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Anal. Chem., **2008**, 80 (15), 5899-5904• DOI: 10.1021/ac800445p • Publication Date (Web): 02 July 2008

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Dip Pen Nanolithography Functionalized Electrical Gaps for Multiplexed DNA Detection

Shifeng Li, Sandra Szegedi, Edgar Goluch, and Chang Liu*

Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign 208 North Wright Street, Urbana, Illinois 61801

Nanoparticle-based, silver-enhanced DNA electrical detection shows great promise for point-of-care diagnostics. In this paper, we demonstrate that the dip pen nanolithography (DPN) method can be used to precisely functionalize multiple electrical gaps for multiplexed DNA detection. With the use of the DPN technique, capture ssDNAs are written inside 5 μm × 10 μm electrical gaps on substrates. The DPN functionalized electrical gaps can specifically hybridize to target ssDNAs in solution. Successful hybridization of the capture-target DNA complex is detected by the use of gold nanoparticles carrying ssDNA, which also hybridize to the target ssDNA, followed by silver enhancement. The drop of resistance across the gaps due to the formation of metal nanoparticle−DNA complexes is measured over time and compared against characteristics of control gaps, which are either left unfunctionalized or functionalized with noncomplementary capture ssDNA. This technique has potential for high-density multiplexed DNA assay chips. Multiplex detection of two different target ssDNAs in solution using DPN functionalized electrical gaps on the same chip is demonstrated. The lowest detection limit is 10 pM.

The detection of DNA sequences is of particular interest in genetics, pathology, criminology, pharmacogenetics, food safety, and many other fields. Many advances have been made in the development of techniques to monitor binding/recognition events of DNA biomolecules in solution and on solid substrates.[1–5] A promising technique is nanoparticle-based electrical DNA detection.[6–15] An electrode gap is made with the gap region functionalized with a capture ssDNA molecule. This detection scheme measures a drop of resistance after the binding of ssDNA-containing gold nanoparticles by specific DNA hybridization interactions. Electrical detection has the potential to simplify detection instruments and minimize their sizes.[13]

Pipetting is usually used to spot capture ssDNA molecules inside the electrical gaps of substrates.[11,15] Spotting by pipetting may result in a large size functionalized area, typically 100 μm × 100 μm. Such large sizes of functionalization area are not amenable for the development of high-density multiplexed detection chips and consumes large amount of capture oligonucleotide material. With the use of pipet spotting, it is impossible to produce functionlization area below 10 μm × 10 μm. Therefore, it is advantageous to develop new functionalization techniques that overcome the aforementioned drawbacks of spotting methodology.

Dip pen nanolithography (DPN) is an emerging technique to fabricate nanoscale chemical patterns. It uses a sharp scanning probe, or an array of scanning probes, to transfer chemical ink onto solid substrates. These inks include small organic molecules, peptides, proteins, oligonucleotides, and inorganic sol−gel.[16–22] In this technique, the AFM tip is brought into close proximity to the substrate under proper conditions. Ink biomolecules transport from the tip of an atomic force microscope (AFM) to a substrate surface via a water meniscus. The typical dimension of biomolecule patterns on the substrates fabricated by DPN is micrometer or submicrometer size.[16,20] Therefore, DPN can easily produce

* Corresponding author. Current address: Chang Liu, Mechanical Engineering Department, Northwestern University, Room L288, Tech Institute, 2145 Sheridan Rd., Evanston, IL 60208. Phone: +1 847 467 0701. E-mail: changliu@northwestern.edu.

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10.1021/ac800445p CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/02/2008

