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Detection of Viable *Cryptosporidium parvum* Using DNA-Modified Liposomes in a Microfluidic Chip

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This paper describes a microfluidic chip that enables the detection of viable *Cryptosporidium parvum* by detecting RNA amplified by nucleic-acid-sequence-based amplification (NASBA). The mRNA serving as the template for NASBA is produced by viable *C. parvum* as a response to heat shock. The chip utilizes sandwich hybridization by hybridizing the NASBA-generated amplicon between capture probes and reporter probes in a microfluidic channel. The reporter probes are tagged with carboxyfluorescein-filled liposomes. These liposomes, which generate fluorescence intensities not obtainable from single fluorophores, allow the detection of very low concentrations of targets. The limit of detection of the chip is 5 fmol of amplicon in 12.5 μL of sample solution. Samples of *C. parvum* that underwent heat shock, extraction, and amplification by NASBA were successfully detected and clearly distinguishable from controls. This was accomplished without having to separate the amplified RNA from the NASBA mixture. The microfluidic chip can easily be modified to detect other pathogens. We envision its use in μ-total analysis systems (μ-TAS) and in DNA-array chips utilized for environmental monitoring of pathogens.

We have developed a sensitive microfluidic chip that detects viable *Cryptosporidium parvum*. Sensors for detecting *C. parvum*, a waterborne pathogen, are important because viable oocysts (infective stages of *C. parvum*) cause serious diseases in infected humans.[13] Well-established methods utilize fluorophore-tagged antibodies or oligonucleotides for detecting waterborne *C. parvum*.[4,5] These methods are often time-consuming and do not distinguish between viable and nonviable organisms.

The microfluidic chip presented detects amplicons generated by NASBA from mRNA templates produced by viable *C. parvum* as a response to heat shock. This response is expected to take place in viable organisms only; therefore, testing for the RNA product of NASBA will allow researchers to distinguish viable from nonviable *C. parvum*. We utilized the extraction and amplification protocols described by Bäumner et al. to specifically amplify the target mRNA from *C. parvum*.[6]

Our approach to detecting RNA utilizes a microfluidic chip in which the amplified RNA can be hybridized as a "sandwich" between capture probes immobilized in a microfluidic channel and reporter probes. To increase the signal derived from the reporter probes, we label them with carboxyfluorescein-filled liposomes rather than with single fluorophores.

Liposomes are lipid vesicles that are used as carriers for substances in a variety of applications.[7−9] When they are used as reporter particles in DNA assays or immunoassays, liposomes generally carry a fluorescent dye. Because each liposome can contain up to 10^5 fluorescent molecules,[10] they generate fluorescent signals greater than those obtainable using single fluorophore molecules. By increasing the signal derived from a single analyte molecule, the liposomes increase the sensitivity of the assay. In a flow injection immunoassay in which liposomes were used to detect the analyte, the sensitivity of the assay was increased 1000-fold in comparison to the same assay conducted using a single fluorophore as the reporter entity.[8]

Our laboratory recently reported a method for tagging liposomes with oligonucleotides.[9] These liposomes were then successfully used in test-strip assays for detecting RNA that was amplified by NASBA.[9,11] The high sensitivity of the test strips can

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in part be attributed to the very close proximity at which the immobilized probes can interact with the target and liposome-labeled probes, because samples and reagents migrate up the strip by microcapillary forces. We, therefore, believe that integrating the method into a microfluidic chip will further improve the method because of the high surface-to-volume ratios achieved in these devices.

Downscaling the assay into a microfluidic format has at least three other advantages: (1) microfluidic devices consume less reagent and sample, (2) multiple analyses can be performed simultaneously, and (3) in a further developed device, the steps for preparing the sample and the steps for detecting the target can be integrated onto one device. The greatest advantage, however, is the low cost at which microfluidic devices can be produced.[12]

The most common method for detecting DNA and RNA in standard laboratories is by means of the "fingerprint" of these molecules in size-dependent separations by electrophoresis. The separated DNA molecules can be stained with an intercalating dye such as ethidium bromide and detected by fluorescence methods. If it is necessary to identify one specific sequence in a mixture of molecules, the DNA can be transferred to solid supports, such as membranes,[13,14] and then hybridized to complementary oligonucleotides that are labeled with molecules that are radioactive,[13,14] color-producing,[15] or chemiluminescent.[16] Electrophoresis and subsequent detection have been successfully scaled down from slab gel assays to capillary electrophoresis and, more recently, to microchannel electrophoresis.[17−19] This radically reduced the time needed for detection from several hours to a few seconds.[20]

