Selective DNA screening in microfluidic channels by electrophoresis through hydrogel matrices

Rebecca A. Zangmeister and Michael J. Tarlov

Process Measurements Division, Chemical Science and Technology Laboratory
National Institute of Standards and Technology, Gaithersburg, MD 20899

Abstract

Advances in microchip design, coupled with novel bioassays have led to advances in the field of genetic diagnostics. Microfluidic devices offer analytical advantages for samples of low concentration and small sample volume due to directed, efficient mass transport of analytes through microfluidic networks. Although significant advances have been made in genetic assays using micro-arrays, there exists a need for a high throughput, DNA detection system for use with microfluidic platforms. One such strategy is to incorporate a selective DNA screening element into a microfluidic device that takes advantage of the aforementioned attributes. We describe here a method for immobilizing single-strand DNA (ss-DNA) probe molecules in polyacrylamide hydrogels within plastic microfluidic channels. Fluorescent-tagged ss-DNA targets are electrophoretically driven through the hydrogel plugs, and hybridize with complementary ss-DNA probes covalently bound in the hydrogel. Here we present results that establish: the reproducibility of the hybridization assay; the effect of base pair mismatches within the target-probe duplex on the amount of target ss-DNA retained by the probe modified hydrogel matrix; and the efficiency of hybridization of target ss-DNA with complementary immobilized probe ss-DNA within the hydrogel plug.

Introduction

Numerous genetic diagnostic systems have been demonstrated in microfluidic chips including cell lysis, PCR amplification, and separation.[1-6] DNA detection elements based on hybridization have been integrated into microfluidic channels based on directed fluid flow,[5,7,8] in two dimensions using chemical immobilization of probe oligomers directly onto the microchannel walls,[9-11] and in three dimensions using probe DNA functionalized microbeads[12-14] and hydrogels.[15-17] We have previously reported the use of polyacrylamide hydrogels formed in microfluidic channels as a DNA hybridization assay platform.[16,17] We have demonstrated that hybridization assays can be performed in minutes or less by exploiting the directed, electrophoretic transport of DNA oligomers to the sensing matrix, an approach first reported using electroactive 2-D arrays.[18-20]

The hybridization assays are based on our ability to immobilize DNA probes in hydrogel matrices within microfluidic channels using a procedure developed by Rehman and coworkers.[16,18] The 5' end of the oligomer is modified with an acrylic acid group that can copolymerize with acrylamide and bis-acrylamide monomers, creating a hydrogel with immobilized probe ss-DNA. Spatial definition of the hydrogel plug is achieved by using a photoinitiator and a photomask. These spatially defined polyacrylamide hydrogel plugs, containing cross linked ss-DNA probes, have been successfully immobilized in poly(methylmethacrylate)/polycarbonate (PMMA/PC) microfluidic channels using photo-initiated polymerization.[16,21] The hydrogel plugs are surface grafted to the PMMA/PC

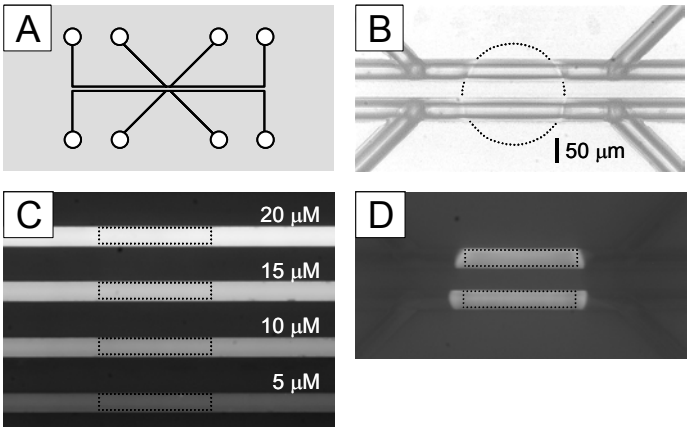

Figure 1: The microfluidic device used in these studies is shown in a line representation (A). The device is composed of two distinct microfluidic channels (top and bottom) that allow for comparative studies. The hydrogel plug is formed in the main channel between two rinse channels that come into the main channel at 45º. A magnified image of this region is shown in B where a dotted line is used to show where the aperture illuminated the main channels where the hydrogel plugs are formed. Standard solutions of target ss-DNA were placed in straight microchannels made of the same composition as the microfluidic channels used for hydrogel plug formation. Average fluorescence intensities of these standard solutions were measured prior to each hybridization assay in representative regions as shown by the dotted lines(C). The fluorescence intensities of the retained target hybrids were measured over the interior area of the hydrogel plug (D), and were compared to the fluorescence intensities of the standard solutions (C).