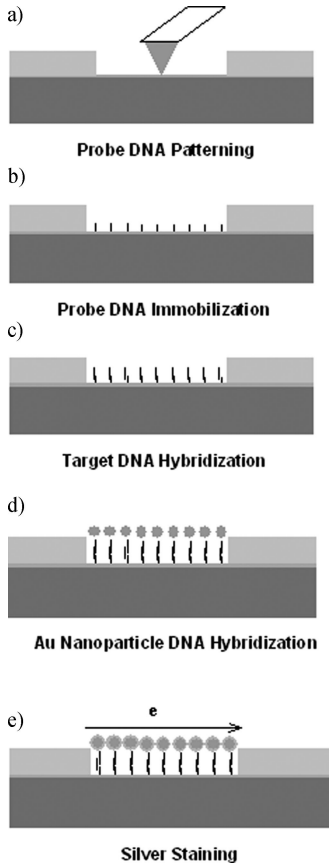

Figure 1. Schematic of the nanoparticle-based, silver-enhanced DNA electrical detection procedure. (a) A DPN probe is used to write chip ssDNAs inside an electrode gap. (b) DPN-written chip ssDNAs are allowed to cross-link with the surface inside the electrical gap chip. (c) Target ssDNAs hybridize with immobilized chip ssDNAs. (d) AuNP probes hybridize with complementary region of the target ssDNAs. (e) Silver staining of the chip results in micrometer-sized silver-coated gold nanoparticles that can conduct an electrical current.

precise and user-defined patterns inside confined, micrometer-sized electrical gaps.

We report the use of DPN as a technique to functionalize electrical gaps for DNA detection instead of pipet spotting. Further, we show the proof of concept of functionalizing multiple gaps in an array for multiplexed detection. Using the DPN technique, specific capture ssDNAs (also known as chip DNAs) are precisely immobilized inside micrometer-sized electrical gaps (Figure 1a). In the presence of target ssDNA molecules, DNA-functionalized gold nanoparticles are hybridized locally inside the electrode gaps. When the proper [chip DNA-target DNA-AuNP probe DNA] hybridization complex is formed, these metal particles can bridge the electrode gap and conduct current. Consequently, the resistance across the gap drops, indicating successful DNA detection. A subsequent silver staining step increases

Figure 2. Schematic of a typical electrical gap DNA chip.

the sizes of nanoparticles, enhancing the drop of resistance measured across the gap for a given target molecule concentration. By leverage of the silver enhancement, a detection limit of 10 pM is established using the DPN functionalized electrical gaps. In contrast, our study shows we can achieve a detection limit of 1 μM readily without the silver enhancement step.

MATERIALS AND METHODS

Oligonucleotides and Silane Chemicals. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by HPLC. The sequence of each oligonucleotide is listed in Table 1. Glycidoxypropyltrimethoxysilane (GOPS), used to functionalize substrates, and 3-aminopropyltrimethoxy silane (APTMS), used to functionalize scanning probes, were purchased from Sigma Aldrich (St. Louis, MO). All other reagents were of the highest purity available from commercial vendors.

Electrical Gap Chip Fabrication and Preparation. An electrical gap chip may contain multiple electrode gaps, each functionalized with different capture DNA sequences or left unfunctionalized intentionally. The chips in this study are designed to contain four gaps (Figure 2). One or two gaps are used for target ssDNA recognition by complementary immobilized capture ssDNA, and the others are used as negative controls (either left unfunctionalized or functionalized with noncomplementary capture ssDNA). The electrical gaps are photolithographically fabricated on a silicon substrate with a 1 μm thermally grown silicon dioxide electrical isolation layer. Gold is chosen as the electrode material due to its chemical stability. The thickness of the gold electrode layer and Cr adhesion layer is 60 and 5 nm, respectively. The dimension of gaps on the substrates is 5 μm wide and 10 μm long. The 1 μm silicon dioxide coated silicon chips with microfabricated

Table 1. Oligonucleotide Sequences

name	sequence
LT68 chip DNA	5′-TCT AAC TTG GCT TCA TTG CAC CGT T-amine-3′
LT68 target DNA	5′-AAC GGT GCA ATG AAG CCA AGT TAG ATT TTT TTT TTT TTT TTT TTT-3′
NP	5′ thiol-AAA AAA AAA AAA AAA AAA AA-3′
LT114 chip DNA	5′-AAT GCT CAA TGG ATA CAT AGA CGA GG-amine-3′
LT114 target DNA	5′-CCT CGT CTA TGT ATC CAT TGA GCA TTT TTT TTT TTT TTT TTT TTT-3′

