Microfluidic platform for isolating nucleic acid targets using sequence specific hybridization

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The separation of target nucleic acid sequences from biological samples has emerged as a significant process in today's diagnostics and detection strategies. In addition to the possible clinical applications, the fundamental understanding of target and sequence specific hybridization on surface modified magnetic beads is of high value. In this paper, we describe a novel microfluidic platform that utilizes a mobile magnetic field in static microfluidic channels, where single stranded DNA (ssDNA) molecules are isolated via nucleic acid hybridization. We first established efficient isolation of biotinylated capture probe (BP) using streptavidin-coated magnetic beads. Subsequently, we investigated the hybridization of target ssDNA with BP bound to beads and explained these hybridization kinetics using a dual-species kinetic model. The number of hybridized target ssDNA molecules was determined to be about 6.5 times less than that of BP on the bead surface, due to steric hindrance effects. The hybridization of target ssDNA with non-complementary BP bound to bead was also examined, and non-specific hybridization was found to be insignificant. Finally, we demonstrated highly efficient capture and isolation of target ssDNA in the presence of non-target ssDNA, where as low as 1% target ssDNA can be detected from mixture. The microfluidic method described in this paper is significantly relevant and is broadly applicable, especially towards point-of-care biological diagnostic platforms that require binding and separation of known target biomolecules, such as RNA, ssDNA, or protein. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4816943>]

INTRODUCTION

The unique specificity of molecular binding between target and probe molecules is the cornerstone of many diagnostic assays, including antibody-antigen coupling^{1,2} and complementary nucleic acid (DNA/RNA) hybridization.^{3,4} In those assays, target is mixed with probe to form a target-to-probe complex. This bound complex is then separated from the free (unbound) probe and non-targets for subsequent detection or amplification. The separation step is the most essential operation to guarantee the sensitivity and accuracy of detection. Microscale magnetic beads are widely employed to capture target molecules. Beads provide a high surface-to-volume ratio, a high density of binding sites, and reduced diffusion time. In traditional assays, the magnetic beads with the target molecules attached were concentrated and immobilized by applying a magnetic field, thus, the target molecules can be separated from other compounds in solution. With the extensive use of magnetic bead in diagnostic research, some specific bead-binding science has been studied and understood for DNA hybridization. Bead size,⁵ hybridization temperature and time,⁵ target sequence length,⁶ and location^{7,8} have all been studied. While these studies help improve the design of studies involving magnetic bead based separation, no unified knowledge has been established due to variable working conditions. Among those traditional platforms, most strategies rely on batch processes, requiring multiple pipetting, tube washing and elution steps that progressively result in signal loss.⁹ New separation platforms, capable of separating minute amounts of target molecules in the presence of large amounts of accompanying compounds from dilute solutions, are needed for the next level of disease detection.

Microfluidic technology provides a rapid and integral platform for sensitive and selective molecular diagnostics that overcome the shortcomings from using bench tubes. Successful assays have been demonstrated for the detection of biomarkers,^{10,11} by transporting magnetic beads with targets in microfluidic devices. Micro-mixers and vacuum pumps are usually required in these assays to promote target hybridization and flush away unbound materials, and multiple thermal conditions are occasionally used for target amplification.

Recently, our lab has developed a microfluidic separation platform that does not require external apparatus and achieves separation by simply moving a magnet to transport beads in static fluids instead of using fluid flow.¹² Similarly, Bordelon *et al.*¹³ developed a RNA extraction cassette that utilized external magnet to transfer the nucleic acid biomarker within 1.6 mm diameter tubing. This flexible approach had also been studied by den Dulk *et al.*¹⁴ to enrich biological target recovery in micro-valves. The idea of using stationary microfluidics is new and believed to bring extensive freedom for designing fully integrated point-of-care systems, but fundamental bead-based capture and separation behavior have not been explored or established for the system. In this paper, we describe our simple microfluidic platform, investigating and understanding bead-based isolation of single stranded DNA (ssDNA) by nucleic acid hybridization.