microchannel walls using a polymer adhesion layer applied prior to hydrogel plug formation.[21] The probe strands retain activity after immobilization, and are able to form duplexes with target strands as they electrophorese through the hydrogel plug. The advantages of such a system are the high probe density that can be achieved in the hydrogel plug, and the enhanced mass transfer inherent to the microfluidic system. These attributes allow for low concentration target scavenging.[16] Our ability to create independent hydrogel plugs within a single microfluidic channel, each containing a different probe sequence, have been shown to be effective for selective multi-target,[16] and screening applications.[17]

The microfluidic device used for these studies is shown in a line drawing (Figure 1A). A full description of the design of the microchannel, the polymerization of the hydrogel plugs, and the steps taken to execute a hybridization assay have been previously described.[16] Briefly, there are two mirror image microchannels on a single device (top and bottom) for comparative studies. The hydrogel plug is formed in the main microchannel between two side channels that intersect the main channel at 45º (Figure 1B). These side channels are used to rinse monomer solution from the channel post polymerization and to introduce sample to the hydrogel interface. The plugs are formed by photopolymerization of a solution containing polyacrylamide/ bisacrylamide (19:1) and ss-DNA modified at the 5' end with an acrylic acid group.[16,18] Saline buffer containing labeled or unlabeled target ss-DNA of 20 bases is loaded onto the device through the fluid access ports (Figure 1A, circles); fluorescently labeled target ss-DNA is electrophoresed through the hydrogel matrix and retained by immobilized probe ss-DNA within the hydrogel as observed using a fluorescence microscope.

We present here the results of a three part investigation to determine: the reproducibility of the hybridization assay; the effect of base pair mismatches within the target-probe duplex on the amount of target ss-DNA retained by the probe modified hydrogel matrix;

and the efficiency of hybridization of target ss-DNA with complementary immobilized probe ss-DNA within the hydrogel plug.

Experimental

Disclaimer. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Materials and Chemicals. The two component microchannel devices are made from polycarbonate (McMaster-Carr) and UV-transparent Acrylite OP-4 (poly(methylmethacrylate), Cyro Industries). An aqueous solution of acrylamide/bis-acrylamide (19:1, nominally 40 g/L), N, N, N', N'-tetraethylmethylenediamine (TEMED), riboflavin, sodium hydroxide, sodium chloride, 10× TE buffer (pH 7.4, 100 mmol/L Tris-HCl, 10 mmol/L EDTA, and Fluoresbrite Polychromatic Red 1.0 μm beads are used as received. The single-strand DNA probe sequence (Table 1) is purchased from a commercial vendor with the Acrydite™ functionality on the 5' end of the sequence. Target sequences (listed in Table 1) are purchased from a commercial vendor with either fluorescein or rhodamine derivative functionalities on the 5' end for fluorescence detection. The estimated values of ΔG for each duplex formation are calculated using the module found online at http://ozone2.chem.wayne.edu/Hyther/hytherm1main.html using 0.5 mol/L NaCl buffer and at a temperature of 25 °C. DNA solutions are made to a final concentration of 10 μmol/L, unless otherwise indicated, using 18.2 MΩ•cm deionized water.

Microchannel Fabrication. A 248 nm excimer laser system is used to ablate ca. 50 μm wide and ca. 95 μm deep microchannels in polycarbonate, as described previously.[16] The polycarbonate microchannel chip is thermally fused at 103 °C for 30 min with an Acrylite OP-4 lid containing ca. 2.5 mm diameter holes that provided fluid access to the microchannels.[16] The Acrylite OP-4 PMMA was specifically chosen because it is transparent down to ca. 300 nm, sufficient to effect UV photopolymerization of the acrylamide precursors.