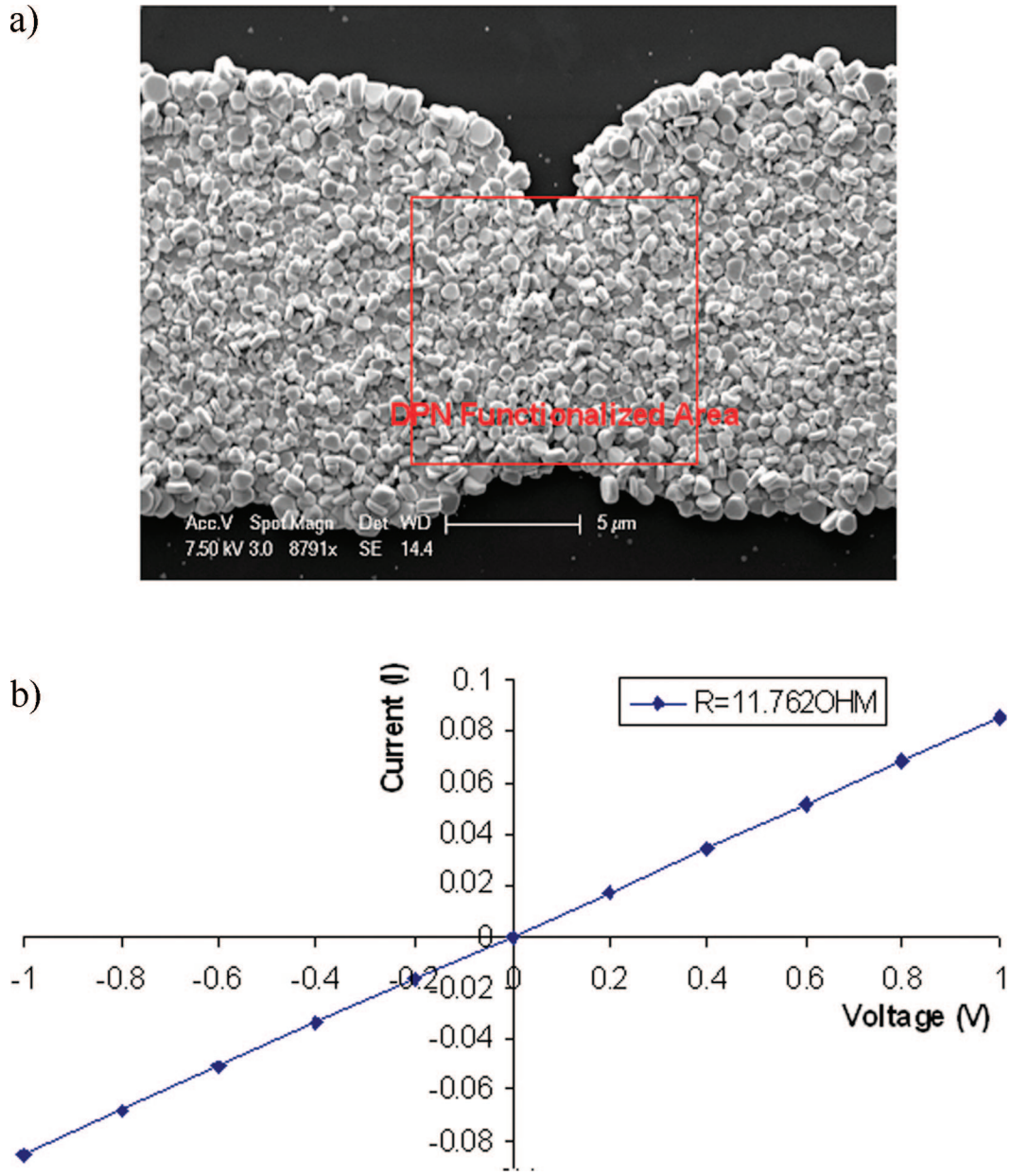

Figure 3. (a) SEM picture showing the closing of two microelectrodes after 15 min silver staining and (b) the *I*−*V* curve measurement for the stained electrical gap shown in Figure 4a.

electrical gaps are cleaned with piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2) and RCA1 (5:1:1 = H_2O/NH_4OH/30% H_2O_2) for 20∼30 min at 120 °C. The cleaned substrates are then immersed into a 1% (v/v) solution of GOPS in toluene for 1 h, followed by washing the substrates with copious toluene and cross-linking at 80 °C for 10 min. Silanization of the electrical gap chips with GOPS results in the generation of epoxy groups over the surface of the chip.