In this work, we performed separation experiments involving the movement of magnetic beads in a new microfluidic system for sensitive capture and isolation of sequence specific target ssDNA. We investigated the isolation ability of target ssDNA through binding to biotinylated capture probe (BP) on magnetic bead surface (Figure 1). We demonstrated superior separation efficiency of target ssDNA when compared to conventional tube washing. We quantified the magnetic bead capacity for capturing target ssDNA and analyzed data on both specific and non-specific hybridizations. A dual-species model was established to explain the hybridization mechanism. Finally, we showed highly efficient isolation and specific detection of target ssDNA in the presence of non-target ssDNA.

FIG. 1. Microchip technique. (a) Illustration of magnetic bead capture complex of target ssDNA hybridized with BP on bead surface. (b) Illustration of thermodynamically simulated hybridization structures of target ssDNA and BP at hybridization condition. BP is highlighted in cyan shadow. (c) Illustration of target ssDNA separation by capture complex movement from reservoir W1 to W2 through a microchannel. (d) Design and photograph of a microfluidic chip. Reservoirs and channels are filled with dyes to enhance contrast. Channels are 3 cm long and 1 cm apart. Channels have tapered openings of 400 μm wide at W1 and end with 50 μm wide opening at W2. Depth of the channels is 120 μm .

MICROCHIP TECHNIQUE

The basic principle of our approach is very simple: Magnetic beads bearing BP are mixed with a sample containing target ssDNA in the microfluidic well 1 (W1). After an incubation period, allowing target ssDNA to bind to BP on magnetic bead surface (Figures 1(a) and 1(b)), the entire magnetic bead-capture complexes are moved by an external magnet underneath the microchannel (Figure 1(c)). The capture complexes travel through a microchannel containing an appropriate buffer towards the reservoir well 2 (W2). This process effectively separates unbound molecules from the capture complex. The capture complexes are finally deposited in W2 before being removed for further detection, if necessary. It is important to discuss the engineering aspect of this separation method. First, the microfluidic channel has to be designed for 100% bead transfer between two wells. Second, the magnet strength has to be optimized to move beads in suspended state rather than clumped or staggered states.

Our microfluidic approach is unique as: (a) the rapid motion of bead transfer through microfluidic channel provides minimal carry-over of non-target molecules ($0.5 \pm 0.3\%$ of fluid volume³⁴); (b) No flow is required for target molecules to hybridize with the capture complex, as the capture/binding of targets occurs in the reservoir (W1) with tunable size. Therefore, our method is capable of processing larger amounts of low concentration clinical samples, as well as increase sensitivity for rapid separation; (c) high efficiency separation is achieved without using any membrane or pumps, which is quite useful in resource limited settings; (d) a counter flow of clean buffer from W2 to W1 can be created by a simple fluid volume differential thus further reduce the carry over or diffusion of non-target molecules; (e) multiple microfluidic separations can be performed as needed by simply fabricating an additional microchannel from W2 to a new well 3 (W3), W3 to a new well 4 (W4), and so on, making this assay customizable for a broader scope of applications.

MATERIALS AND METHODS

Microfluidic chip preparation

The polydimethylsiloxane (PDMS) microchips were fabricated using soft lithography following a method previously reported.¹² Sylgard 184 elastomer base mixed with curing agent (Dow Corning, Midland, MI) was poured over a master mold (fabricated SU-8 structure on a silicon wafer) and incubated at 70 °C to polymerize. The solid PDMS was cut out, punched with holes to generate wells, then cleaned and bonded to a Gold Seal® glass slide (1.2 mm thick) with oxygen plasma (PDC-32G, Harrick Plasma Ithaca, NY). The chip was baked at 70 °C for at least 1 h right after bonding to enhance the irreversible bond between plasma treated PDMS and the glass. As shown in Figure 1(d), each microchip can simultaneously process four samples in parallel. The PDMS chip was cleaned with RNase decontamination solution and incubated overnight with priming buffer (0.02% Tween-20 and 1 mg/ml bovine serum albumin (BSA)) before use.

