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A Robust Technique for Assembly of Nucleic Acid Hybridization Chips Based on Electrochemically Templated Chitosan

Hyunmin Yi,[†,‡] Li-Qun Wu,[†,§] Reza Ghodssi,[∥,⊥] Gary W. Rubloff,[∥,#] Gregory F. Payne,[†,§] and William E. Bentley*[,†,‡]

Center for Biosystems Research, University of Maryland Biotechnology Institute, 5115 Plant Sciences Building, Department of Chemical Engineering, The Institute for Systems Research, Department of Electrical and Computer Engineering, and Department of Materials and Nuclear Engineering, University of Maryland at College Park, College Park, Maryland 20742, and Department of Chemical and Biochemical Engineering, University of Maryland, Baltimore County, Maryland 21250

A nucleic acid hybridization assay was assembled onto a robust and readily addressable silicon-based chip using polysaccharide chitosan as a scaffold for the covalent coupling of probe DNA to the chip's surface. Chitosan is a unique polymer, ideally suited for this application because its net charge and solubility are pH dependent. Specifically in this work, gold-patterned electrodes were created using standard photolithographic techniques, chitosan was electrodeposited in a spatially resolved manner onto the polarized electrodes, probe DNA was covalently assembled onto the chitosan, and both DNA:DNA and DNA:mRNA hybridization detection schemes were evaluated. Hybridization of target nucleic acid was quantifiable, reproducible, and robust; the surface was regenerated and rehybridized up to eight times without loss of signal. Finally, transcriptional upregulation of the *Escherichia coli* chaperone, DnaK, which is an indicator of cellular stress, was observed using the hybridization chip sandwich assay. Thus, this method enables rapid and facile monitoring of gene expression in a format that is reusable and requires minimal reagent quantities.

Recent advances in genetic engineering and biosensor technologies have opened the door for direct, simple, and rapid monitoring of bioprocesses using multiple analytes (e.g., metabolites, nucleic acids, and proteins).[1−7] Although there have been successful attempts to monitor transcription of a large number of genes from cells cultivated in bioreactors,[8−12] the practice is not widespread, perhaps due to assay limitations as well as long processing times. For example, DNA microarrays require numerous preparation and analysis steps such as sample amplification and labeling, as well as data acquisition and processing. Also, these microarrays are generally not reusable. A microscale hybridization assay is needed that is low-cost and that enables detection of a small number of transcripts in a reusable and portable format. We suggest that a readily addressable area-based assembly of probe species could improve the likelihood of on-line mRNA monitoring. Our concept combines the resolution and spatial organization made available through microfabrication with the rapidly evolving hybridization detection chemistries that continually improve selectivity and sensitivity.[13−16] Although there has been a significant progress in photolithographic microfabrication, assembling biological species (e.g., hybridization probes) onto microfabricated surfaces in a simple, robust, and reproducible manner remains problematic. For example, soft lithographic techniques such as microcontact printing of oligonucleotides onto

* To whom correspondence should be addressed. Phone: (301) 405-4321. Fax: (301) 314-9075. E-mail: bentley@eng.umd.edu.

† Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park.

‡ Department of Chemical Engineering, University of Maryland, College Park.

§ Department of Chemical and Biochemical Engineering, University of Maryland, Baltimore County.

∥ The Institute for Systems Research, University of Maryland, College Park.

⊥ Department of Electrical and Computer Engineering, University of Maryland, College Park.

Department of Materials and Nuclear Engineering, University of Maryland, College Park.

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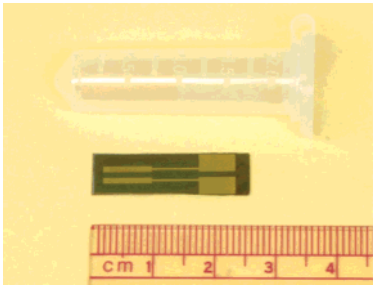

Figure 1. Nucleic acid hybridization chip composed of two gold patterns on a silicon nitride surface. Each chip fits within a 2-mL microcentrifuge tube.

spotted or otherwise spatially arranged arrays require complex surface preparation and robotics.[17−19] In addition, area-based chemical surface modification techniques, such as silanization and silyation, do not offer spatial resolution.[20] Further, covalent coupling of biomolecules to such functionalized surfaces is preferred for ensuring repeated use through cycles of hybridization/denaturation.[16,21]

We use the aminopolysaccharide chitosan as an integral component of our biosensor because it has two unique properties that enable simple covalent assembly of biomolecules in a spatially selective manner. First, chitosan has pH-dependent electrostatic properties. At low pH, chitosan's primary amines are protonated, and chitosan becomes a water-soluble, cationic polyelectrolyte. At high pH (>∼6.3), these amines become deprotonated, and chitosan loses its charge and becomes insoluble. This pH-dependent electrostatic behavior allows chitosan to be deposited onto a cathode surface in response to an applied voltage, and this deposition is spatially selective at the microscale.[22,23] Chitosan's second unique property also comes from its primary amine groups—such amines are desirable surface substituents because a range of simple and standard chemistries exist to couple biomolecules to surfaces through surface amines.[24−26] The reactivity of these amines has been exploited in previous studies to covalently tether ssDNA probes to chitosan.[27] We believe these two properties make chitosan a unique material to serve as an interface to link labile sensing biomolecules to microfabricated surfaces. Here we report the first use of chitosan for the facile assembly of a nucleic acid hybridization "chip" that provides simple and robust target detection.