Gold Nanoparticle Functionalization with Probe ssDNA. Gold nanoparticles (13 nm in diameter) functionalized with NP ssDNA ("AuNP probes") were prepared as described.[23–26] First, freshly prepared 0.1 M dithiothreitol (DTT) in 0.17 M sodium phosphate buffer (pH 8.0) was added into lyophilized NP ssDNA to cleave the disulfide bonds. After sitting at room temperature for 2 h, the cleaved DNA was separated from the DTT solution using an Amersham NAP-5 column (catalog no. 17-0853) equilibrated in nuclease-free water. Column fractions containing freshly cleaved thiol-containing NP ssDNA were quantitated using a Nanodrop spectrometer and immediately added to Au nanoparticles in water using the ratio of 1 OD of thiol-containing NP ssDNA to 1 mL of Au colloid. The DNA−Au colloid mix was placed in an Eppendorf Thermomixer shaker at 300 rpm and allowed to mix undisturbed for 16 h at room temperature. Subsequently, colloid salting was done in a stepwise fashion to 0.3 M NaCl, 10 mM phosphate buffer, 0.01% SDS, pH = 7.2 with shaking over 1 day. The AuNP probe was purified from excess DNA by centrifugation at 14 000 rpm at 20 °C for 25 min. The AuNP probe preparation was washed and centrifuged three more times using 0.5 M NaCl, 10 mM phosphate buffer, 0.01% Tween 20, pH = 7.2 (scanometric buffer) as a wash buffer. The AuNP probe preparation was then resuspended in scanometric buffer at a concentration of ∼10 nM and stored at 4 °C prior to use.

Functionalization of Electrical Gaps Using DPN. Commercial AFM tips (Nanoink, Chicago, IL) were cleaned with

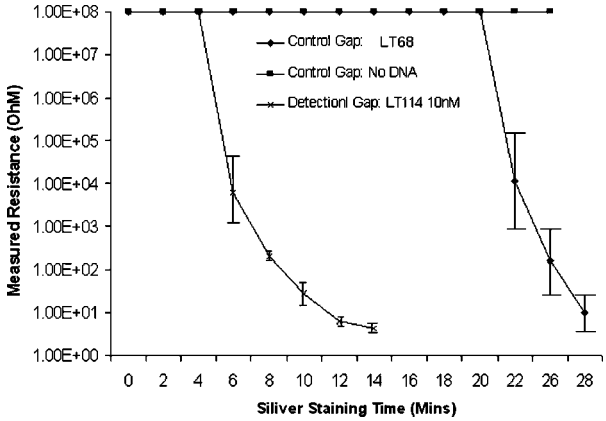

Figure 4. Silver-staining time course study of a chip containing gaps filled with either complementary or noncomplementary chip DNA or no DNA. Resistance measurements were taken every 2 min.

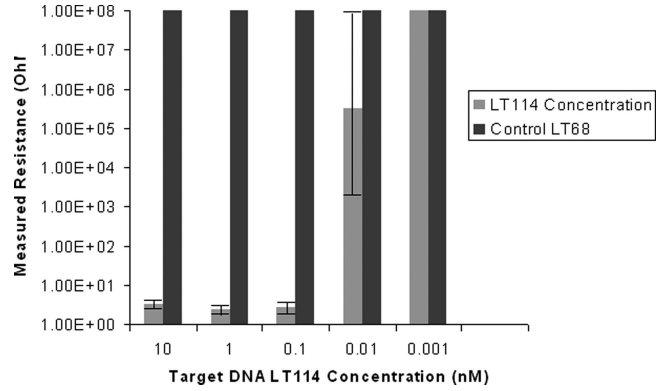

Figure 5. Graph of resistance measurements of electrical gap chips treated with different concentrations of LT114 target DNA. The silver-staining time for each chip was 20 min.

Table 2. Arrangement of Chip DNAs for Multiplex DNA Electrical Detection

chip	gap 1Chip DNA	gap 2Chip DNA	gap 3Chip DNA	gap 4Chip DNA	target DNA(s)
1	LT68	LT68	LT114	none	LT68
2	LT114	LT114	LT68	none	LT114
3	LT114	LT68	none	none	LT68 and LT114
4	LT114	LT68	none	none	none

piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2) followed by immersion into 1% (v/v) 3-aminopropyltrimethoxy silane (APTMS) in 95% ethanol to cover the surface of the tips with positively charged amino functional groups. The silanized tips were then immersed in 10 nM chip ssDNA solution to "ink" the tip. Since the DNA backbone is negatively charged, the positive charge of the surface of the silanized tips can easily absorb a sufficient amount of the chip ssDNA.