Design of oligonucleotide sequences

Target ssDNA was designed based on sequences obtained through Los Alamos HIV sequence database and synthesized by Integrated DNA technologies (IDT, IA). 6-FAMTM, a single isomer derivative of fluorescein (IDT, IA) was attached to the 5'-end. BP was designed to have a total length of 26 nt with or without 6-FAM attachment. BP sequence is completely complementary to the 26 nt region (bold and underlined in Table I) near 3'-end of the target ssDNA (Figure 1(b)). BP-2 was designed to be non-complementary to target ssDNA while it has a same length to BP. The amplifiable probe was designed consisting of three parts from the 5' end to 3' end: a 25 nt segment duplicating the sequence of primer 2, a 19 nt segment (written in italic letters in Table I) that is reverse complement to the target ssDNA but excluded the region that hybridizes for BP, and a 25 nt segment that is reverse complementary to the primer 1. It is important to note that primer 1 contains a T7 promoter sequence (20 nt, lowercase in Table I) at its 5' end. The molecular beacon was designed to be a hairpin structure containing a

TABLE I. Oligonucleotide sequences used in this study.

Oligo	Sequence	Length (nt)
Capture		
Biotinylated capture probe	5'-BioTEG/CTTAATATTGACGCTCTCGCACCCAT-3'	26
	5'-BioTEG/56-FAM/CTTAATATTGACGCTCTCGCACCCAT-3'	
Target ssDNA	5'-/56-FAM/aaaa CAGGGTTAAAGAA AACAAATCGGT AACAG aaaaaaaaaa <u>ATGGGGTG</u> <u>CGAGAGCGTCAATATTAAG</u> aaaa-3'	74
Biotinylated capture probe-2	5'-BioTEG/AATTTTGATGCCTGAAACCGTACCA-3'	25
NASBA		
Amplifiable probe	5'-TCAAGAGTAGACAC CTGTTACCGAT TTGTTCTTCTTAACCTG GTAATCAGATCAGAGCAATAGGTCA-3'	69
Primer 1	5'-taatacgactcactataggg TGACCTATTGCTCTGATCTGATTAC-3'	45
Primer 2	5'-TCAAGAGTAGACAC CTGTTACCGAT-3'	25
Beacon	5'-/6-FAM/cgcg TCAAGAGTAGACACCTGTTACCGAT cgcg /3IABlk_FQ/-3'	33

stem and a loop sequence identical to the middle region of the amplifiable probe, so as to target the RNA transcribed from amplifiable probe. All oligonucleotide names, sequence compositions, and lengths are listed in Table I.

Capture and isolation of target ssDNA

BP and 1 μg of streptavidin-coated magnetic beads (Dynabead[®] M-280, Invitrogen[™]) were mixed in hybridization buffer (composed of 20 mM Tris pH 8.0, 20 mM MgCl_2 , 150 mM NaCl, 0.02% Tween-20, and 1 mg/ml BSA) and incubated for 30 min at room temperature before adding target ssDNA. The mixture was then heated to 40 °C for 2 min and cooled to 4 °C for 5 min. The mixture was then incubated at room temperature for 15 min to allow adequate hybridization. The total mixture volume of 50 μl was transferred into W1 with microchannel pre-filled with hybridization buffer. Beads were collected at the bottom of the well using cubic neodymium magnets (N42, K&J Magnetics, Inc. Jamison, PA), and pulled into W2 along the channel at an approximate speed of 0.7 mm/s (Figure 1(d)). The channel was kept horizontal or slightly tilted towards W1 throughout the whole process to eliminate unwanted flow-driven transport. The number of beads were chosen after an optimization process, ensured by microscopic observation >95% bead transfer efficiency from W1 to W2 without any jamming in the microfluidic channel.

Isolation experiments were also performed using conventional manual tube washes. Instead of transferring mixture volume to W1, three washes were conducted and the washed solutions were collected for further measurement.