Figure 1 shows our hybridization chip, prepared using standard photolithography starting from a silicon substrate that is patterned to have two gold surfaces. This chip is small enough to fit within a 2-mL microcentrifuge tube, illustrating the potential for both simplicity and portability. In Scheme 1, we show the steps used to assemble the ssDNA probe onto the fabricated surface and outline a series of experiments that test the chip performance. In the top row, chitosan is deposited onto gold surfaces that are polarized to have a negative bias (i.e., serve as a cathode). Next, the deposited chitosan is activated for ssDNA assembly by reacting with glutaraldehyde. The second row of Scheme 1 depicts the assembly of amine-terminated and fluorescein-labeled ssDNA. The third row shows the protocol for generating the hybridization probe surface by assembling amine-terminated ssDNA (not fluorescein-labeled). To examine selectivity and sensitivity, we then performed hybridization assays using the generated probe surface with fluorescently labeled target ssDNAs. The third row also shows experiments where we performed multiple hybridization/denaturation tests. Finally, we tested this chip for its ability to rapidly detect changes in stress gene transcription in shake-flask cultivated recombinant *Escherichia coli* (bottom row of Scheme 1). Specifically, we induced recombinant *E. coli* to produce a foreign protein and measured changes in transcription of a global regulatory gene, *dnaK*. The *dnaK* gene encodes the molecular chaperon protein, DnaK (also referred to as hsp70), and its expression is often used as a measure of cellular stress. Thus, in our application, the chip enabled detection of cellular stress.

EXPERIMENTAL SECTION

Reagents. Chitosan (deacetylated chitin, 85% minimum) from crab shells, glutaraldehyde (grade I 50% aqueous solution), urea (SigmaUltra grade), PerfectHyb Plus hybridization buffer, saline sodium citrate (SSC) buffer (20× concentrated, molecular biology grade), Tris-EDTA (TE) buffer (100× concentrated), lysozyme, and ampicillin were all purchased from Sigma (St. Louis, MO). Sodium hydroxide pellets, $MgCl_2 \cdot 6H_2O$ (enzyme grade), and sodium borohydride powder ($NaBH_4$) were purchased from Fisher Chemical (Fair Lawn, NJ). An RNeasy mini kit was purchased from Qiagen (Valencia, CA). Distilled and deionized water (ddH_2O, 18 MΩ·cm, Milli-Q) was also autoclaved before use. Otherwise noted, 1× SSC buffer with 0.1 M $MgCl_2$ was used for equilibration and rinsing throughout this study. To prepare this SSC buffer, we first diluted the SSC buffer 20× concentrated with ddH_2O. After autoclaving, we added autoclaved 4 M $MgCl_2$ solution and adjusted the pH to 7.1.

Silicon wafers (4-in. diameter) were obtained from MEMC Electronic Materials (St. Peters, MO). The gold and chromium used for sputtering onto the wafer were purchased from Kurt J. Lesker Co. (Clairton, PA). The primer was hexamethyldisilazane (HMDS, Microelectronic Materials). The photoresist (Microposit Photoresist S1813) and developer (Microposit Developer 352) were purchased from Shipley Co. (Marlborough, MA). The etchants (TFA for gold and TFD for chromium) were obtained from Transene Co. (Danvers, MA).

ssDNA Oligonucleotides. HPLC-purified ssDNAs of 20 bases with various modifications (Table 1) were purchased from either Gene Probe Technologies (Rockville, MD) or Integrated DNA Technologies, Inc. (Coralville, IA) and used without further purification. Two target DNAs are 20-base sequences of *E. coli dnaK* and *groEL* genes that have little homology between them. Surface probe DNA is amine-modified at the 5′ end, and the 20-