Microchannel Hydrogel Polymerization. Prior to hydrogel polymerization, the microchannel devices are surface modified with a polymer adhesion layer.[21] The

Table1. Primer sequences used to study hybridization in the hydrogel plug matrix (underlines indicate mismatches between the probe and target sequences).

	Sequence		
Probe:	5'-AGGCCCGGGAACGTATTCAC-3'		$\Delta G^{\dagger}$ (kJ mol^{-1})
Target:	T1	5'-GTGAATACGTTCCCGGGCCT-3'	-133.6
	T2	5'-GTGAATAGCATCCCGGGCCT-3'	-84.9
	T3	5'-GTGAATAGCAAGCCGGGCCT-3'	-71.6
	T4	5'-GATGGTACATGACAAGGTGC-3'	6.7

$^{\ddagger}\Delta G$ of formation calculated using: http://ozone2.chem.wayne.edu/Hyther/hytherm1main.htm

microchannels are then rinsed with 18MΩ-cm water to remove unreacted silane prior to introduction of the monomeric polymerization solution. The polymerization solution is made by diluting a commercially available aqueous solution of acrylamide/bis-acrylamide (19:1, nominally 40 g/L) 4 times into a solution containing 15 μmol/L riboflavin, 15 μmol/L acrylamide-modified oligomer, 10 mmol/L TEMED, and 0.001 g/L Fluoresbrite beads in 1× TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4). Equal volumes of this solution are placed into each of the fluid reservoirs. Fluoresbrite beads (λ_{ex} = 591, λ_{ex} = 657) are used as visualization markers to minimize fluid flow during photopolymerization due to unequal volumes in the fluid reservoirs. An aperture, located within the microscope, is used to define the illumination area for polymerization. Hydrogel plugs ca. 300 μm in length are polymerized in the microchannel by exposure to 340 nm to 380 nm light for 5 min. Post polymerization, the monomer solution is rinsed through the open channels on either side of the hydrogel plugs using pressure driven flow. Excess monomer species are removed by electrophoresis using a neutral buffer solution containing 0.5 mol/L NaCl and 1× TE. All microfluidic chips are filled with neutral buffer and refrigerated (40 °C) when not in use.

Hybridization Assay. Most hybridization assays are performed on microfluidic chips immediately following photopolymerization of the immobilized probe containing hydrogel; refrigerated microfluidic chips are allowed to come to room temperature before use. A detailed description of the hybridization process has been previously published.[16] A brief description is given here. The neutral buffer solution is removed from one side of the microchannel, and replaced with a hybridization solution containing the target ss-DNA using pressure. Platinum wire electrodes are placed in fluid access wells at either end of the microchannels and connected to a ± 40 V power supply. The magnitude of the current flowing through the microchannel is determined by measuring the voltage drop across a 100 kΩ resistor connected in series to the power supply and the microchannel. Typical currents measured at 25 V varied from 10 μA to 20 μA. The voltage (25 V) is applied such that the negatively charged ss-DNA targets would electrophorese through the hydrogel plug.

Image Acquisition. Imaging of the microchannels is performed using a standard fluorescence microscope equipped with a 100× objective, mercury arc lamp, and appropriate filters. Frame grabber software connected to a black and white CCD camera is used to capture fluorescence images.

Results and discussion

Hybridization and fluorescence intensities. Based on previously published work, it is known that complementary target ss-DNA will hybridize with immobilized probe ss-DNA as it is electrophoresed through a hydrogel plug matrix formed in a microfluidic channel.[16] This hybridization event is signaled by measured fluorescence within the hydrogel plug due to retained target ss-DNA molecules that have been fluorescently tagged. The study presented here investigates whether the magnitude of the fluorescence intensity reflects the retention of target ss-DNA based on either complementarity of a specific target-probe duplex, or based on the amount of probe ss-DNA immobilized in the hydrogel plug.