For medical applications, miniaturized detection schemes have been developed in which the DNA of interest is first amplified by polymerase chain reaction (PCR) in a microchip[21−23] and then detected by microchannel electrophoresis.[24,25] PCR amplicons produced in a microchip can also be detected by hybridizing them to immobilized probes similar to those used in DNA microarrays.[26,27]

In this study, we use a microfluidic chip that is fabricated by casting a microchannel in poly(dimethyl siloxane) (PDMS) using a micromachined silicon template as the molding tool. The PDMS channel seals to a gold-coated glass slide that serves as the bottom of the channel. Thiolated capture probes are immobilized on the gold by means of sulfur−gold linkages as previously described.[28,29] A self-assembled layer of mercaptohexanol and blocking liposomes reduces nonspecific adsorption of target and of liposomes to the gold. The small dimensions of the microchannel allow us to reduce the amount of liposomes needed for detecting the target, thereby conserving the supply of this reagent. A constant flow of liposomes through the channel reduced the limitation on the rate of hybridization imposed by diffusion processes. The accelerated reaction enabled us to detect the target far more rapidly. By exchanging the oligonucleotides used as capture and reporter probes, researchers can easily modify the chip to detect other pathogens; therefore, the sensor can be applied in DNA array chips used for multianalyte detection.

EXPERIMENTAL SECTION

Oligomers. The oligonucleotide molecule that is detected in our system is the 103-basepair RNA product generated by nucleic-acid-sequence-based amplification (NASBA) from the mRNA coding for *C. parvum*'s heat-shock protein (Hsp70).[6] Using appropriate primers, this oligonucleotide is specifically amplified by NASBA from *C. parvum* extracts.[6] Because DNA is more stable than RNA, we used the oligonucleotide sequence of a 103-mer synthetic target that has the same sequence as the NASBA product (5′-aga agg acc agc atc ctt gag tac ttt ctc aac tgg agc taa agt tgc acg gaa gta atc agc gca gag ttc ttc gaa tct agc tct act gat ggc aac tga a-3′) in order to optimize the background signal and to determine the limit of detection. The reporter probe, a 20-mer oligonucleotide (5′-gtg caa ctt tag ctc cag tt-3′) complementary to a part of the amplified RNA, was modified using a C3 amino linker at the 3′ end. The capture probe, which is also complementary to a part of the amplified RNA, was modified with a $-(CH_2)_6-S-S-(CH_2)_6OH$ at its 5′ end. When the target RNA hybridizes to the reporter probe and the capture probe, a sandwich complex is yielded. All of the oligonucleotides were synthesized by the BioResource Center, Cornell University (Ithaca, NY).

Preparation of Acetylthioacetate (ATA)-Tagged Liposomes Containing Carboxyfluorescein. The liposome encapsulant, a 100 mmol/L carboxyfluorescein solution, was prepared in 20 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.5, 421 mOsmol/kg). Although carboxyfluorescein is not particularly photostable, we used it as the encapsulant, because in previous applications, we observed good encapsulation efficiencies and excellent retention of the dye when carboxyfluorescein was incorporated into liposomes. We have determined that carboxyfluorescein is stably retained in liposomes for almost a year when appropriately stored.

The liposomes were prepared using a modified version of the reverse phase evaporation method as published by Siebert and colleagues.[30] We obtained the phospholipids dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidyl glycerol (DPPG), and dipalmitoylphosphatidylethanolamine (DPPE) from Avanti Polar Lipids Inc. (Alabaster, AL). We prepared a solution containing 7.2 μmol of DPPE and a volume fraction of 0.7% triethylamine in chloroform. We reacted this solution with 14.3 μmol of *N*-succinimidyl-*S*-acetylthioacetate (SATA; purchased from Pierce; Rockford, IL) to form DPPE-acetylthioacetate (DPPE-ATA). We then prepared a lipid solution containing 40.3 μmol of DPPC, 4.2 μmol of DPPG, and 40.9 μmol of cholesterol dissolved in 8 mL of a solvent mixture consisting of chloroform, isopropyl ether, and methanol in a volume fraction ratio of 6:6:1. To this lipid solution, we added an aliquot of DPPE-ATA so that the final solution contained a mole fraction of 4% DPPE-ATA. While sonicating the lipid suspension under a low stream of nitrogen at 45 °C, we added 2 mL of encapsulant. We then removed the organic solvent using