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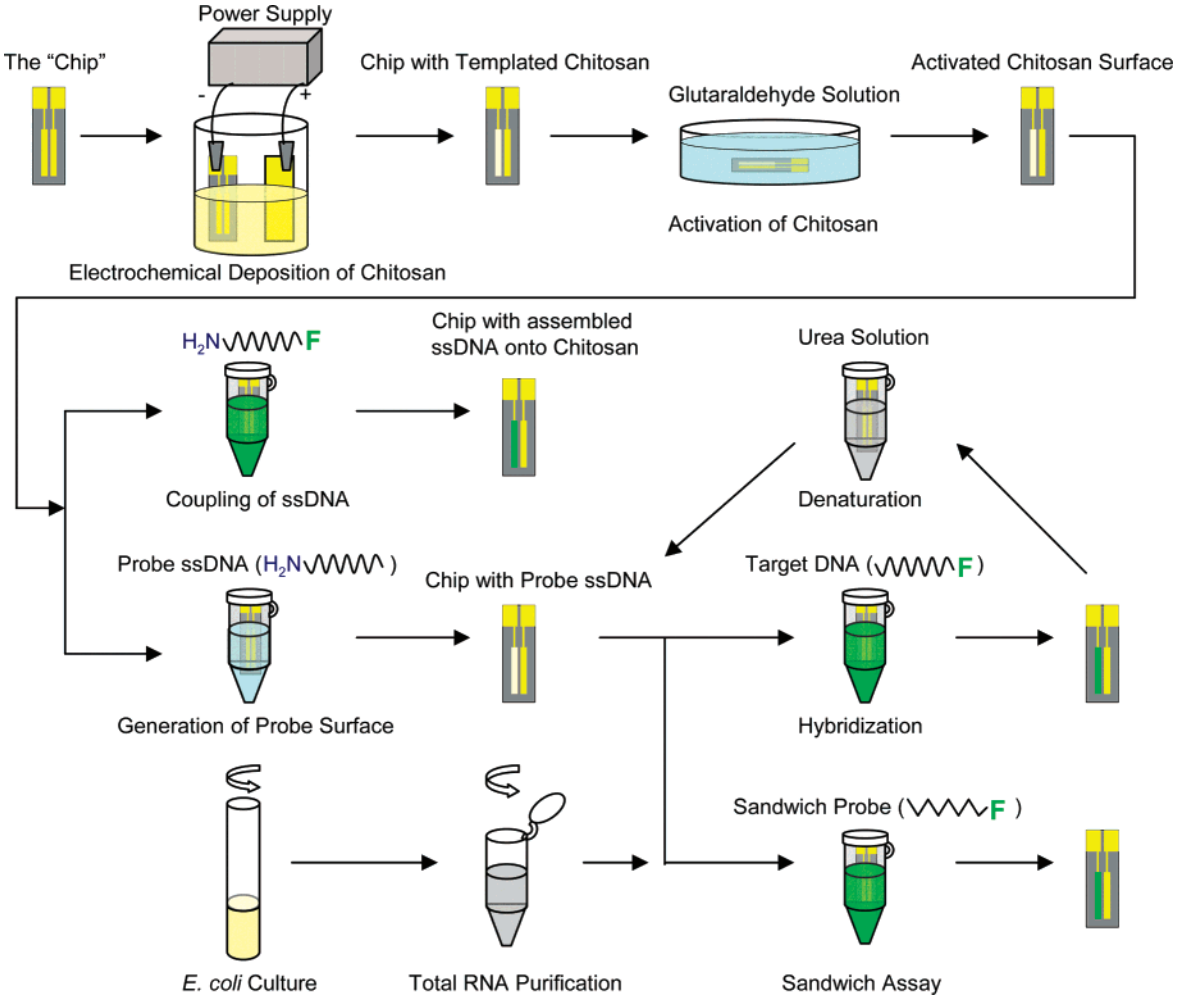
Scheme 1. Schematic Diagram of Assembling the Hybridization Chip and of Hybridization Reactions[a]



[a] The first row represents deposition and activation of the chitosan scaffold. The following rows represent ssDNA assembly, hybridization, and sandwich assay, respectively.

Table 1. ssDNA Sequences and Modifications

ssDNA	5′ end modification	sequence	3′ end modification
fluorescein-labeled and amine-terminated ssDNA	NH_2	CTT TCG CGT TGT TTG CAG AA	FITC
probe DNA (*dnaK*)	NH_2	CTT TCG CGT TGT TTG CAG AA	(none)
fluorescein-labeled target (*dnaK*)	FITC[a]	TTC TGC AAA CAA CGC GAA AG	(none)
mismatched target (*groEL*)	FITC	GTT ACC GAC CTG CCG AAA AA	(none)
sandwich probe	FITC	TAC CAG CTC TTC AAA CTT AC	(none)

[a] FITC, fluorescein isothiocyanate.

base sequence complementary to the target DNA (*dnaK*) sequences extends from the anchoring amine. For rapid visualization, some ssDNA was also fluorescein-labeled at the 3′ end. For mRNA sandwich hybridization assays, the surface probe and sandwich probe DNA are complementary to different regions of *E. coli dnaK* mRNA that are separated by 308 bases. Sandwich probe DNA has a fluorescein label at the 5′ end.

Chip Preparation. The hybridization chip (Figure 1) consists of two gold rectangular patterns (8 mm × 1 mm) separated by a 1-mm gap. Each pattern is linked to a 3-mm × 4-mm rectangular region for alligator clip connection by an 8-mm-long gold connecting line. The patterned surfaces were fabricated by depositing 50-Å-thick chromium and then 2000-Å-thick gold films on 4-in.-diameter silicon wafers, which had previously been coated with 1-μm-thick silicon nitride film. Patterning was achieved using photolithography, in which a primer and then a photoresist were spin-coated onto the gold surface. After soft-baking of the coated wafer at 100 °C for 1 min, a specially designed mask was placed over the surface, and the wafer was exposed to UV light (total dosage, ~190 mJ/cm^2). After 30 s of development, the wafer was then hard-baked at 120 °C for 10 min. The exposed areas were then etched away by gold and chromium etchants, and the photoresist was removed using acetone.[23]

Deposition of Chitosan. Chitosan solution was prepared by adding chitosan flakes to water and incrementally adding small amounts of HCl to the solution to maintain the pH near 3. After

mixing overnight, the chitosan solutions were filtered to remove undissolved material, and the pH was adjusted as indicated using NaOH (1 M).