Quantification of bead captured oligonucleotide concentration

The ssDNA concentration was determined by analyzing the measured fluorescence (Photon Technology International) from tagged 6-FAM. First for every experiment, a standard curve of measured fluorescence versus oligonucleotide concentration was plotted with linear fit, where the fluorescence of the hybridization buffer was subtracted as the background noise. After pulling beads from W1 to W2, the remaining solution in W1 was collected and measured for fluorescence. The concentration of the leftover/unbound solution was subsequently determined by superimposing the fluorescence measurement onto the standard curve of fluorescence vs. concentration and interpolating the concentration value. Finally, the amount of ssDNA captured/hybridized by beads was calculated by subtracting the leftover/unbound

concentration from the starting concentration in W1. For off-chip wash measurements, fluorescence of collected washed solutions was measured, and the final concentration was normalized with diluted volumes.

Amplification and detection of target ssDNA

We employed a well-known amplification method: nucleic acid sequence based amplification (NASBA),¹⁵ which occurs isothermally at 41 °C using three enzymes: T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase (AMV-RT), and RNaseH. The reagents used for NASBA and their concentrations at the final reaction volume were 40 mmol/l Tris (pH 8.0), 12.5 mmol/l MgCl₂, 73.5 mmol/l CH₃COOK, 5.25 mmol/l dithiothreitol, 1.05 mmol/l dNTP, 2.1 mmol/l rNTP, 0.21 mmol/l each primer, 52.5 nmol/l molecular beacon, 15.75% dimethyl sulfoxide, 1.68 U/μl T7 RNA polymerase, 0.34 U/μl AMV-RT, 0.005 U/μl RNase-H, and 0.11 mg/ml bovine serum albumin. Enzymes and reagents were purchased from Promega (Madison, WI).

RESULTS AND DISCUSSION

Theoretical estimates of bead binding capacity for BP

We carefully performed calculations of the surface binding capacity of streptavidin-coated bead for BP. Since a streptavidin molecule has a diameter of 4 nm,¹⁶ suppose full coverage of streptavidin molecules on beads, the total number of streptavidin molecules coated on one 2800 nm diameter bead can be estimated as the surface area of the bead divided by the cross-sectional area of the spherical streptavidin molecule: $4\pi(1400\text{ nm})^2/\pi(2\text{ nm})^2 = 1.96 \times 10^6$. Note that a stricter calculation can be done by assuming hexagonal packing of spheres on a flat surface, where the area occupied by each streptavidin molecule is $8\sqrt{3}\text{ nm}^2$. This leads to $4\pi(1400\text{ nm})^2/8\sqrt{3}\text{ nm}^2 = 1.78 \times 10^6$ streptavidin molecules occupy a single bead, which is within 10% of the previous estimation. Since a streptavidin molecule contains four binding sites for biotin, the single bead binding capacity for BP may be between 1.96×10^6 and 7.84×10^6 . Thus, 1 μg of bead consisting of 6.5×10^4 individual bead can capture at least $(1.96 \times 10^6) \times (6.5 \times 10^4) = 1.27 \times 10^{11}$ biotinylated molecules or $1.27 \times 10^{11}/(50 \times 10^{-6})/6.02 \times 10^{23} = 4.2 \times 10^{-9}\text{ M}$ of biotinylated molecules in 50 μl total volume. Hence, 1 μg bead would theoretically capture 4.2 to 16.8 nM of BP in the same volume. To validate these calculations, we performed bead capture experiments using our microchip technique.

Experimental examination of BP binding to bead

Using the method detailed above, we measured the surface binding capacity of streptavidin-coated beads for BP. Figure 2(a) shows a plot of measured fluorescence for BP remaining in W1 at different starting concentrations C_{BP}^0 . For standard curve with no bead in W1, the fluorescence measurements resulted in an expected linear relation between fluorescence and C_{BP}^0 (R-square = 0.993). Clearly, for a fixed amount of 1 μg bead transferred, the measured amount of leftover BP increased with the initial loading concentration. Below a critical concentration of about 4 nM, there was no signal detected in W1 after the bead separation. Hence, the bead capacity for BP was found to be about 4 nM. Theoretically, 1 μg bead was decorated with 4.2 nM of streptavidin molecules in 50 μl solution; our data suggest that the BP binding is restricted to roughly one per streptavidin molecule.