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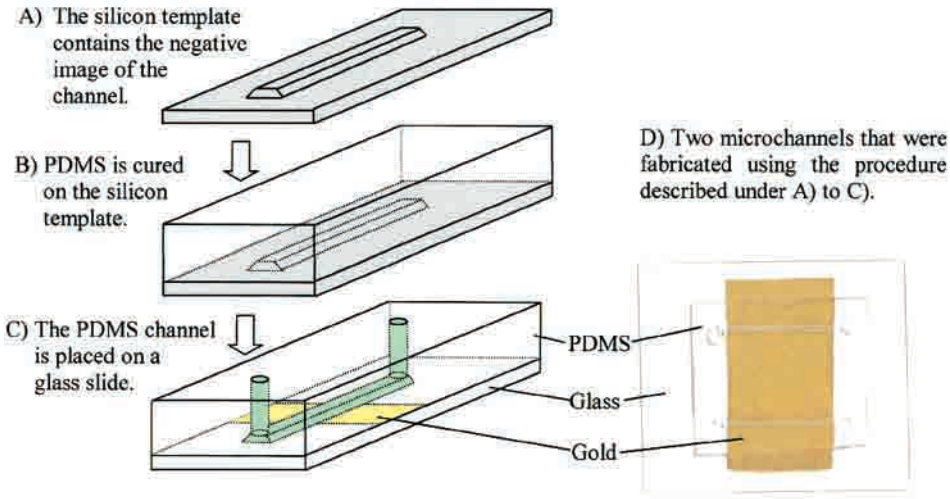

Figure 1. Fabrication of a microchannel that consists of PDMS side and top walls and a glass bottom. (A) The negative image of the channel was fabricated in silicon by standard photolithography and a KOH etch. (B) The silicon wafer was then used as a template on which PDMS can be cured. (C) After curing, the PDMS channel was taken off the template and placed on a glass slide that contained a region on which gold had been deposited. The inlet and outlet were cut into the PDMS. (D) Photograph of two microchannels fabricated using the described procedure.

a vacuum rotary evaporator. Each of the last two steps was repeated once. After the liposomes were formed, we let them remain under nitrogen for 10 min at 45 °C, then forced them through a final series of polycarbonate syringe filters with pore sizes of 3.0 μm, 0.4 μm, and 0.2 μm. We separated unencapsulated dye from liposomes by size-exclusion chromatography using Sephadex G-50-150 (Sigma Chemical Co.; St. Louis, MO). To prevent liposomes from lysing during the separation, we used sucrose to adjust the osmolality of the buffer (10 mmol/L HEPES, 200 mmol/L NaCl, pH 7.5) to 520 mOsmol/kg. The liposomes were then dialyzed for 12 h at 4 °C against the same buffer and recovered in approximately 13 mL of buffer solution. We used dynamic light-scattering to measure the diameter of the prepared liposomes, finding it to be 349 nm with a standard deviation of 120.

Conjugating Reporter Probes to ATA-Tagged Liposomes. Two simultaneous preparations were required. First, we derivatized the C3 amino-linker-modified reporter probes (dissolved in 50 mmol/L phosphate buffer, pH 7.8, with 1 mmol/L EDTA) with maleimide groups by incubating them with three times the molar quantity of *N*-(κ-maleimidoundecanoyloxy)sulfosuccinimide ester (sulfo-KMUS) dissolved in dimethyl sulfoxide (DMSO). The reagents were allowed to react for 3 h at room temperature. Second, and at the same time, we deacetylated the ATA groups on the liposome surface to yield unprotected thiol groups. For this reaction, we prepared a 500 mmol/L hydroxylamine hydrochloride solution with 25 mmol/L EDTA in 100 mmol/L HEPES buffer. We then gently mixed a 1.4 mL aliquot of the liposome solution recovered from dialysis with 140 μL of the hydroxylamine hydrochloride solution. The reaction was allowed to proceed at room temperature in the dark for 2 h. For conjugation, we reacted the thiol groups on the liposome surface with the reporter probes we had derivatized using the maleimide groups for 4 h at room temperature and then overnight at 4 °C at a pH of 7.0 throughout. All unconjugated thiol groups were quenched with ethylmaleimide solution isotonic to the encapsulant. The liposomes were then purified on a Sepharose CL-4B column (Sigma Chemical Co.; St. Louis, MO) equilibrated with 20 mmol/L Tris−HCl buffer pH 7.0 (containing 150 mmol/L NaCl and sucrose) with an osmolality of 520 mOsmol/kg. The recovered liposomes were stored at 4 °C in the dark. When stored under these conditions, we were able to use the liposomes for up to 9 months without observing high losses of dye (due to possible leaking) or capture probes.