For deposition, Scheme 1 shows that the patterned chip was immersed in a chitosan solution (pH = 3.7, 0.5 wt % polymer), and one of the patterned gold surfaces was polarized to serve as a negative electrode (i.e., the cathode). The positive electrode in these experiments was an unpatterned gold-coated silicon wafer. The two electrodes were connected to a dc power supply (model 6614C, Agilent Technologies) using alligator clips. Deposition was performed by applying a voltage of 2.1 V for 1 min. After deposition, the chip was removed from the chitosan solution, rinsed briefly with distilled water, disconnected from the power supply, and immersed in 1 M NaOH for 5 min to neutralize the chitosan. After neutralization, the chip was rinsed with distilled water and either equilibrated in 5 mL of buffer at room temperature or stored in buffer at 4 °C until use.

Assembly of ssDNA. Scheme 1 shows that chitosan was activated for ssDNA assembly by placing the chip with templated chitosan in a Petri dish containing 5 mL of 0.1% glutaraldehyde solution for 30 min at room temperature, with 75 rpm gyratory shaking. After glutaraldehyde activation, the chip was rinsed with buffer for 10 min and then immersed in 1 mL of a solution containing 20 μg/mL of the amine-terminated ssDNA surface probe (in a 2-mL microcentrifuge tube). After reaction for 2 h at room temperature, the chip was rinsed with buffer and observed using the fluorescence microscope. To confirm that the ssDNA was covalently tethered, we then rinsed the chip in 4 M urea solution extensively and observed it again. In experiments where we studied the hybridization characteristics of the chip, we performed an additional sodium borohydride reduction step after the buffer wash. For this reduction, we immersed the thoroughly rinsed chip in 5 mL of buffer, added 200 μL of a sodium borohydride solution, and allowed the reaction to proceed for 5 min. The borohydride solution was prepared by dissolving 10 mg of sodium borohydride ($NaBH_4$) powder in 1 mL of buffer.

Hybridization/Denaturation. Hybridization reactions were performed either with purified 20-base ssDNA targets (either a complementary 20-base *dnaK* target or a mismatched 20-base *groEL* target) or with total RNA extracts (for the sandwich assay). In all cases, hybridization was carried out by immersing the chip (with the tethered surface probe) into 1 mL of Sigma PerfectHyb hybridization buffer containing nucleic acid. In some experiments, the nucleic acid was simply the purified targets, while the nucleic acid for the sandwich assay studies included both the total RNA extract (15 μg) and the fluorescently labeled sandwich probe (6 μM). Hybridization was performed for 30 min at room temperature, after which the chip was removed and briefly rinsed with buffer.

For denaturation, the chip was immersed in 1 mL of 4 M urea solution and heated at 65 °C for 30 min, again in a microcentrifuge tube. After denaturation, the chip was briefly washed with buffer.

***E. coli* Culture and Total RNA Preparation.** *E. coli* strain JM105 (*F′ Δlac-pro thi strA endA sbcB15 hspR4 tra36 pro AB*$^+$ *lacI*^{q}*-ZΔM15*),[28] harboring the plasmid pBAD-GFP::CAT,[4] was used to prepare RNA samples. Incubation was initiated by adding 200 μL of seed culture to a test tube containing 5 mL of Luria-Bertonelli (LB) media with ampicillin (100 μg/mL). After 6 h of incubation at 30 °C and 225 rpm (OD_{600} = 0.85), the culture was split in two. One of the cultures was then induced with sterile filtered L-arabinose (0.2%), while no additions were made to the second culture. After 20 min of further incubation, 1-mL samples were collected from each culture and immediately centrifuged (5000*g* at 4 °C). A standard total RNA purification protocol for bacterial cells was used with the Qiagen RNeasy mini kit per the manufacturer's manual. Briefly, centrifuged cells were lysed in a 100-μL lysozyme solution in TE buffer for 5 min at room temperature. After the cell lysate was loaded onto the RNeasy mini spin column, several washing steps were performed by adding different buffers and centrifuging the column. Finally, the total RNA sample was eluted in 40 μL of distilled water. After elution, the total RNA concentration was measured using a UV/vis spectrophotometer.

Analysis. A fluorescence microscope (Leica, MZFLIII) using an excitation wavelength of 480 nm, with 40-nm bandwidth and an emission cutoff filter at 510 nm (FluoIII fluorescence filter), was used to observe fluorescence throughout the study. Photomicrographs were prepared from the fluorescent microscope using a digital camera (Spot 32, Diagnostic Instruments). Scion image analysis software was used to obtain image profiles and to determine mean fluorescence values with standard deviations over the entire patterned area. A UV/vis spectrophotometer (DU 640, Beckman, Fullerton, CA) was used to measure cell growth (i.e., optical density; OD) at 600 nm (OD_{600}), total RNA concentration at 260 nm, and RNA purity at 260/280 nm. For OD_{600} measurement, samples were diluted with distilled water to obtain OD readings in the linear range (0−0.25 OD unit).

RESULTS AND DISCUSSION

Selective Deposition of Chitosan and Subsequent Assembly of ssDNA. To demonstrate assembly of ssDNA onto a patterned surface, we used the chip shown in Figures 1 and 2a. Figure 2a shows a photomicrograph of the chip under the fluorescence microscope with bright field. This chip consists of two gold electrodes that were patterned onto a silicon nitride surface. To selectively deposit chitosan onto the left-most electrode, the chip was immersed into a 0.5% chitosan solution, and the left-most pattern was polarized (negative) at 2.1 V for 1 min. After deposition (i.e., templating) of chitosan onto the cathode, the chip was rinsed with distilled water, neutralized with NaOH, and equilibrated with buffer (pH = 7). The templated chitosan film was then activated by immersing the chip in a glutaraldehyde solution (0.1%). Figure 2b shows that when this "activated" chip was illuminated with 480-nm excitation light, no image was visible (i.e., none of the chip's surfaces are fluorescent).