A microfluidic device composed of four discrete microchannels of the same size and composition as the microchannels used for the hydrogel plug device is used for fluorescence

calibration. These microchannels were filled with standard solutions of target ss-DNA molecules ranging in concentration from 5 μmol/L to 20 μmol/L. The average fluorescence intensities of these standard solutions is measured over a representative area as shown in Figure 1C (dotted lines). These fluorescence intensities are used to create a calibration curve for quantitation of retained target ss-DNA captured in the hydrogel plug by hybridization with immobilized probe ss-DNA. Fluorescence intensities of standard solutions were taken in conjunction with all experimental runs to eliminate error associated with lamp intensity fluctuations.

Replicate hybridization assays. Replicate hybridization assays are possible because the probe ss-DNA is covalently bound as a copolymer in the hydrogel. The hydrogel system can be reused multiple times by electrophoresing with a denaturing buffer (0.5 mol/L NaOH and 0.5 mol/L NaCl) to remove hybridized target sequences from the system. Following denaturation, the pH of the system is adjusted to neutral by electrophoresing with buffer (0.5 mol/L NaCl and 1× TE, pH = 7.4). At this point, the microchannel device is loaded with new target solution for the next assay. Three successive hybridization assays of complementary 20mer target sequence are shown in Figure 2A. The average fluorescence intensity is monitored over a homogeneous area of the hydrogel plug (Figure 1D, dotted lines) during the three stages of the assay.

The first stage, filling, is achieved by electrophoresing the target ss-DNA through the hydrogel plug. The fluorescence intensity measured within the hydrogel plug increases during this process, and levels off at a maximum level (Figure 2A, squares). The next stage, a stringency rinse, removes excess target from the hydrogel plug by electrophoresing with buffer solution containing no DNA. The fluorescence intensity measured within the hydrogel plug decreases as excess target electrophoreses out of the hydrogel (Figure 2A, circles), and plateaus when only hybridized target remains in the hydrogel plug. To regenerate the sensing matrix, a denaturing buffer is used for electrophoresis, and the hybridized target strands electrophorese out of the hydrogel plug, (Figure 2A, triangles), whereby the fluorescence intensity returns to baseline. The hydrogel matrix is conditioned with neutral hybridization buffer before a new target sample is introduced. Each complete hybridization assay run (fill, rinse, denature) requires approximately 15 min for completion.

It was observed that successive runs of a single target species in a single hydrogel sensing matrix gave reproducible fluorescence intensities with ± 5 % relative error. Replicate

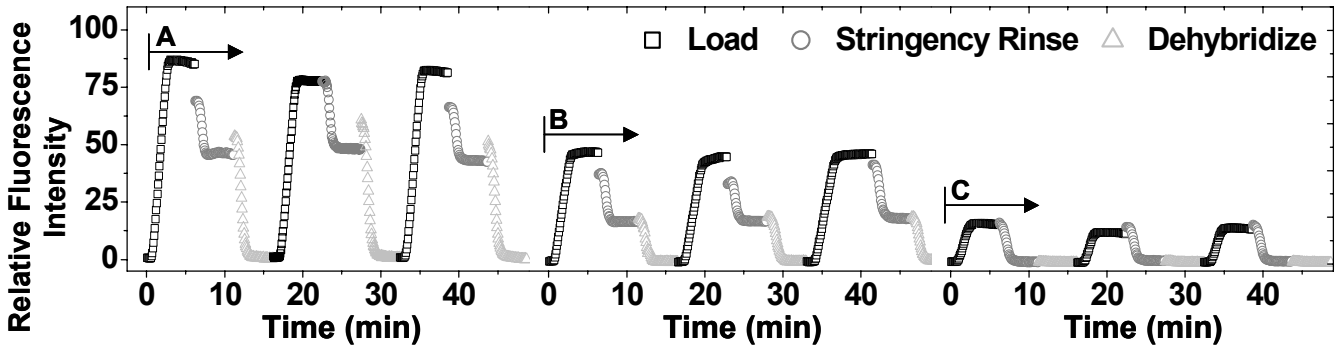

Figure 2: Replicate hybridization assays monitored by fluorescence intensity over time of target ss-DNA molecules with varying degrees of complimentarity to the immobilized probe sequence. (A) 100 % complementary; (B) 85 % complementary; and (C) 40 % complementary.

hybridization assays were used to investigate whether the magnitude of this fluorescence intensity would reflect the retention of target ss-DNA based on either complementarity of a specific target-probe duplex, or on the amount of immobilized probe ss-DNA present in the hydrogel plug.