This was quite a surprising result at first, given that each streptavidin molecule contains four binding sites for biotin. However, in our model, we assume that streptavidin molecules are tightly packed on bead surface, this leads to the number of streptavidin molecules estimated to be 1 780 000 by space occupation, which is close to the maximal number of BP (1 750 000) bound to beads from experimental result. It is likely that streptavidin molecules are not engineered to tightly packed on beads, thus, with less streptavidin to capture same amount of BP, multiple binding sites may be occupied.¹⁷ Although other studies^{5,6,18–21} were performed using similar bead-based streptavidin-biotin complexes, they did not report or quantify this finding.

FIG. 2. Bead exposed with BP. (a) Fluorescence measurements in reservoir W1 before and after beads were removed. (b) Concentration of BP adsorbed by the bead shown as a saturation curve. Y axis: number of BP per unit area (μm^2) on bead surface.

We further evaluated the surface concentration of streptavidin bound BP Γ_{SBP} on bead (radius $R_b = 1.4 \mu\text{m}$, numbers $N_b = 65\,000$) by subtracting the final concentration of BP C_{BP}^f from the initial concentration C_{BP}^0 in W1

$$\Gamma_{SBP} = 6.02 \times 10^{23} \times (C_{BP}^0 - C_{BP}^f) \times V / 4\pi R_b^2 N_b. \quad (1)$$

Here, Γ_{SBP} value was obtained in the unit of number of molecules per μm^2 . Figure 2(b) shows a plot between measured Γ_{SBP} for various loading concentrations of BP. We observed that the binding of capture probe onto streptavidin-coated beads followed similar characteristics using both microfluidic channel separation (in blue) and conventional tube washing (in red). The higher apparent bead surface concentration Γ_{SBP} for capture probe in the tube wash assay perhaps resulted from probe loss during manual washes. This establishes that microfluidic approach results in highly efficient isolation of ssDNA. The data further suggest that the binding of BP to streptavidin (S) follows the Langmuir isotherm like behavior⁷ which can be described by the binding reaction

At steady state, the number of adsorbed BP molecules Γ_{SBP} on the streptavidin coated magnetic bead surface can be calculated as

$$\Gamma_{SBP} = \frac{\Gamma_{\infty} \times C_{BP}^0}{C_{BP}^0 + k}. \quad (3)$$

Equation (3) can also be rewritten as

$$\frac{1}{\Gamma_{SBP}} = \frac{1}{\Gamma_{\infty}} + \frac{k}{\Gamma_{\infty} \times C_{BP}^0}, \quad (4)$$

Γ_{∞} is the maximum number of adsorbed BP on streptavidin coated magnetic bead surface. Note that bead surface is blocked with BSA during production; non-specific binding of probe to bead is negligible even if at slightly lower streptavidin surface coverage. By fitting $1/\Gamma_{SBP}$ linearly with $1/C_{BP}^0$ (as shown in Figure 2(b) inset, which yields $1/\Gamma_{SBP} = 8.71 \times 10^{-6} + 1.92 \times 10^{-5}/C_{BP}^0$, R-Square = 0.997), we obtained Γ_{∞} and k as $114\,688/\mu\text{m}^2$ (6.1 nM based on 50 μl total volume) and 0.22/nM, respectively. Given 1 μg bead, our experimental measurements (Figure 2(b) in blue) agree well with the model prediction (Figure 2(b) in green). The maximum capture of biotinylated probe was predicted to be $(114\,688/\mu\text{m}^2) \times 4\pi(1.4\,\mu\text{m})^2 \times 65\,000/(6.02 \times 10^{23}/\text{mol}) = 0.31\text{pmol}$. According to Invitrogen, 1mg of Streptavidin Dynabead M-280 typically binds approximately 20pmol ss-oligonucleotide, which is 0.02pmol for 1 μg bead used. Our data suggest that biotinylation improves ss-oligonucleotide binding by 15-fold.