Preparation of Capture-Probe/Hexane Monolayers on Gold-Covered Glass Slides. Microscope glass slides (Corning Inc.; Corning, NY) were cleaned by soaking them for 30 min in 70% (volume fraction) concentrated sulfuric acid and 30% (volume fraction) hydrogen peroxide [30% (volume fraction) H_2O_2 in H_2O]. *Warning! This cleaning solution is extremely oxidizing, reacts violently with organics, and should be stored only in containers that are hand-tightened only loosely so that no pressure builds up.* The glass slides were then rinsed with 18 MΩ deionized water and dried under a nitrogen stream. Using a thermal evaporator and a metal mask, we deposited a 15-Å chromium layer and a 450-Å gold layer on a 15 × 15 mm area of the slides. After the deposition, we cleaned the slides thoroughly with the cleaning solution described above. We then immersed the slides for 60 min in a 1 mmol/L solution of the disulfide-modified capture probe (diluted in 1 mmol/L potassium phosphate buffer, pH 7.0). We washed the slides with 18 MΩ water and immediately immersed them into a 1 mmol/L mercaptohexane solution diluted in ethanol. After 60 min, we washed the slides with ethanol and carefully dried them with nitrogen.

Preparation of the Microfluidic Chip. The microfluidic channels were fabricated using the technique shown in Figure 1. First, we used contact-photolithographic and wet-chemical etching methods to produce a silicon template containing negative three-dimensional images of the channels. The "inverted" channels had a trapezoidal profile with the following dimensions; top width, 300 μm; bottom width, 440 μm; depth, 50 μm. We then used the finished silicon template to mold microchannels. To prepare the microchannels, we covered the silicon template with liquid poly(dimethyl siloxane) (PDMS; Sylgard, 184 Silicone Elastomer, Dow Corning Co.; Midland, MI). After curing the PDMS for 60 min at 60 °C, we peeled off the channels from the template. We then sealed the PDMS channels nonpermanently to the glass slides

that we had modified with capture probes and hexane, as described above.

Assay Protocol. To induce the flow of aqueous solutions by gravity, we first primed the microfluidic channels with ethanol and subsequently slowly replaced the ethanol solution with aqueous buffer. After the substitution, we filled the inlet well with 12.5 μL of buffer and the outlet well with 5 μL of buffer. We tilted the channels to an angle of 30° so that the resulting flow would be driven by gravity. The flow was 92.4 μm/s with a standard deviation of 0.5 μm/s. A solution of blocking liposomes was then introduced into the channels for 30 min. The blocking liposomes were filled with buffer instead of carboxyfluorescein and did not have the reporter probe tag on their surface.

We filled the inlet well with 12.5 μL of sample solution containing either synthetic DNA or RNA amplified by NASBA in hybridization buffer. The final concentrations of the components in this solution were 600 mmol/L NaCl, 60 mmol/L NaH_2PO_4, 2 mmol/L EDTA [2× concentrated standard saline phosphate buffer with EDTA (2× SSPE)] and a volume fraction of 40% formamide. The hybridization was allowed to proceed for 30 min. We then washed the channels with 2× SSPE buffer. Next, we introduced the reporter-probe-tagged liposomes into the channel. The final concentrations of the components in this solution were a volume fraction of 5% of the liposome solution (as recovered from the Sepharose column), SSPE concentrated four times (4×), a volume fraction of 20% formamide, a volume fraction of 0.2% ficoll (type 400), and 125 mmol/L sucrose. This buffer had been previously optimized for the use of liposomes in hybridization studies.[11] After 30 min of hybridization, we washed the channel with a buffer containing 2× SSPE and a volume fraction of 50% formamide for 5 min.

Detection and Quantification. Using a standard fluorescence microscope equipped with a 20× long-distance working objective and a digital camera (C4742-95, Hamamatsu, NJ), we acquired images of the liposomes bound in the channel. The fluorescence was quantified using software acquired from Carl Zeiss Incorporation (Thornwood, NY). To obtain accurate measurements of fluorescence, we acquired all of the images using the same acquisition time and avoided bleaching of the samples by not exposing them to the light source before acquiring images.

Nucleic Acid Sequence-Based Amplification (NASBA). The extraction of nucleic acids from *C. parvum* and the amplification of the mRNA coding for the heat shock protein (Hsp70) by NASBA was conducted as described by Bäumner and co-workers.[6] In short, the mRNA production in oocysts was stimulated by heating them for 20 min at 42 °C. The oocysts' membranes were disrupted by incubation in lysis buffer (provided in a Qiagen RNeasy kit and a Organon Teknika Boom extraction kit). NASBA reactions were performed on either Hsp70 mRNA isolated from *C. parvum* oocysts or H_2O (as negative controls) using the NASBA kit and instructions from Organon Teknika (Boxtel, Netherlands). The primers for specific amplification are described elsewhere.[6] It should be noted that the product of this amplification method is complementary RNA to the mRNA that *C. parvum* produces as a response to heat shock.