Effects of base pair mismatch. The effect of base pair mismatches on measured fluorescence intensity was examined by performing hybridization assays with a 20-mer target containing 0, 3, 5, or 12 base pair mismatches (see Table 1, T1 – T4). Because the immobilized probe ss-DNA is in an aqueous-like environment within the hydrogel, the amount of target retained in the hydrogel plug should correlate with the thermodynamic stability of each duplex. Replicate measurements of T1, T2, and T4 are shown in Figure 2A, 2B, and 2C, respectively. The fluorescence intensity measured after the stringency rinses were consistent within each target ss-DNA group. The fluorescence intensities of T1 – T4, measured in the plateau region (open circles) were plotted versus a calculated ΔG of hybrid formation,[22] shown in Figure 3. There is a distinct downward trend in fluorescence intensities as the ΔG of hybrid formation becomes less favorable. Therefore, the fluorescence intensities measured within the hydrogel matrix can be used as a measure of the relative complementarity of a probe-target duplex within the types of short sequences and variations in sequence presented here.

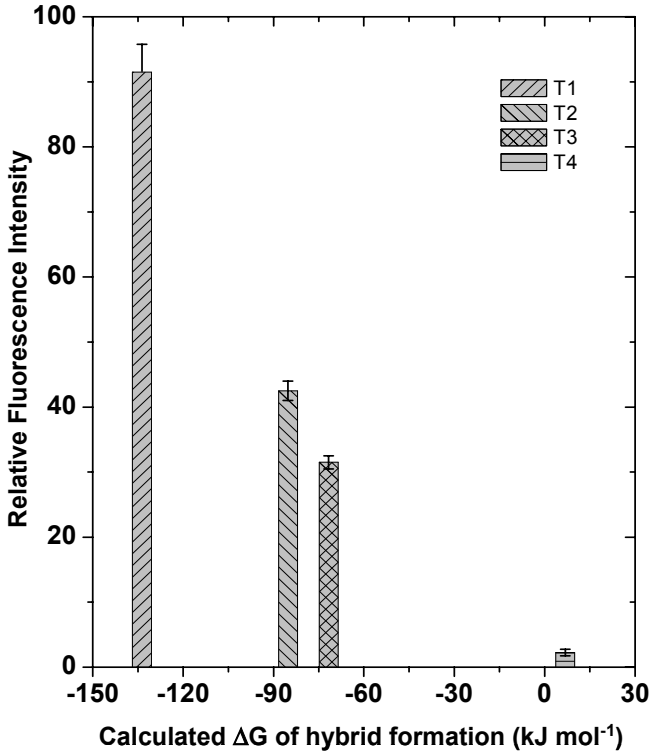

Figure 3: Relative fluorescence intensity of retained target ss-DNA in the hydrogel plug plotted versus a calculated ΔG of hybrid formation for each duplex pair. Error bars represent one standard deviation of repeat measurements.

Hybridization Efficiency. The hybridization efficiency of the complementary probe-target (T1) duplex was examined over a range of immobilized probe concentrations (5 μmol/L, 10 μmol/L, 15 μmol/L, and 20 μmol/L). Monomer solutions, containing 5 μmol/L, 10 μmol/L, 15 μmol/L, and 20 μmol/L of probe ss-DNA were used to create hydrogel plugs in four different hydrogel plug devices. It was assumed that the concentration of probe ss-DNA in the monomer solution would remain fairly constant during the polymerization step, and that the concentration of probe in the hydrogel would equal the concentration of probe in the monomer solution. Direct quantitation of the amount of immobilized probe within the hydrogel plug is complicated by the small amount of oligomers present in the hydrogel (ca. 200 picomoles). Attempts at using standard intercalator dyes within the hydrogels to quantitate both the amount of immobilized probe and the amount of hybridized target were unsuccessful because of non-specific binding of intercalator dyes to the hydrogel plugs.

