of 200 nm/15 nm) were patterned using evaporation and then lift-off and were then cut into separate pieces. The electrodes were 10 μm in width. Au/Cr electrodes were micropatterned on a glass wafer using traditional photolithography, sputtering, and then lift-off processing and then cut into separate chips using a wafer saw. The microchannels were fabricated in PDMS. The master mold for the microchannels was patterned onto a silicon substrate using SU-8 photoresist (fabricated by the Stanford Microfluidics Foundry). PDMS (10:1 prepolymer:curing agent) was poured onto the master mold and allowed to cure for 2 h at 80 $^{\circ}\text{C}$. Holes of 1 mm in size were punched through the pdms

to make inlets and outlets of the channels. The glass chips and the PDMS slabs were aligned and then bonded together after oxygen plasma treatment.

Tygon tubing was attached to the inlet and was connected to a 50 μ l syringe, where the flow was controlled by a syringe pump (Harvard Apparatus Model 11, Instech Solomon, Plymouth Meeting, PA).

2.2. Measurement apparatus

Electrical impedance measurements were collected across the channel in the region between electrodes A and C. We applied a voltage signal to electrode A and a low noise current preamplifier (Stanford Research Systems Model SR570) to electrode C in order to measure the current across the channel and then the data was collected with a National Instruments data acquisition card and read by a Labview program. The channels were also monitored using optical microscopy in order to confirm that the electrical signal changes were due to beads binding in between the electrodes.

Given that we want to detect the presence of the microspheres due to the resulting change in channel resistance, we want to minimize the effect on the impedance measurement resulting from all impedances except for the bulk solution resistance. This can be achieved by working at sufficiently high frequencies, where from our previous work we measured to be 20 kHz. For our channel and electrode geometry, 30 kHz was an optimum frequency for device operation. At frequencies below this, the impedance due to the double layer capacitance had not yet completely diminished, however as the frequency of the excitation voltage signal was increased above 30 kHz, the output signal became noisier.

In this assay the target DNA consisted of the following sequence, 5'-AGGT GTGG GGTG A TCA TTTG TCAG TGTG AGGG AGTG TGGT AGTG C-3', and the probe DNA consisted of 5'-ACAC CTGC ACTA CCAC ACTC CCTC ACAC TGAC AAAT GATC ACCCC-3'. The target DNA sequence, of 45 base pairs, was biotinilated at the 5' end.

2.3. Channel surface chemistry

The probe DNA sequence was biotinilated at the 5' end. Biotinilated Bovine Serum Albumin was immobilized on the bottom of the surface channel by physical adsorption. Afterwards, Streptavidin was incubated in the channel for 15 min in order to bind onto the biotinilated BSA. The biotinilated probe DNA was then incubated in the channel to be captured by the streptavidin molecules. Incubation times of at least 15 min were necessary to produce optimal immobilization results. The channel surface was then blocked using 1 mg/ml of Bovine Serum Albumin (BSA).

2.4. Bead washing

Fifty microliters of solution (PBS buffer) containing 0.5% (m/v) 20 μ m polystyrene beads precoated with streptavidin (Spherotech Inc., Lake Forest, IL) was prepared. The solution was rotated for 15 min in order to prevent precipitant from forming. The solution was then centrifuged, the supernatant removed, and the beads were again re-suspended in PBS. The PBS buffer had a salt concentration of 700 mM NaCl which is required for rapid hybridization of DNA strands. This process was repeated three times in order to ensure that all free target DNA strands were removed from the solution. The same procedure was also used to coat 10 μ m polystyrene beads precoated with streptavidin (Spherotech Inc., Lake Forest, IL) with DNA.