After the functionalization of the AFM tips and the electrical gap chips, the inked AFM tips were loaded on the Nscriptor machine (Nanoink, Skokie, IL) to directly write chip ssDNA in the gaps of the chip at 50% relative humidity (RH). The dimension of the DPN design pattern was 10 μm and bigger than the length of the electrical gaps (5 μm) to ensure that the DPN DNA pattern on the substrate connected the two separated electrodes. After DPN patterning, the substrates were incubated at 26 °C and 50% RH for 1 h to allow cross-linking between the amine group of the chip ssDNA (LT68 or LT114) and the epoxy functional group on the silanized surface of the electrical gap chip. The DPN patterned electrical gap chips were then washed with copious nuclease-free water to remove residual nonreacted chip ssDNA, followed by a 20 min incubation in nuclease-free water at room temperature to quench unreacted epoxy groups.

To functionalize multiple gaps with different chip DNA molecules, we used individual DPN probes and functionalized them differently. Each DPN probe was mounted on the DPN instrument separately and used to functionalize a gap. Successive DPN functionlization results in a multiplexed chip.

DPN Functionalized Electrical Gaps DNA Bioassay. After the DNA functionalized electrical gap chips were rinsed with phosphate buffered saline (PBS) solution, pH 7.4, they were immersed into 1% BSA in PBS solution for 20 min at room temperature to block the entire surface of the chip to prevent nonspecific binding of target ssDNA or the AuNP probe during the electrical DNA detection procedure. The substrates were then washed with 0.3 M PBS three times and incubated with 10 nM LT68 or LT114 target ssDNA in scanometric buffer for 4 h at room temperature. After target ssDNA incubation, the chips were washed three times with 0.3 M PBS to remove nonhybridized target ssDNA. This was followed by incubation with 0.1 nM AuNP probe solution for 2 h at room temperature. Unhybridized AuNP probe was then rinsed away with copious amounts of 0.3 M PBS solution. The chips were then washed three times with 0.3 $NaNO_3$ in 10 mM phosphate buffer (pH 7) to remove chloride ions. Finally, the 0.3 $NaNO_3$ solution was removed and silver stain solution (mixing an equal volume of Nanosphere silver stain solution A with solution B) was added. The substrates were stained for up to 30 min, washed three times with Nanopure water, and dried under a stream of nitrogen.

RESULTS AND DISCUSSION

Electrical Characteristics of Gaps. Figure 3a is a representative SEM image of an electrode gap with cDNA hybridization [LT114 chip DNA-LT114 target DNA-AuNP probe] complexes after 15 min silver staining. An $I-V$ curve (Figure 3b) of the filled gap indicates that the resistance of the filled gap is 11.76 Ω. The resistance measurement of parallel control gaps containing noncomplementary LT68 chip DNA or no chip DNA was infinity, indicating that proper DNA complexes did not form and nanoparticle-based, silver-enhanced DNA detection did not occur (data not shown).

We investigated the effect of silver staining time on the differentiation of the detection gap and the control gaps next. The resistance of each gap and the differentiation among gaps is a function of staining time. We conducted a silver-staining time-course study over 30 min (Figure 4). The chips used in the study contained two gaps functionalized with LT114 chip DNA, one gap functionalized with LT68 chip DNA, and one gap with no chip DNA functionalization. A solution containing LT114 target DNA (at a concentration of 10 nM) was used in this time course study. It can be observed from Figure 4 that when a proper nanoparticle-containing DNA hybridization complex is formed ([LT114 chip