RESULTS AND DISCUSSION

Minimizing the Background Binding of Liposomes. Initial experiments showed that the sensitivity of our sensor is limited by the background signal the sensor generates when no target is present if the gold surface has been modified with capture probes only. Figure 2A,B shows fluorescent images of channels in which the assay was conducted with a positive sample (containing synthetic target at a concentration of 400 fmol/μL) and a control. The fluorescence obtained from these two images does not differ, because target and liposomes adsorb nonspecifically to the gold surface.

A study by Levicky et al. suggests that in mixed monolayers consisting of capture probes and mercaptohexanol (both bound to gold by means of a sulfur−gold linkage), the mercaptohexanol can act as a passivating reagent that prevents nonspecific adsorption of the target oligonucleotide.[28] Besides passivating the gold surface, the mercaptohexanol also forces the sulfur-bound capture probes to rise upward from the surface, making them more accessible for hybridization.[28]

To test the effectiveness of such a mixed monolayer to prevent nonspecific adsorption of targets and liposomes in our chip, we conducted assays in channels modified with capture probes and mercaptohexanol. Figure 2C,D shows that the fluorescence obtained from a positive sample and a control remained the same, thus suggesting that the mixed layer did not prevent the liposomes from absorbing nonspecifically. We, therefore, prepared mixed layers of capture probes and various other passivating reagents and conducted assays in which we measured the fluorescence generated by triplicates of positive samples (solutions containing synthetic target at a concentration of 400 fmol/μL) and negative controls. The differences of the fluorescence intensities of the controls and the positive samples that are obtained using different blocking reagents are listed in Table 1. The greatest difference in fluorescence was generated using a blocking layer that combined mercaptohexane and untagged liposomes that contain buffer instead of dye ("blocking liposomes"). Figure 2E,F shows fluorescent images of channels that are treated with mercaptohexane and blocking liposomes. There are two ways in which this blocking layer passivates the gold. The capture probes and mercaptohexane yield a mixed monolayer that appears to resist the nonspecific adsorption of the negatively charged liposomes. Nonspecific binding is further decreased by presaturating the surface with blocking liposomes. Additionally, we found that the mercaptohexane and blocking liposomes worked very well as passivating reagents when each was used independently of the other.

We also tested bovine serum albumin (BSA) as a passivating reagent. BSA has been successfully employed for blocking nonspecific binding in immunoassays[31] and on test strips.[11] Our experiments showed that treating the gold surface with BSA reduces nonspecific liposome adsorption to approximately the same degree as a mixed monolayer of mercaptohexane and capture probes.

Although monolayers consisting of alkylated 1-thiahexa(ethylene oxide) compounds, and oligo(ethylene glycol)-terminated layers are known to resist the adsorption of certain proteins on gold,[32,33] these reagents did not generate great differences in