Examination of target ssDNA isolation using bead-streptavidin-BP complex

Figure 3 shows a plot of the concentration of hybridized target ssDNA on bead surface Γ_{ssDNA} versus initial concentration of target ssDNA C_{ssDNA}^0 in the bulk. Bead-streptavidin-BP complexes were produced by saturating bead with BP before adding target ssDNA. At low concentrations of target ssDNA $C_{ssDNA}^0 < 2.5\text{ nM}$, only a few molecules of target ssDNA hybridized to bead. At concentrations of $C_{ssDNA}^0 > 2.5\text{ nM}$, the hybridization value increased rapidly and reached a saturated level when $C_{ssDNA}^0 \sim 10\text{ nM}$. At this concentration, a total of $\Gamma_{ssDNA}A N_b = 0.026\text{ pmol}$ (where A = surface area of single bead) of target ssDNA molecules successfully hybridized. This is smaller by a factor of 6.5 than the amount of BP $\Gamma_{bp}A N_b = 0.17\text{ pmol}$ captured on bead surface, which demonstrates a 6.5:1 ratio of hybridization between BP and target ssDNA.

We explored the role of steric effects to explain this hybridization relationship and evaluated hybridization mechanism with a dual-species kinetic model. We note that the radius of gyration of DNA is a dynamic property that changes based on local environment and solution

FIG. 3. Number of target ssDNA on bead with specific hybridization via BP (experimental data in red, model simulation in green) and non-specific adsorption to BP-2 (experimental data in magenta, model simulation in blue). Concentration of target ssDNA bond to bead is shown as number of target ssDNA per unit area (μm^2) on bead surface.

conditions, such that it is merely an estimation of the space occupied by DNA in its minimal free energy state.²² To keep the model simple, we evaluated the radius of gyration in free solution rather than being bound to a surface in an attempt to address the spatial accessibility of target ssDNA that could occupy the beads surface. The wormlike chain model gives an approximate radius of gyration²³

$$R_g = \sqrt{\frac{2a(L - a + ae^{-L/a})}{6}}. \quad (5)$$

Here, L is the contour length of target ssDNA $L = 0.43 \text{ nm} \times 74 = 31.82 \text{ nm}$ given unstretched ssDNA single base size to be 0.43 nm .²⁴ $a = 2.5 \text{ nm}$ ²⁵ is the persistence length of target ssDNA with the salt condition of 150 mM in our experiments. Using Eq. (5), we calculated $R_g = 5 \text{ nm}$. This is also in good agreement with experimental measurement recently reported.²⁶

Hence the total number of target ssDNA that can possibly occupy the bead surface is $N_{ssDNA} = A/\pi R_g^2 = 313\,600$. As evaluated earlier, the number of BP on bead surface is $N_{bp} = \Gamma_{\infty} A = 1\,750\,000$. Therefore, the BP to ssDNA ratio becomes $\frac{N_{BP}}{N_{ssDNA}} = \frac{1\,750\,000}{313\,600} = 5.6$. This is in remarkably good agreement with our experimentally determined ratio of 6.5:1. Note that since BP has high coverage on beads surface (the area per BP, Σ is about 9.1 nm^2), brush forms when $R_g^2 > \Sigma$. The possible polymer-polymer interactions will decrease the number of BP available to hybridize to target ssDNA. Nevertheless, we speculate weak polymer-polymer interactions, with $\Sigma > R_g^2$ (gyration of BP is estimated to be $R_g = 2.7 \text{ nm}$ using Eq. (5)).²⁷

With the understanding of restriction of target ssDNA hybridization on bead surfaces, a dual-species hybridization model was built to explain the sigmoidal shape of experimental data. We assume the hybridization of the target ssDNA as follows:

Here, SBP and BP stand for streptavidin-bead bound BP and freely available BP, respectively. At steady state, the concentrations of the hybridized target ssDNA with SBP and BP are

$$C_{SBP-ssDNA} = \frac{C_{SBP}C_{ssDNA}}{K_2} \quad (8)$$

and

$$C_{BP-ssDNA} = \frac{C_{BP}C_{ssDNA}}{K_3}, \quad (9)$$

respectively. The total concentration of BP and target ssDNA are conserved, hence

$$C_{BP} + C_{SBP} + C_{SBP-ssDNA} + C_{BP-ssDNA} = C_{BP}^0 \quad (10)$$

and

$$C_{ssDNA} + C_{SBP-ssDNA} + C_{BP-ssDNA} = C_{ssDNA}^0. \quad (11)$$

Here $C_{ssDNA}^0 = C_{initial}$ and C_{BP}^0 are the initial concentrations of target ssDNA and BP in the bulk, respectively. $C_{SBP-ssDNA} = C_{initial} - C_{final}$ is the measured concentration of target ssDNA hybridized to bead. Equations (8)–(11) were solved for $C_{SBP-ssDNA}$ using

$$\begin{aligned} & C_{SBP-ssDNA}^2 - (C_{BP}^0 + C_{ssDNA}^0 - 2C_{BP-ssDNA} + K_2)C_{SBP-ssDNA} \\ & + C_{BP-ssDNA}(C_{BP-ssDNA} - K_3 - C_{BP}^0 - C_{ssDNA}^0) + C_{BP}^0C_{ssDNA}^0 = 0. \end{aligned} \quad (12)$$

Figure 3 (in green) shows model fit to the experimental data (in red). Here, we used $K_2 = 3.5/\text{nM}$ and $K_3 = 6.5/\text{nM}$ as dissociation constants. The two-species model agrees very well with the experiments.

Examination of non-target ssDNA adsorption to bead

Non-complementary BP-2 (Table I) were saturated on beads, which were subsequently incubated with original target ssDNA. Note that this experiment presented the original target ssDNA as non-target molecules, and the incubation time was kept at 15 min as the same to incubation of BP with target ssDNA. Figure 3 (colored in magenta) shows non-specifically adsorbed target ssDNA concentration on bead surface Γ_{ssDNA} versus initial target ssDNA concentration C_{ssDNA}^0 in the bulk. When concentration of target ssDNA $C_{ssDNA}^0 < 4 \text{ nM}$, few molecules of target ssDNA attached to bead surface with non-specific capture probe. At concentrations larger than $C_{ssDNA}^0 > 4 \text{ nM}$, some target ssDNA molecules began being adsorbed and quickly reached a saturation level as C_{ssDNA}^0 reached $\sim 6 \text{ nM}$. At this concentration, a total of $\Gamma_{ssDNA} A N_b = 0.009 \text{ pmol}$ target ssDNA successfully hybridized. The sigmoidal shape of experimental data was also fitted with a dual-species hybridization model (Figure 3, blue). In this case, the dissociation constants of target ssDNA to BP and target ssDNA to SBP were both fitted at very low values ($0.04/\text{nM}$), confirming our experimental design for non-specific binding.

Effect of specific detection of target ssDNA mixed with non-target probe

Figure 4 shows the NASBA real-time detection of the target ssDNA within mixtures of non-target ssDNA probe. There was no significant change in baseline fluorescence for samples containing no ssDNA and for samples containing only non-target ssDNA, confirming that capture of the target ssDNA is highly specific. In mixtures of target ssDNA and other ssDNA types, as low as 1% target ssDNA can be detected with our microfluidic technique. With a higher starting concentration of the target ssDNA (100% and 50% of target mixed with non-target probe), the amplification reaction confirms detection of the target ssDNA. This establishes the remarkable capability of our microfluidic isolation technique.

FINAL DISCUSSION AND CONCLUSIONS

Although many researchers have reported successful protocols for DNA and RNA isolation via hybridization onto functionalized bead surfaces, there is still limited knowledge on the

FIG. 4. NASBA-amplified detection of isolated target ssDNA with mixture of non-target ssDNA probe shown in real-time fluorescence as a function of time. Standard deviation is within 5% of results (error bars not shown). Note that 50% “contamination” of non-target ssDNA in sample solution made no difference in signal values compared to that of pure target ssDNA in detection.

quantitative immobilization efficiency and on the application of pertinent theoretical models. Our microfluidic separation technique is novel for understanding bead based binding characteristics. The method now can become a quality control method for quantifying specific hybridization, non-specific binding and binding affinities.