To couple ssDNA to the activated chitosan surface, we immersed the chip in 1 mL of solution containing 20 μg/mL ssDNA that was amine-terminated at the 5′ end (for coupling) and fluorescein-labeled at the 3′ end (for visualization). Specifically, the ssDNA was a 20-base sequence complementary to a region in the *dnaK* gene of *E. coli*, as shown in Table 1. After reacting for 2 h, the chip was rinsed with buffer. Figure 2c shows the fluorescence image of the resulting assembly of ssDNA. The left-most electrode in Figure 2c is observed to be highly fluorescent, and the fluorescence is spatially well-resolved. Figure 2c shows no noticeable fluorescence for either the right-most electrode (that

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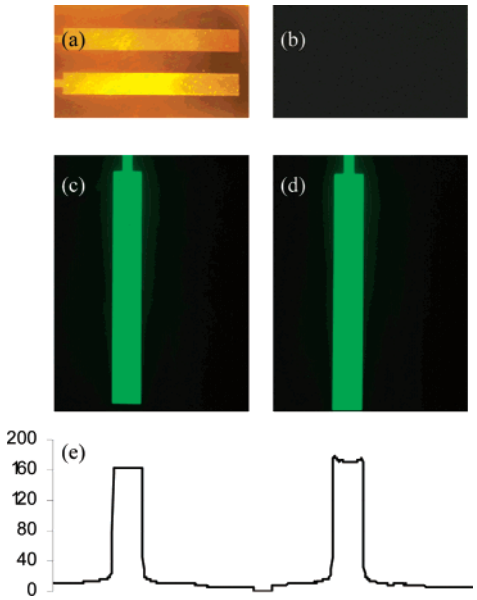

Figure 2. Assembly of ssDNA onto templated chitosan. (a) Bright-field image of gold patterns. (b) Fluorescence image of the chip after glutaraldehyde activation. (c) Fluorescence image of the chip after ssDNA assembly and brief buffer wash. (d) Fluorescence image of the chip after extensive urea wash. (e) Quantified fluorescence profile of (c) and (d), obtained by using Scion image analysis software.

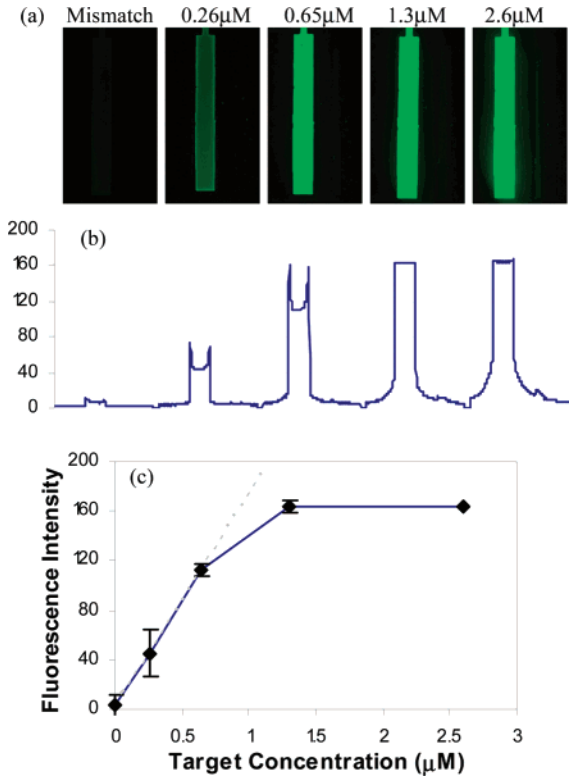

Figure 3. Hybridization of fluorescently labeled target DNA to surface probe DNA tethered to chip. (a) Fluorescence images of DNA−DNA hybridization with corresponding target DNA concentration. All images were obtained after 30 min of hybridization and a 30-s exposure time. Mismatch target DNA concentration was 2.5 μM. (b) Fluorescence profile of images in (a). (c) Mean fluorescence obtained from image analysis of the total fluorescent area. Error bar represents ±one standard deviation.

was not polarized during the chitosan deposition step) or the silicon nitride surface. This observation demonstrates that the individual steps of chitosan deposition, activation, and nucleic acid assembly are well-resolved spatially. As a final test, we performed a thorough urea wash to remove non-covalently bound nucleic acid. Figure 2d shows that the assembled ssDNA was not removed by this urea wash, suggesting that the ssDNA was covalently tethered to the chitosan template.

To further analyze the results in Figure 2, we used standard image analysis software to quantify the fluorescence intensities of the images. Figure 2e shows that the fluorescence profiles have a low and stable background, sharp interfaces, and large signals. Additionally, the urea wash had little effect on the fluorescence intensity profiles (i.e., the profiles from parts c and d of Figure 2 are similar), indicating that the conjugated ssDNA is stable. In conclusion, Figure 2 shows that ssDNA can be assembled onto a templated chitosan pattern with high spatial resolution, and the assembled ssDNA is stable to denaturing conditions.