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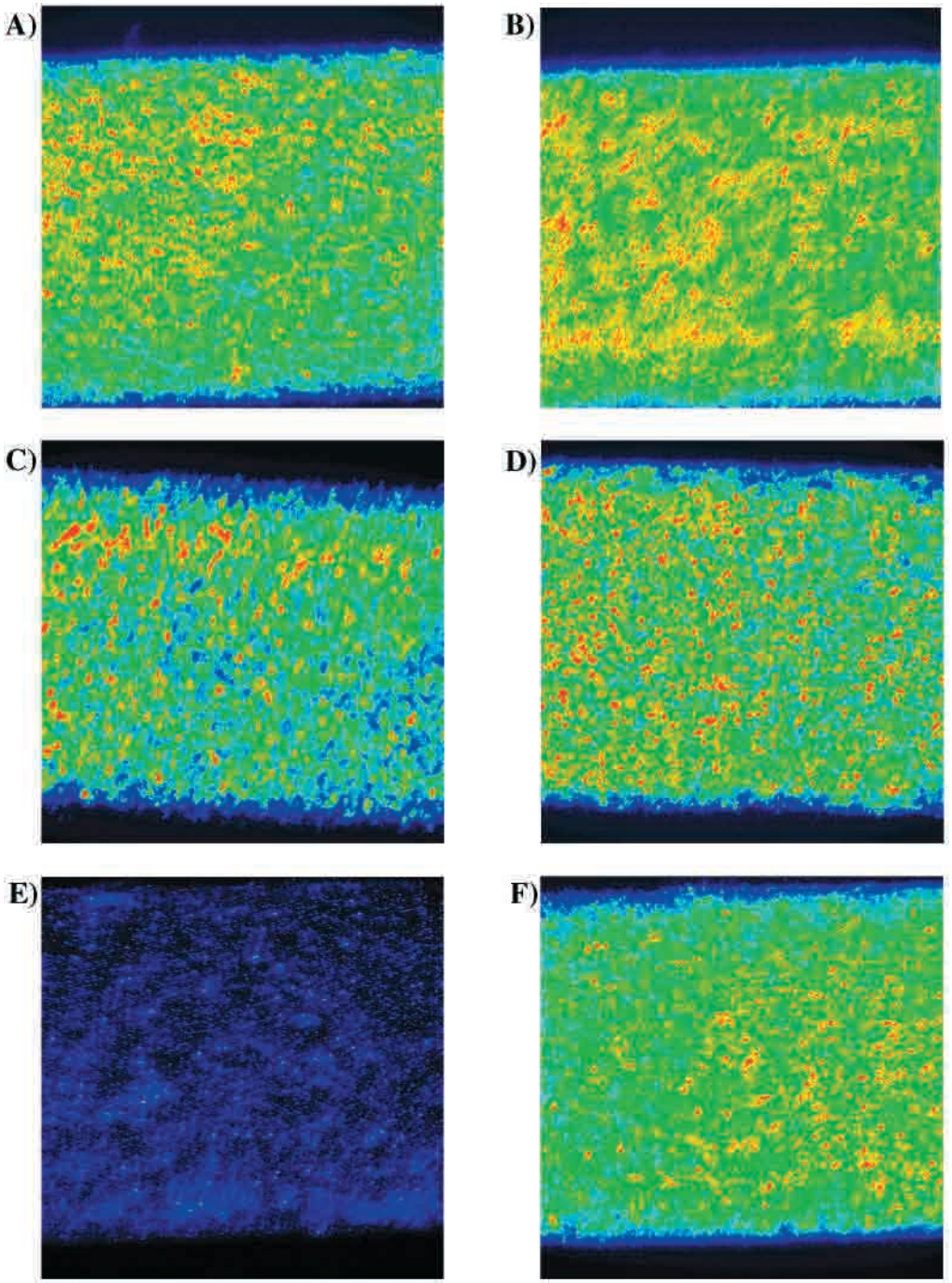

Figure 2. Fluorescent images of channels that were not treated with any blocking reagent (A, no analyte present; B, analyte concentration is 400 fmol/μL), images of channels treated with mercaptohexanol (C, no analyte present; D, analyte concentration is 400 fmol/μL), and images of channels that were treated with mercaptohexane and blocking liposomes (E, no analyte present; F, analyte concentration is 400 fmol/μL).

fluorescence intensity between the controls and the positive samples. A similar result was found for mixed monolayers with capture probe and 3-mercaptopropionic acid.

Determining the Limit of Detection. The limit of detection was determined by exposing the chip modified with a mixed layer of capture probe, mercaptohexane, and blocking liposomes to 12.5 μL of sample solutions that contained synthetic target oligonucleotide in concentrations ranging from 0 to 800 fmol/μL. Plotting the measured fluorescence against the analyte concentration yielded the dose−response curve shown in Figure 3. The lowest detectable target concentration was 0.4 fmol/μL. We define the limit of detection as the analyte concentration at which the interval yielded by doubling the calculated standard deviation of the fluorescence obtained for this concentration does not overlap with the same interval from controls.[34,35] This detection limit compares very well with the RNA-sensing test strip we recently developed for *C. parvum*.[11] The test strip reported previously had a detection limit for measuring RNA concentration of 3 fmol/μL with a sample volume of 30 μL. The high sensitivities achieved with the

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Table 1. Differences in Fluorescence Obtained for Sample Solutions Containing Synthetic Target in Concentrations of 0 fmol/μL and 400 fmol/μL Solution in Sensors Modified Using Different Blocking Reagents.[a]

blocking reagent	Difference in the fluorescence obtained for solns with target concns of 0 and 400 fmol/μL (arbitrary units)
no blocking	65 ± 540
mercaptohexane and blocking liposomes	1610 ± 181
blocking liposomes	1556 ± 207
mercaptohexane $HS(CH_2)_5CH_3$	1307 ± 567
bovine serum albumin (BSA)	1305 ± 337
alkylated 1-thiahexa(ethylene oxide) $HS(CH_2CH_2O)_6C_{10}H_{21}$	916 ± 1047
oligo(ethylene glycol)-terminated thiol $HS(CH_2)_{11}(OCH_2CH_2)_6OH$	484 ± 372
mercaptohexanol $HS(CH_2)_6OH$	110 ± 254
mercaptopropionic acid $HS(CH_2)_2COOH$	−138 ± 247