Complementary target ss-DNA is electrophoresed into each of these distinct hydrogel plug devices. The fluorescence intensities of retained target are measured and converted to target concentration by comparison to fluorescence intensities measured for standard solutions (see Figure 1C). The results of this study, average of four measurements, are presented in Figure 4. Ideally, one would expect that the concentration of retained target ss-DNA should equal the concentration of immobilized probe ss-DNA for this complementary case (dashed line). The data do not deviate far from this ideal case. At lower concentrations, the amount of target is comparable to the amount of immobilized probe; at higher concentrations the amount of target deviates from the ideal case, and falls off at the highest concentration (20 μmol/L). A possible explanation for this observation is that at lower concentrations (up to 15 μmol/L) the immobilized probe ss-DNA is able to hybridize with target ss-DNA up to a maximum capacity. At the highest concentration, the ss-DNA may be sterically hindered, and not able to hybridize up to a maximum capacity, or the amount of probe incorporated in the hydrogel may limit at the higher concentrations.

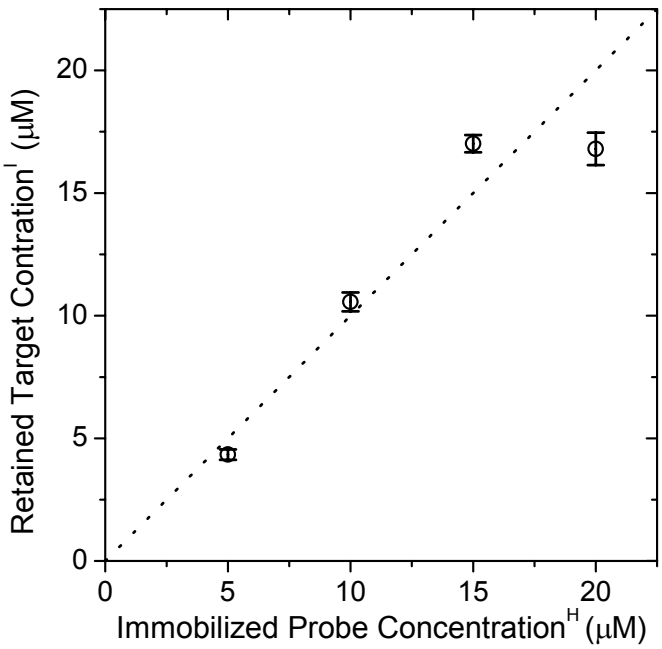

Figure 4: Plot of the concentration of retained target ss-DNA versus concentration of probe ss-DNA immobilized in the hydrogel plug. Dashed line represents ideal case. Error bars represent one standard deviation of repeat measurements.

† Immobilized probe concentration is defined as the concentration of probe ss-DNA in the monomer solution prior to hydrogel plug formation.

‡ Retained target concentration is calculated from measured fluorescence intensities in the hydrogel plug compared to external fluorescence calibration standards (Figure 1C).

Conclusion

We presented the results of a three part investigation focused on: the reproducibility of the hybridization assay; the effect of base pair mismatches within the target-probe duplex on the amount of target ss-DNA retained by the probe modified hydrogel matrix; and the efficiency of hybridization of target ss-DNA with complementary immobilized probe ss-DNA within the hydrogel plug.

Replicate measurements of hybridization assays revealed that successive runs of a single target species in a single hydrogel sensing matrix gave reproducible fluorescence intensities with ± 5 % relative error. Replicate measurements of hybridization assays where base pair mismatches were introduced revealed that the fluorescence intensities measured within the hydrogel matrix can be used as a measure of the relative complementarity of a probe-target duplex within the types of short sequences and variations in sequence presented here.

Quantitation of retained target ss-DNA in a hydrogel plug containing complementary immobilized probe of concentrations varying from 5 μmol/L to 20 μmol/L was used to estimate the efficiency of hybridization. At lower concentrations, the amount of target is comparable to the amount of immobilized probe; at higher concentrations the amount of target deviates from the ideal case, and falls off at the highest concentration (20 μmol/L). Therefore, at lower concentrations, up to 15 μmol/L, the efficiency of hybridization of a complementary 20mer duplex pair within the hydrogel plug environment appears closer to ideal.

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22. The estimated values of ΔG for each duplex formation were calculated using the module found online at http://ozone2.chem.wayne.edu/Hyther/hytherm1main.html using 0.5 M NaCl, 25^{o}C and the Fotin et al. correction factor.