Selectivity, Sensitivity, and Responsiveness of the Hybridization Chip. In the next series of studies, we examined the selectivity, sensitivity, and responsiveness of our nucleic acid hybridization chip. As described in Scheme 1, the chip was prepared by templating the chitosan onto the cathode surface, activating the templated chitosan with glutaraldehyde, and conjugating an amine-terminated ssDNA surface probe onto the activated chitosan. In contrast to the previous experiment, the ssDNA surface probe was not fluorescently labeled. To enhance the chip's long-term stability, we further treated the chip with sodium borohydride, so the linkages that tether the nucleic acid probe to chitosan were converted from Schiff base to more stable secondary amine linkages.[27]

The selectivity of the nucleic acid hybridization chip was first examined by challenging the probe with a solution containing a high concentration (2.5 μM) of ssDNA that had mismatching sequences. This 20-base mismatched "target" has sequences that are a part of the *groEL* gene from *E. coli*, with only five consecutive bases that are complementary to the surface probe ssDNA. Additionally, this mismatched "target" was fluorescently labeled at the 5′ end. In the experiment, the chip was immersed in the challenge solution for 30 min, after which it was rinsed and observed using a fluorescence microscope. The left-most photograph in Figure 3a shows that little fluorescence appears on this chip. Thus, little hybridization occurred between the fluorescently labeled mismatched ssDNA and the assembled probe. The fluorescence intensity profile of Figure 3b further confirms that hybridization between the probe and the mismatching ssDNA is small.

To examine the chip's sensitivity, we then exposed the probe to a solution containing a low concentration (0.26 μM) of target ssDNA. This target had a 20-base sequence that is fully complementary to the surface probe's sequence, and the target was also fluorescently labeled at the 5′ end. As shown in the second photograph in Figure 3a, detectable fluorescence is observed after exposure of the probe to a low concentration of this labeled target. The results from this photograph are also supported by the fluorescence intensity profile in Figure 3b, which indicates that the fluorescence signal is significantly above background noise when the chip is exposed to subnanomolar quantities of target (the 0.26 μM concentration corresponds to 0.26 nmol of target

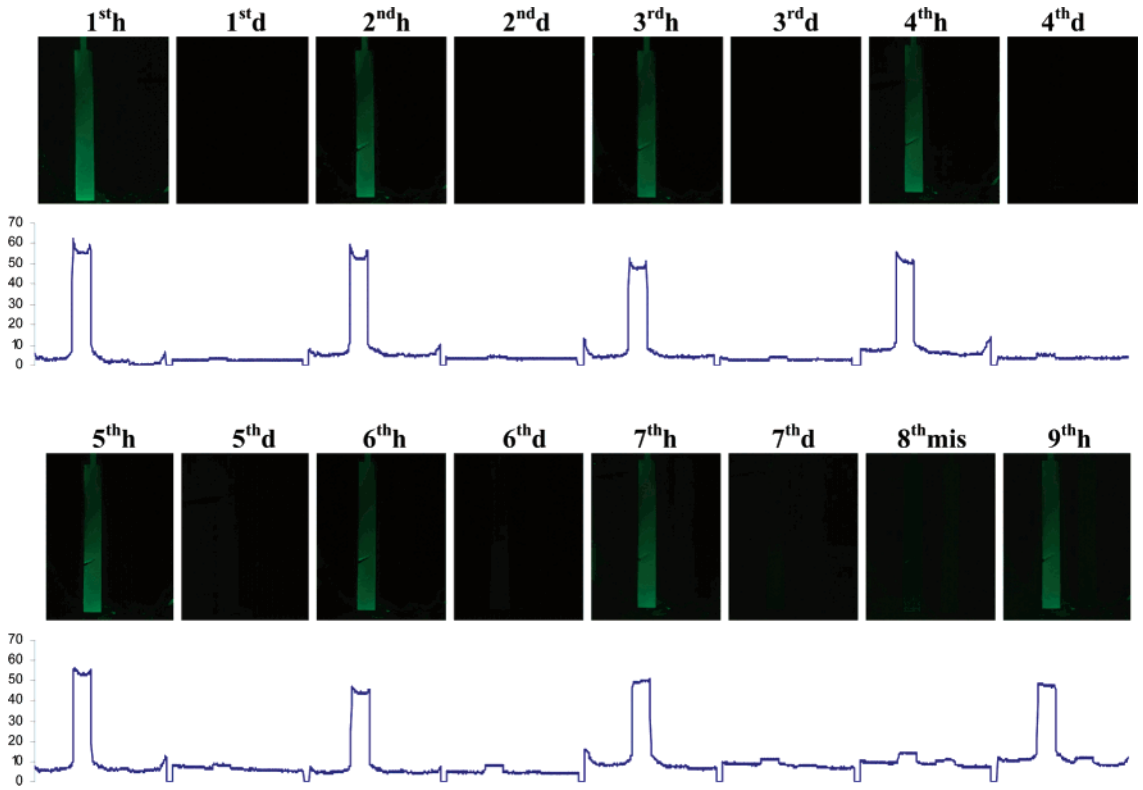

Figure 4. Repeated hybridization/denaturation of fluorescently labeled target DNA to surface probe DNA tethered to chip. "h" and "d" denote hybridization and denaturation, respectively. "8thmis" denotes eighth hybridization using 2.5 μM mismatched target DNA. Final hybridization was performed using matched target DNA after 8th hybridization.

molecules). It is important to note that this target concentration yielded a high signal-to-noise ratio (~12), suggesting that our actual sensitivity could be substantially improved without diminishing statistical significance.