3. DNA quantification assay

In order to characterize the ability of our sensor to quantify target DNA and to determine the detection limit we optically examined the percentage of beads captured in the microchannel as a result of DNA hybridization. For this assay we used a 200 μm wide 50 μm deep channel. The test sample, which contained the target DNA, was injected and incubated for 30 min in the channels which were immobilized with probe oligonucleotides, allowing for enough time for the target DNA to hybridize to the probes. Afterwards, the 10 μm beads which are precoated with streptavidin are injected into the channel until the surface is completely covered with beads. The beads are incubated in the channel for 1 min, which was sufficient time to allow them to come to rest so that the streptavidin on the bead can bind to the biotin on the 5' end of the target DNA which should be facing upward. A flow of 90 nl/min was applied to the channel which provided enough shear force to pull off the beads which had not bound to the surface of the channel, while those which the streptavidin and biotin were bound together remained intact. We performed this assay for various concentrations of target DNA suspended in PBS buffer. The NaCl concentration of the PBS buffer was increased to 800 mM, which is necessary for the hybridization reaction to occur in a timely manner. The channels were monitored under an optical microscope. The number of beads both before and after the application of the flow was counted manually (by eye) and the results are plotted in Fig. 3 along with the standard error bars. The beads all tend to settle to the bottom of the channel, so it is clear for one who looks under the microscope that the beads that are counted both before and after application of flow are only a monolayer. As the concentration of the target DNA increases, the number of beads attached increases until it reaches saturation at 10 μM . When no target DNA is present in the test sample, the maximum percentage of beads ever binding was 20% however, the average was around five percent.

4. Minimizing false positive signals

It is important to minimize the false positive signals which arise from beads nonspecifically binding to the surface of the channel. This result can be due to electrostatic interactions between the beads and the channel surface, the nonspecific interactions between the DNA and the surface, beads coming to rest on the surface, and many other causes. In general bead capture due to DNA strands hybridizing is higher in affinity compared to nonspecifically bound beads. By adjusting the flow rate such that the drag force on the beads is strong enough such that nonspecifically bound beads can be pulled off while the beads bound due to DNA hybridization remain attached; it is possible to minimize the nonspecific interactions.

In this assay the target DNA consisted of the following sequence, Biotin-5'-AGGTGTGGGGTGATCATTTGTCAGTGTGAGGGAGTGTGGTAGTGC-3', and the probe DNA consisted of Biotin-5'-ACAC CTGC ACTA CCAC ACTC CCTC ACAC TGAC AAAT GATC ACCCC-3'. We also examined a target DNA strand with a single base pair mismatch in the 27th position of the sequence. In this assay we used 10 μm beads.

The beads for each assay were separately incubated in the channel for 1 min. The flow rate was incrementally increased as the beads were pulled off. The average flow rates and the standard error required to detach the beads from the surface of the channel are shown in Fig. 4. In the first column the target DNA on the beads and the probe DNA on the channel surface were non-complementary with each other, and were not expected to hybridize. A flow rate of 100 nl/min was required to wash off the beads. In the second column, the target DNA and the probe DNA were mismatched by a single base pair, requiring a high flow rate of 1000 nl/min to wash off the beads. In the third column the target DNA and the probe

DNA were complementary to each other also requiring a high flow rate of 900 nl/min to wash off the beads. In the second case, the single base pair mismatched DNA unexpectedly has a higher affinity compared to the perfectly matched DNA, however their error bars overlap with each other. With more experiments, it is expected that these two average flow rates will converge. With lengths of DNA as long as those we have examined in this study, we are unable to distinguish between single base pair mismatches and perfectly complementary DNA. This may however be more feasible with shorter length DNA molecules. We will explore these issues in future studies.

One of the advantages of this technique lies in the fact that by operating at the optimal flow rate the nonspecifically bound beads will not get a chance to become permanently attached to the channel surface, while the specifically bound beads will get captured and remain attached. A flow rate window between 70 and 350 nl/min will minimize the false positive signals.

DNA microarrays typically require overnight incubation before the hybridization can be detected. Using our biochip we are able to achieve detection of hybridization within a half hour. Given that in DNA microarrays tens of thousands of different probe DNA molecules are being tested against a mixed soup of DNA molecules, it is expected that the overall hybridization time will be much larger than the case which we tested. However, a half hour is still more rapid than that which can be achieved with fluorescent detection, which is on the order of several hours. The reason for this great decrease in analysis time is a result of the number of molecules required to hybridize before being detectable by the sensing apparatus. For fluorescent detection technologies, at least several thousand molecules are required to hybridize before producing enough optical signal to be detected by the fluorescent scanners. In the case of our assay, this number can be determined by calculating the affinity of the beads to the surface of the microchannel, and then determining the number of hybridized DNA molecules by dividing the total force by the force holding a single molecule together.