[a] Each number represents the mean of three experiments ± one std dev

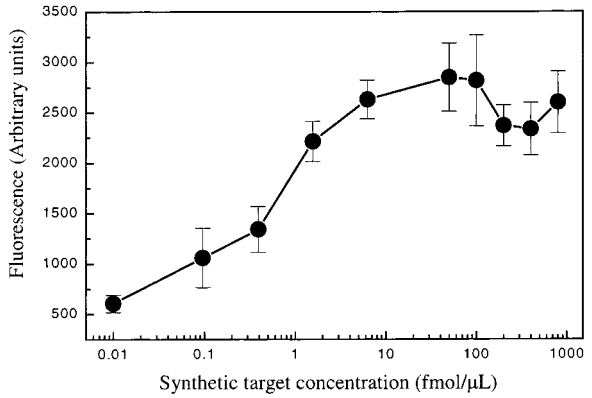

Figure 3. Dose−response curve for the synthetic target. Each point represents the mean of three measurements. Error bars represent ±1 std dev.

microfluidic chip and the test strip are in part attributable to the advantages offered by dye-filled liposomes. Because one liposome contains many fluorescein molecules in its aqueous cavity[36] the signal generated from one binding event is quite large; however, because of their size, the liposomes may occupy several target sites. At high analyte concentrations this limits the number of liposomes that bind to the surface. On the other hand, because of the two-dimensional fluidic character of the liposome membrane, reporter probes are mobile and may migrate, so that several reporter probes on a single liposome can hybridize simultaneously with immobilized target (each liposome theoretically contains reporter probes at a mole fraction of 2% on the outside of heir membrane). This results in strong binding of liposomes in the presence of the high concentrations of target.[36]

An advantage of the microfluidic format is that the steady flow of liposomes moving through the microchannel constantly places

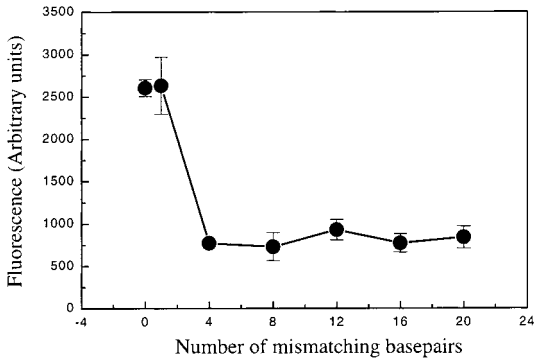

Figure 4. Fluorescence obtained in experiments conducted with targets that contained mismatched basepairs ranging from 1 to 20 within each of the 20-mer sequences that hybridized with the capture and reporter probes. Each point represents the mean of three measurements. Error bars represent ±1 std dev.

liposomes with free reporter probes within close dimensions of the immobilized target. This close juxtaposition speeds up the reaction by minimizing the limitation on the rate at which hybridization can occur under normal diffusion processes.

The dose−response curve shows that the fluorescence intensity initially increases dramatically as target concentrations increase, but then intensity reaches a maximum value at 25 fmol/μL when the capture probe becomes saturated. By introducing the sample solution and the liposomes sequentially into the channel of the microfluidic chip, the reporter and capture probes cannot simultaneously be saturated with target (even at high target concentrations), because the reporter probes are not exposed to the bound targets until all of the unbound target is washed out of the channel; therefore, false-negative results, as observed in other assay formats in which sandwich hybridization is utilized,[9] do not occur.

Determining Specificity. It is particularly important that positive signals are measured only in response to the target of interest. We conducted experiments to prove that the developed microfluidic chip does not give false-positive results with targets from organisms other than *C. parvum*. We tested targets that were modified so that they contained mismatched basepairs ranging from a single-base mismatch up to a 20-base mismatch (100%) within each of the 20 mer regions that under ideal conditions are complementary to the capture probe and the reporter probe. The mismatches were evenly distributed throughout these regions. The fluorescence values yielded by testing these modified targets in samples with a concentration of 52 fmol/μL are shown in Figure 4. We compared the fluorescence obtained for the mismatched targets with the fluorescence obtained for the perfect match. The targets in which mismatches ranged between 4 (20%) and 20 (100%) generated fluorescence intensities slightly higher than that obtained by the target concentration that was earlier defined as the limit of detection for perfectly complementary targets. The single-base mismatched target could not be readily distinguished from the perfect complement. However, we consider this result as sufficient, because we chose a target sequence that occurs only in *C. parvum*. (Researchers should be aware of a recently published study that identified this particular sequence or a very similar sequence in two other strains of *Cryptosporidium*.[37])