To test the responsiveness of the surface probe, we then performed a second hybridization by immersing the same chip in a solution containing 0.65 μM of the fluorescently labeled complementary target. As indicated by the photograph (Figure 3a) and intensity profile plot (Figure 3b), the fluorescence of the chip was increased by exposing it to this higher target concentration. As indicated by the plot in Figure 3c, the increase in fluorescence intensity is proportional to concentration for these target concentrations (up to 0.65 μM). Figure 3 visually indicates that, when this chip was exposed to higher target concentrations, more target hybridized to the chip, although the measured fluorescence intensity did not increase proportionally. Visual observation of the chip indicated that the chip was more fluorescent after it had been exposed to 2.6 μM than after it had been exposed to 1.3 μM, as suggested by the higher fluorescence in the electrode-flanking region in the profile for 2.6 μM in Figure 3b. We believe the apparent saturation behavior observed in Figure 3c is due to either saturation of available surface probe or limitations in the digital camera used to capture the images in Figure 3. In addition to this apparent saturation, fluorescence images at the lower target concentrations showed enhanced fluorescence along the pattern edges. This could be due to uneven chitosan deposition at the edges and higher probe DNA densities in these regions. In summary, the results in Figure 3 (which represent spatially integrated signal) demonstrate that, when the probe is sequentially exposed to higher target concentrations, hybridization increases.

Stability and Reliability of Chip for Repeated Use. To examine the stability and reliability of the nucleic acid hybridization chip, we exposed our chip to a sequence of hybridization and denaturation steps. Initially, the chip was immersed in a solution containing 0.35 μM of the fluorescently labeled and complementary 20-base target DNA. After 30 min of hybridization, the fluorescence of the chip was examined. The image and intensity profile (Figure 4) show significant fluorescence results from this initial hybridization step (designated "1sth"). To remove target nucleic acid from the probe, the chip was subjected to denaturing conditions of 4 M urea and 65 °C for 30 min. After a brief rinse with buffer, the fluorescence of the chip was examined. The photograph and intensity profile after this first denaturation step (designated "1std" in Figure 4) indicate that the hybridization was completely reversed by this treatment.

This sequence of hybridization and denaturation was repeated for seven cycles. Hybridization was performed using the *same* target solution, while fresh urea solutions were used for each denaturation step. As illustrated in Figure 4, the average fluorescence intensity varied from 44 to 55 after hybridization and from 3 to 11 after denaturation. These results indicate that hybridization is reversible and the capacity of the probe does not decay upon repeated exposure to denaturing conditions. It is interesting to note that we accidentally scratched the left edge of the probe during the second hybridization step, and this scratch remains visible in all subsequent images.

To ensure that the probe surface retains selectivity even after these repeated cycles, we then exposed the chip to a high concentration (2.5 μM) of mismatching target DNA (the 20-base sequence of *groEL* that was fluorescently labeled). Although identical hybridization conditions were used, the results designated "8thmis" in Figure 4 indicate that no hybridization had occurred with this mismatched sequence. Immediately after challenging this probe with the mismatched sequence, we finally exposed the chip to the *same* complementary target DNA solution as used for the first hybridization cycles. As indicated in Figure 4, "9thh", the probe was still able to hybridize to its complementary target, and hybridization was qualitatively and quantitatively similar to that observed in the first seven cycles.

In conclusion, the nucleic acid hybridization probe was shown to be robust and retained its selectivity and capacity after repeated exposures to hybridization and denaturation conditions. That is, the electrodeposited chitosan, when coupled with covalently attached oligo probes, exhibited sufficient mechanical binding strength for repeated use.

Sandwich Assay of Total RNA Samples. To examine if our nucleic acid hybridization chip can be used to detect changes in gene expression at the transcription level, we performed a sandwich hybridization assay using total RNA samples obtained from *E. coli* cultures. As shown in Figure 5a, our sandwich assay consists of three components. First, surface probe DNA is assembled onto the chitosan-coated electrode. This surface probe has a 20-base sequence that is complementary to a part of *dnaK* gene mRNA. The second component in this assay is the target mRNA from the total RNA fraction that was prepared from the *E. coli* cultures. The final component is the fluorescently labeled sandwich probe DNA that served as the reporter molecules. This sandwich probe is 20-base ssDNA, with sequences complementary to a region in *dnaK* mRNA. The surface probe and sandwich probe are complementary to regions of *dnaK* mRNA that are 308 bases apart.

In our initial study, we demonstrated that excess sandwich probe DNA does not interfere with our assay to give false positives. To show this, we exposed the chip's surface probe to a solution containing a high concentration (6 μM) of the fluorescently labeled sandwich probe for 30 min under hybridization conditions. The left-most photograph in Figure 5a shows that hybridization between the surface and sandwich probe is not significant.