5. Calculation of the affinity of the beads and the channel surface

The flow rate in the channel is directly proportional to the drag force applied to the beads. The drag force required to detach the beads from the surface of the channel is equal to the binding force between the hybridized DNA molecules. In order to determine the binding force between the hybridized DNA molecules accurately using the flow rates in Fig. 4, it would be necessary to perform a rigorous calculation of the relationship between the flow rate and the drag force on a sphere on the bottom of a microchannel with the dimensions of our fabricated channels. However, in order to get a quick order of magnitude estimate of the drag force, it is possible to use the sphere-drag formula of Stokes [9]:

$$F=6\pi\mu Ua \quad (1)$$

where U is the mean velocity at which the sphere travels, and a is the radius of the sphere.

An average flow rate of roughly 900 nl/min was required to pull the beads off the surface of the channel which corresponds to a drag force of 126 pN. The rupture forces for larger molecules of DNA tends to saturate at around 70pN [4]. This means that on average the beads are held attached to the base of the channel by the force of one or two DNA molecules. Given that the biotin streptavidin bond is in general much stronger than that of two hybridized DNA strands, we assume that the two complementary DNA strands are being pulled apart before the biotin streptavidin interaction breaks.

This confirms our initial hypothesis regarding the reason for the rapid hybridization detection rates. This is due to the fact that a single DNA molecule hybridizing is sufficient to cause the bead to get captured, compared to fluorescent detection technologies which require several thousand DNA molecules to hybridize in order to generate enough optical signal to be detectable by the fluorescent scanners.

6. DNA real-time assay

The 20 μm beads coated with streptavidin were injected into the non-activated microchannel at a flow rate of 175 nl/min. Here we have used a two electrode configuration, where two electrodes surround the pore. We measure the impedance across the pore at a frequency high enough such that the impedance contribution from the parasitic double layer capacitance is minimized, and the impedance is dominated by the bulk resistance across the pore. We have found this optimum frequency to be 30 kHz as described in our previous work [7]. We perform this measurement by tying the left electrode to a 30 kHz waveform generator, and the right electrode to a current preamplifier, and from this the current passing through the pore is obtained. The current was monitored across the micro-pore as shown in Fig. 5(A). An optical micrograph showing the beads passing through the channel is shown in Fig. 5(B). The downward spikes correspond to beads passing through the channel. The reason for the variance in the pulse heights is due to the fact that the drop in current is proportional to the location of the bead relative the central axis as it passes through the pore. Multiple beads passing across the pore will also result in a larger current drop. The widths of the pulses are a function of the time of flight across the pore.

In Fig. 6, we show representative data of a positive experiment where hybridized DNA is attached to the surface and the biotin is exposed to the channel so that the streptavidin coated beads can get captured at the surface. The sharp downward peaks in the current result in beads passing through the pore. At roughly $t = 65$ s, as a bead is captured beside the pore, a permanent drop in the current results until roughly $t = 95$ s where the bead again detaches and exits the pore. In Fig. 7(A), an image is shown of a bead in the vicinity of the pore, which is close enough (to the pore) to cause a steady state current drop as seen in Fig. 6. Fig. 7(B) shows an image of the bead exiting the pore.

7. Conclusion

In this paper we have demonstrated the ability of our biochip to detect the hybridization of complementary DNA molecules within a half hour. Our system is advantageous over conventional fluorescent detection technologies because the readout time is reduced by at least an order of magnitude without the need for any fluorescent labels. The costs required for labelling on a generic fluorescent DNA chip average at roughly \$20, whereas the use of polystyrene microspheres for labelling averages at about \$3 per chip, a decrease of one order of magnitude. A sample volume of roughly 0.1 μl is required to perform this assay. The ability to detect DNA hybridization on chip electrically opens up the potential for multiplexed detection of nucleic acid biomarkers on a portable device which can be a good candidate for use in the clinical setting. Although, this type of system will not be able to compete with DNA microarrays, which can achieve multiplexed detection of hundreds of thousands of genes, our platform is useful in the clinical setting where one would need a portable platform to inexpensively analyze less than 100 genes for biomarker detection.