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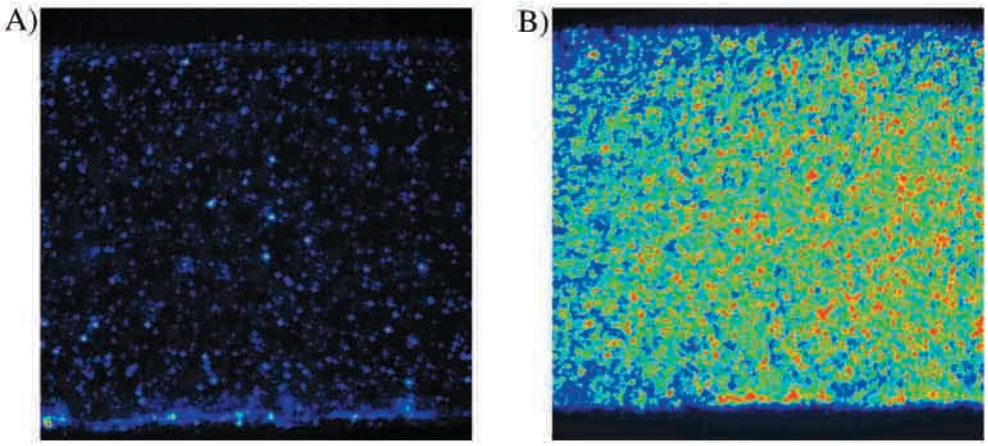

Figure 5. Fluorescence images of channels in which samples from the NASBA reaction were tested: A, negative NASBA sample; B, positive NASBA sample.

Testing of RNA Amplified by NASBA. The detection of *C. parvum* in water treatment plants requires that organisms be collected from several liters of sample water and then separated from contaminating debris.[38] Such procedures sometimes recover only a few organisms, and the amount of RNA extracted from them cannot readily be detected without amplification. Because even very low numbers of *C. parvum* can cause life-threatening conditions in immunocompromised people, it is necessary to develop test schemes that can detect this small amount of RNA.[3] Many researchers have developed protocols to facilitate the amplification of the recovered DNA or RNA.[39,40] It has been shown that the target RNA for which our sensor is developed can be specifically and reliably amplified from as few as 10 organisms by NASBA when proper primers are used.[6] To test the compatibility of our chip with NASBA, we conducted experiments with varying amounts of RNA generated by NASBA from *C. parvum* extracts. We found that the fluorescent signals obtained for all of the positive samples were higher than those obtained for the limit of detection, and they were readily distinguishable from those obtained for control samples that did not contain *C. parvum* extracts (see Figure 5). To accomplish this, it was not necessary to separate the amplified RNA from the components of the NASBA.

Reusability of the Chip. We examined whether the result of a measurement was reproducible if we reused the same chip for multiple consecutive detections of target. After conducting tests with control solutions and positive samples (containing synthetic target at a concentration of 8 fmol/μL) on six different chips (three loaded with controls, three loaded with positive samples) simultaneously, we dehybridized the probes in each chip by treatment with deionized water, then repeated the tests using the same chips two more times. The average of the three fluorescence values obtained as averages of the three simultaneous conducted experiments for the control is 880 $\pm$ 230 (an arbitrary unit), and for the sample containing the target, we obtained an average fluorescence of 1900 $\pm$ 130 (an arbitrary unit). These results indicate that the chip is not altered after the first set of experiments, and therefore, it is reusable.

CONCLUSIONS

We have developed a sensitive microfluidic RNA sensor that uses oligonucleotide-tagged liposomes as hybridization markers in a sandwich-hybridization assay. Detection conducted with the presented chip is specific, and the chip is capable of measuring target concentrations as low as 0.4 fmol/μL with a 12.5-μL aliquot of sample solution. RNA that was extracted from viable *C. parvum* oocysts was amplified by NASBA and successfully detected. NASBA-generated amplicons can be detected without separating them from the NASBA−enzyme mixture.

We successfully demonstrated the feasibility of dye-entrapped liposomes as reporter particles in a microfluidic system. Because the liposomes do not need to be lysed to measure the fluorescent signal, the developed format can be used in microfluidic array chips in which a number of spatially separated binding sites may be used to indicate the presence or absence of multiple targets in one sample. We are planning to further improve the performance of this assay.

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