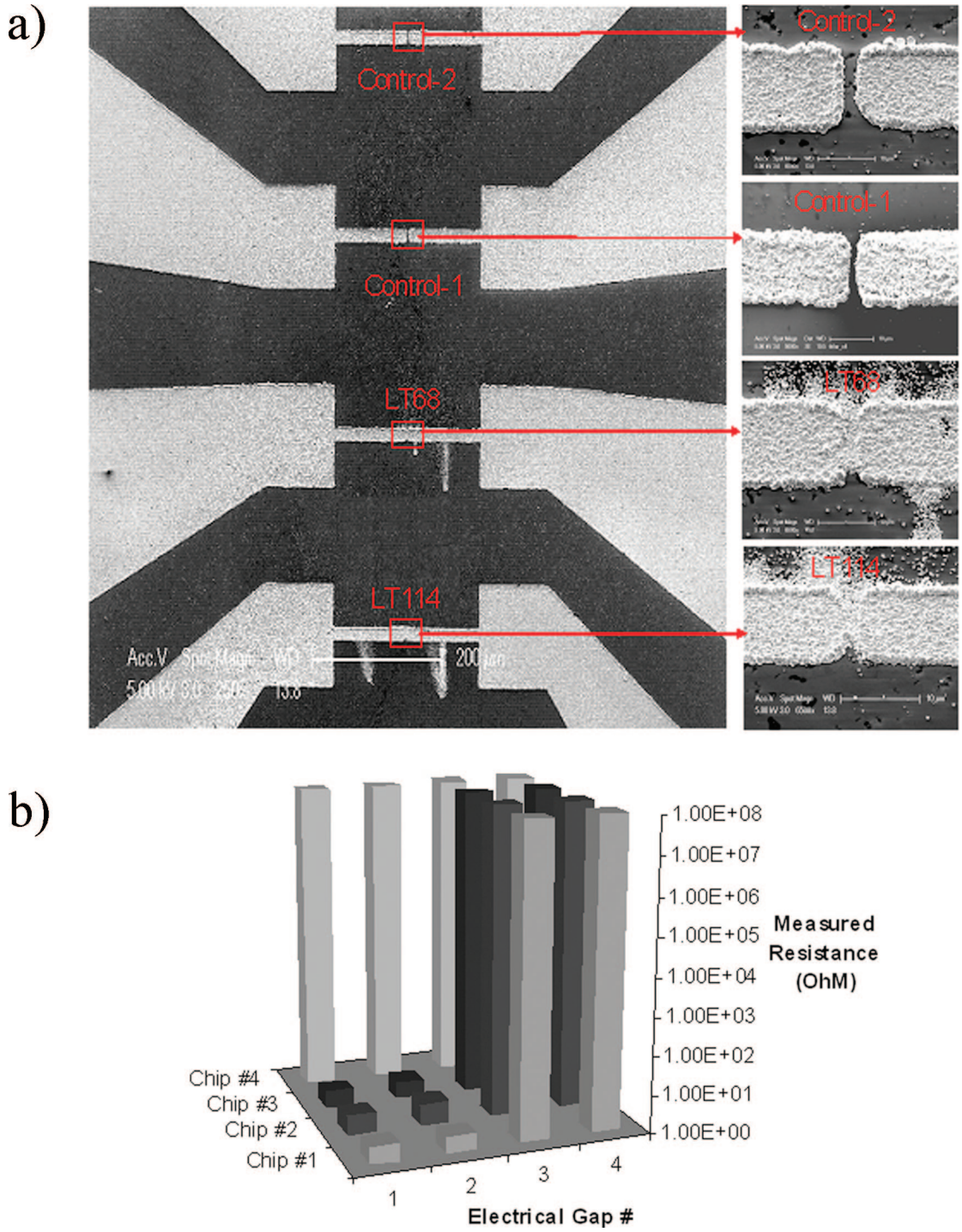

Figure 6. (a) SEM picture of chip no. 3 for multiplex detection of LT68 and LT114 (Insets are the large view of four gaps on chip no. 3 after assay.) and (b) graph of resistance measurements for electrical gap chips used for DNA multiplex electrical gap detection experiments.

DNA-LT114 target DNA-AuNP probe]), a measurable drop in resistance is seen from 6−12 min silver-staining time. During this time, the control gaps containing noncomplementary chip DNA (LT68 chip DNA) or no DNA exhibit infinity resistance readings. By 26 min, nonspecific silver-staining of the gap containing LT68 chip DNA occurs as indicated by a drop in resistance, whereas the gap with no chip DNA shows measurable resistance even at 30 min staining time. This study shows the optimal time for silver staining to differentiate detection gaps and control gaps. Under-staining (e.g., <6 min) will not differentiate various gaps, whereas overstaining (e.g., over 20 min) will result in nonspecific detection as well.