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Biographies

Mehdi Javanmard received his BS degree with highest honors from the Georgia Institute of Technology in 2002, and the MS degree from Stanford University in 2004, and the PhD degree from Stanford University in 2008 all in Electrical Engineering. He has held research positions in Georgia Tech Research Institute, Lawrence Livermore National Laboratory, Stanford Linear Accelerator Center, and Stanford Genome Technology Center. His main research areas are biosensors, bioelectronics, and microfluidics with an emphasis on detection of pathogenic bacteria, genetic biomarkers, and protein biomarkers. He is currently an Engineering Research Associate at Stanford University.

Ronald W. Davis received the BS degree in Mathematics, Physics, Chemistry, and Botany from East Illinois University in 1964, and the PhD in Chemistry from California Institute of Technology in 1970. He is considered to be a world leader in biotechnology, and the development and application of recombinant DNA and genomic methodology to biological systems. His laboratory has developed many of the techniques currently used in academic and industrial biotechnology laboratories. He is also considered to be a world expert in the

electron microscopy of nucleic acids and has developed many of the mapping methods for which he received the Eli Lilly Award in Microbiology in 1976. His laboratory was also instrumental in the development of lambda vectors, which were commonly used for the primary cloning of DNA molecules in *E. coli*. His laboratory also developed many of the yeast vectors and helped to develop yeast as a host for recombinant DNA for which he received the United States Steel Award in 1981, presented by the National Academy of Sciences. In 1983 he became a member of The National Academy of Sciences. He was a co-author on a publication that first described a new approach for conducting human genetics and for the construction of a human genetic linkage map for which he received the Rosentiel Award for Work in Basic Medical Research. His laboratory is now conducting genomic analysis of *Saccharomyces cerevisiae* for which he received the 2004 Lifetime Achievement Award from the Yeast Genetics Society. His laboratory is developing many new technologies for the genetic, genomic, and molecular analysis of model organisms and human with a focus on clinical medicine for which he received the 2004 Sober Award from the American Society for Biochemistry and Molecular Biology (ASBMB/IUBMB).

Fig. 1.

(A) Surface of the microchannel is activated with oligonucleotide probes. Target DNA strands are immobilized on the surface of polystyrene beads, which are injected into the microchannel. (B) Hybridization of the DNA strands causes capture of beads resulting in (C) an increase in the channel resistance. Alternatively, if the target DNA can be functionalized with a biotin molecule, the test sample can be injected into the channel and incubated. After the hybridization is completed, streptavidin coated beads can be injected into the channel to bind onto the biotinylated target DNA.

Fig. 2.

Schematic of the microfluidic biosensor and the setup used for measuring the current across the pore. A function generator is tied to the left electrode, and a current preamplifier to the right electrode for converting the current to a voltage.

Fig. 3. Optical results for DNA quantification. As target DNA concentration increases, the percentage of the beads which remain attached also increases, and our detection limit is about 1 nM.

Fig. 4.

The average flow rate required to pull off all of the beads attached to the base of the channel, and also the standard error bars. In the first column, the target DNA and the probe DNA were completely mismatched, thus a negligible flow rate was sufficient to pull off the beads. In the second column, the DNA on the beads was mismatched with the probe DNA on the channel surface by a single base pair, and a flow rate of 1000 nl/min was necessary to pull off the beads. In the third column, where the target and probe DNA were expected to hybridize a flow rate greater than 900 nl/min was required to pull the beads off. In order to minimize the false positive signals due to beads nonspecifically binding, one must operate within the flow rate window between 100 and 900 nl/min.

Fig. 5.

(A) Control experiment. Current across the pore being monitored as beads pass through the pore in a control experiment with no probe DNA immobilized on the surface. The downward spikes seen after $t = 10$ s are due to beads passing through the pore. (Inset) A closeup of the data between $t = 60$ s and $t = 70$ s. (B) Optical image of 20 μm beads passing through the pore.

Fig. 6. Current across the pore being monitored in a functionalized channel. The downward peaks correspond to beads passing through the channel. At time $t = 60$ s, the sharp drop corresponds to a bead getting capture, which is why the current does not rise up again instantaneously. At $t = 90$ s the bead becomes detached from the channel surface rising up again near it's original level.

Fig. 7. Image of a single bead situated in the pore. A single 20 μm bead trapped in this vicinity of the pore will cause the permanent current drop similar to that seen in Fig. 6. (B) Image of bead released and exiting the pore.