In this study, we first provided fundamental insights into the efficiency of immobilizing a short capture probe onto bead surface using biotin-streptavidin chemistry. We have described the adsorption of BP onto bead surface using the Langmuir curve (Figure 2(b)). We have established that our microfluidic approach resulted in DNA isolation with efficiency better than the conventional tube washing approach (Figure 2, in red). The higher apparent bead surface concentration Γ_{BP} for capture probe in the tube assay suggested significant capture probe loss during tube wash. This problem has been previously reported.^{28,29}

We have also shown successful isolation of bead-probe-target complex using our microfluidic approach. We have quantified the efficiency of hybridizing target ssDNA with BP on bead. Sequence based hybridization is generally believed to be specific and a useful analytical technique in molecular biology.^{30–32} However, we observed non-negligible carry-over of non-target ssDNA adsorbed on solid surface covered with non-specific capture probes (Figure 3). The non-specific capture probe was energetically unfavorable for hybridization (supplemental Table II);³⁵ however, there existed moderate non-specific ionic interactions and/or physical adsorption. These weak interactions can be possibly eliminated by changing pH and ionic strength of the solution. In addition, motion of beads through microchannels involves complex motion of high shear fluid past spheres. We believe this complex motion reduces, if it does not eliminate, the non-specific binding. Since it is not trivial to estimate the shear forces on an assembly of loosely packed spheres in this work, we defer this important aspect to future studies. On the other hand, when we have considerable non-target ssDNA mixed with target ssDNA, we can achieve desired separation with our microfluidic technique; where as low as 1% target in solution can be detected (Figure 4). Chan *et al.*³³ theoretically predicted that hybridization could be enhanced when a solid surface is sparsely covered with specific capture probe while an uncovered region adsorbed non-selective DNA, such that the target DNA can find the complementary strand faster by diffusing on 2D surface rather than 3D.

Several different factors that affect hybridization were investigated in our study. One is temperature. Temperature affects the free energy profile of hybridization for DNA in different structures. Chen *et al.*³⁴ pointed out nucleation and hairpin opening as two rate-limiting steps that influence DNA hybridization with secondary structure. At higher temperatures, hybridization is independent of secondary structures, but if the temperature is too high, it will melt the hybridization structure. We found that beads capture most target ssDNA at 40 °C incubation (supplemental Figure 3).³⁵ We also looked into how hybridization locations affect DNA hybridization. While the accessibility may be influenced by the length of the free hanging tail on solid surfaces, as reported by Peytavi *et al.*,⁸ Peeters *et al.*⁵ observed no impact on 50 nt ssDNA hybridized to different locations of 169 nt target. In our study, we also observed no difference on hybridization when the probe had the complementary sequence on different locations of free target (supplemental Figure 2).³⁵ This provides helpful insight into biological sequence separation development, where the target sequence location cannot be shifted, but the decision on probe design can be independent of targeting locations.

To conclude, a simple microfluidic platform, as the one demonstrated in this work, can be used to investigate and understand the isolation of ssDNA by nucleic acid hybridization. A sensitive capture of sequence specific ssDNA can be performed by bead movement using a mobile magnetic field. Our chip design shows an insignificant amount of non-target capture, highly specific target capture and transfer, despite the presence of non-target molecules in solution. Our chip is easy to make. The PDMS material is cheap for mass production. Thus the device can be made disposable. More importantly, the platform requires $\sim 50 \mu\text{l}$ or less sample volume to work with. Even though we are still at early stage of technology development, we believe this fundamental understanding will provide insightful guidance for point-of-care biological diagnostic platforms that require binding and separation of known target biomolecules, such as RNA, ssDNA, or protein.

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