To prepare total RNA samples, *E. coli* JM105 was grown in LB medium for 6 h until OD_{600} reached 0.85. At that time, the culture was split in two—one part was induced with a 0.2% final concentration of arabinose while the other was uninduced. At 20 min after induction, 1 mL of each culture was harvested and immediately centrifuged at 4 °C. Total RNA was purified by standard procedure for bacterial samples using a Qiagen RNeasy mini kit. For the sandwich assay, 15 μg of this total RNA fraction was added to 1 mL of a hybridization solution containing 6 μM sandwich probe DNA in a 2-mL microcentrifuge tube. This solution was then contacted with the hybridization chip coated with the surface probe DNA. The chip and solution were incubated for 30 min for hybridization. The second photograph in Figure 5a shows that significant fluorescence was detected when the induced sample was examined using this sandwich assay format. For comparison, the right-most photograph in Figure 5a shows

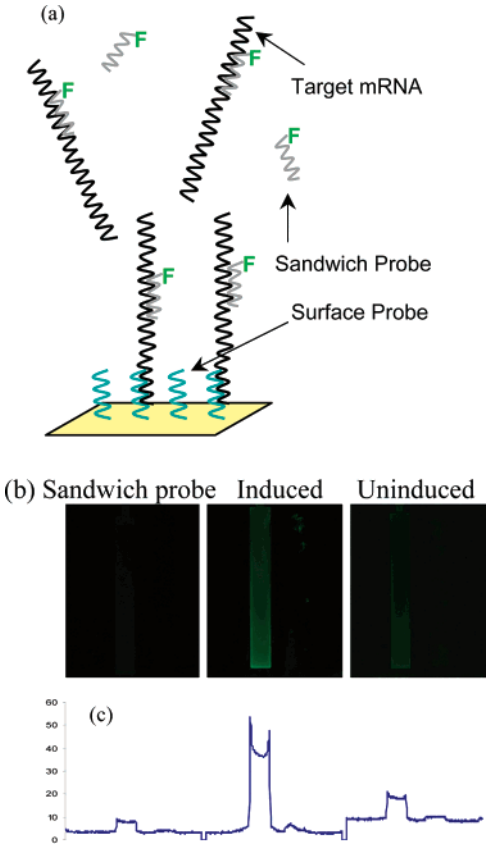

Figure 5. Sandwich hybridization assay using total RNA samples. The hybridization mixture consisted of 15 μg of purified total RNA from *E. coli* culture (see Experimental Section) and 6 μM sandwich probe DNA in 1 mL of hybridization buffer. (a) Schematic diagram of the sandwich assay, comprising surface probe DNA assembled onto templated chitosan, target mRNA, and labeled sandwich probe DNA. (b) Fluorescence images upon hybridization for sandwich probe DNA solution without an RNA sample, and for induced samples and uninduced samples, respectively. (c) Corresponding fluorescence intensity profiles for (b).

considerably less fluorescence for the uninduced sample. *E. coli* are known to express low, constitutive levels of *dnaK*, and that expression is increased by the stress associated with induction for high-level expression of a foreign protein.[12] The corresponding fluorescence intensity profiles for samples are shown in Figure 5b and confirm the differences in *dnaK* transcription between the induced and uninduced samples.

In conclusion, Figure 5 shows that the sandwich hybridization assay with our nucleic acid hybridization chip provided a simple and rapid approach for detecting gene transcription. The total processing time was less than 1 h, and this includes 20 min for total RNA preparation and 30 min for hybridization. This approach allows target mRNA to be detected without the need for sample labeling or amplification, and analysis was performed in the presence of many potential interfering species, such as other gene transcripts, ribosomal and transfer RNAs, and excess amounts of sandwich probe.

CONCLUSIONS

Chitosan is a unique material that facilitates the assembly of labile biological sensing molecules onto microfabricated surfaces. We exploit chitosan's pH-responsive electrostatic properties to

selectively deposit chitosan onto patterned cathode surfaces, while chitosan's nucleophilic properties allow the amine-terminated ssDNA oligonucleotide probe to be readily conjugated to the "templated" chitosan. In this study, we used standard glutaraldehyde coupling chemistries to covalently tether the ssDNA at its 5′ terminus. We believe this chitosan-based assembly method is simple compared to other spatially selective assembly approaches. Chitosan-based assembly does not require the complex printing equipment characteristic of arrayer systems. Additionally, chitosan-based assembly does not require careful surface preparation, as is the case when microcontact printing[17] is used to covalently anchor a probe molecule, or when common covalent coupling procedures are used to couple DNA onto solid surfaces.[13,29] We must note that the initial patterning of our chip does require microfabrication under controlled conditions; however, these operations are fairly routine and can be performed far "upstream" from the end-user (i.e., the deposition, activation, and conjugation steps do not require that the end-user possess specialized skills or equipment).

The results demonstrate that the nucleic acid hybridization chip is sensitive and robust, and facilitates rapid analysis. The probe is sufficiently sensitive to measure subnanomole quantities of target molecules and to detect differences in stress response between induced and uninduced bacterial cultures. The probe is robust and provides reproducible measurements with a high signal-to-noise ratio—even after repeated hybridization and denaturation cycles. Hybridization and denaturation with this chip was fast and occurred within the 30 min we allowed. Although we did not study other times, we anticipate that analysis time could be reduced if necessary. Additionally, we believe that the ideas presented in this paper can be implemented within microfluidic devices such as BioMEMS for simultaneous sample processing and detection, among other applications. In summary, chitosan-based assembly is simple, and the covalently tethered probes are robust.

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