Detection Limit of DPN Functionalized Electrical Gap DNA Detection Chips. After the optimal silver-staining time was determined, we characterized the detection limit (lowest target

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DNA concentration) that would give a resistance measurement. We used the same experimental conditions as described for the silver-staining time course study. For these experiments, five electrical chips were generated in a batch, each containing four gaps. In two gaps, LT114 chip DNAs were present, while the other two gaps contained LT68 chip DNA and no DNA. Each of the five chips was used to detect 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM LT114 target DNA, respectively. Two batches of DPN functionalized chips were used for the experiment. The measured resistances of the detection gaps and the control gaps after 20 min of silver staining are plotted in Figure 5. The chips treated with 100 pM, 1 nM, and 10 nM LT114 target DNA gave measurable resistance readings (~10 Ω) in gaps with immobilized LT114 chip DNA, whereas the control gaps (LT68 chip DNA and no DNA) gave no measurable readings. Thus, at these concentrations of target DNA, the gaps were filled with enough silver-coated nanoparticle complexes to generate a current across the electrode. At a target DNA concentration of 10 pM, a relatively large fluctuation in resistance was observed, suggesting that the threshold of detection was reached. At 1 pM target DNA, no measurable drop of the resistance was found. The 10 pM target DNA detection limit of the DPN-functionalized electrical gap chip is comparable to detection limits found in other studies using electrical DNA detection.[12,13,27]

Multiplex DNA Detection Using DPN Functionalized Electrical Gaps. With the use of the DPN technique, it is possible to functionalize multiple gaps in close physical proximity with different molecules, taking advantage of the small functionalized area and spatial registration of the DPN instrument. We tested the capability of DPN-functionalized electrical gap chips to perform multiplex DNA detection of two target DNAs, LT68 and LT114, at a concentration of 10 nM. Assay conditions were identical to those used for the silver-staining time course experiment. Four chips were used in this study. The functionalization of each gap with chip DNA for each chip is indicated in Table 2. Chip no. 1 was used to detect LT68 target DNA. Chip no. 2 was used to detect LT114 target DNA. Chip no. 3 was designed to perform multiplex detection of both LT68 and LT114 target DNAs. Chip no. 4 was functionalized exactly as chip no. 3 but was treated with buffer solution without target DNA present. After the DNA hybridization procedure and a 20 min silver enhancement step, the resistance of all gaps was measured. As expected, chip no. 1 specifically detected the presence of LT68 target DNA with a resistance of approximately 10 Ω. Conversely, the two control gaps (one functionalized with LT114 chip DNA and the other without chip DNA) gave an infinity resistance reading. Likewise, chip no. 2 detected the presence of LT114 target DNA with a resistance of ~10 Ω. Again, the two control gaps (one functionalized with LT68 chip DNA and the other without chip DNA) were open, indicating that no nanoparticle-specific or nonspecific silver staining occurred. Chip no. 3 successfully detected the presence of both LT68 and LT114 target DNAs, thus indicating multiplex DNA detection, whereas the control gaps were open (Figure 6a). No measurable resistance was detected for the gaps on chip no. 4, indicating that the multiplex DNA detection on chip no. 3 was specific. The measured resistance of all the gaps is plotted in Figure 6b.

CONCLUSION

We have demonstrated that DPN can be used to functionalize multiple electrical gaps for sensitive and specific multiplex DNA detection. The chip ssDNAs were precisely written inside micrometer-sized electrical gaps area using DPN. We found that the DPN-functionalized electrode gaps specifically detected as low as 10 pM target ssDNA in solution. Finally, this technique can be further developed for high-density multiplex electrical gap chips using DPN for highly sensitive disease diagnostics and genetic research.

ACKNOWLEDGMENT

The authors wish to thank the financial support of the NSF Nanoscale Science and Engineering Center (NSEC) headquartered at the International Nanotechnology Institute at the Northwestern University, Evanston, IL.

Received for review March 3, 2008. Accepted May 30, 2008.

AC800445P

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