Gold nanoparticle-assisted single base-pair mismatch discrimination on a microfluidic microarray device

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Two simple gold nanoparticle (GNP)-based DNA analysis methods using a microfluidic device are presented. In the first method, probe DNA molecules are immobilized on the surface of a self-assembled submonolayer of GNPs. The hybridization efficiency of the target oligonucleotides was improved due to nanoscale spacing between probe molecules. In the second method, target DNA molecules, oligonucleotides or polymerase chain reaction (PCR) amplicons, are first bound to GNPs and then hybridized to the immobilized probe DNA on a glass slide. With the aid of GNPs, we have successfully discriminated, at room temperature, between two PCR amplicons (derived from closely related fungal pathogens, *Botrytis cinerea* and *Botrytis squamosa*) with one base-pair difference. DNA analysis on the microfluidic chip avoids the use of large sample volumes, and only a small amount of oligonucleotides (8 fmol) or PCR products (3 ng), was needed in the experiment. The whole procedure was accomplished at room temperature in 1 h, and apparatus for high temperature stringency was not required. © 2010 American Institute of Physics.

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I. INTRODUCTION

For many years, gold nanoparticles (GNPs) have been used in the fields of biodiagnostics based on different formats.¹ In the first format, GNPs are labeled to thiol-modified oligonucleotide probes which are used for the specific recognition of target DNA. Signal read-out is achieved based on the unique properties of GNPs. For example, scanometrical methods were developed using the catalytic effect of GNPs on silver staining;^{2–4} numerous molecular-beacon methods were constructed based on the strong quenching effect of GNPs on fluorescence;^{5–8} colorimetric methods were also developed based on the hybridization-induced interlinkage of GNP-labeled probes.^{9–12}

Recently, the noncovalent binding of single-strand DNA molecules (ssDNA) to GNPs was discovered, and this was applied to DNA analysis.¹³ It is because GNPs are stabilized via a layer of adsorbed negative ions (such as citrate ions) on their surface¹⁴ since GNPs are usually synthesized by citrate reduction of HAuCl₄ in water.^{15,16} Therefore, uncoiled ss-DNA could bind to GNP surface through attractive van der Waals forces between the bases and the negatively charged GNPs.¹³ This binding is tight and GNPs could be released only upon the hybridization with complementary target DNA. Therefore, in a second format, GNPs acted as competitors to discriminate between complementary and noncomplementary DNA targets. Subsequent duplex detection can be achieved either by colorimetric method from salt-induced GNP aggregation or by fluorescence-resonance-energy-transfer method.^{13,17–19} Since no thiol modification is involved in this second format, the method is simple and it has been used in oligonucleotide detection, single base mismatch discrimination, as well as aptamer-based applications in bulk solutions.^{13,19–22}

Microfluidic technology offers the advantages of less sample consumption and fast detection processes. Nevertheless, its applications to GNP-assisted bioanalysis are mostly focused on immunoassay with GNP catalyzed silver staining.²³ In terms of DNA analysis, a polydimethylsilox-

FIG. 1. PDMS microchannel plate on a 3×2 in.² glass slide. (a) Vertical PDMS channel plate used for probe immobilization. (b) Horizontal PDMS channel plate used for sample hybridization. (c) Cross-sectional view of the 16 microchannels along line AB in (b).

ane (PDMS) microfluidic device was developed for DNA discrimination by hybridization-induced GNP aggregation-related color change.¹¹ However, only 15-mer oligonucleotides were analyzed and concentration was tested at 6 μM .

In our paper, two GNP-assisted methods for DNA analysis were evaluated. In the first method, the glass surface was modified with a submonolayer of GNPs, and probe DNA molecules were then applied and allowed to bind to GNPs. The nanoscale spacing between GNPs on the surface resembles the distance created from dendrimer coating, which allows better hybridization efficiency and higher signals.^{24,25} In the second method, we found that even when the probe DNAs were immobilized on a glass surface, they are also able to hybridize with the GNP-bound target DNAs in solutions. GNP will then be released and washed away. We thus combined the use of GNPs with microfluidic technology and applied the method for the detection of PCR amplicons. Moreover, we found that the mismatched target DNAs showed less hybridization with immobilized probes, as compared to the matched ones. Therefore, the mismatched DNAs remained bound with GNPs in solution and were washed away later. We have successfully achieved the room-temperature discrimination of two 260 bp PCR products with one base-pair difference using this second method.

II. EXPERIMENTAL

A. Fabrication of the PDMS-glass microchip

PDMS channel plates consisting of 16 parallel microchannels were fabricated, as described in our previous work.²⁶ The width of the straight channels was 300 μm and the channel height was 30 μm . Solution reservoirs (1 mm in diameter) were punched at both ends of channels on the PDMS channel plate using a flat-end syringe needle. The glass slides were chemically modified to produce amine or aldehyde-functionalized surfaces using an established procedure.²⁷ As shown in Fig. 1, the PDMS channel plate was sealed against a glass slide to assemble the microchip used in the later work.

B. DNA samples

Oligonucleotides were synthesized and modified by Sigma-Genosys (Oakville, ON, Canada) or International DNA Technologies (Coralville, IA). The 21-mer DNA probes were modified with an amine group at the 5'-end. Target oligonucleotides are either 21-mer or 50-mer with Cy5 dye at the 5'-ends. The central 21 bases are complementary (perfect match) or one base-pair mismatch to the sequences of the probe molecules.

Two 264 bp PCR products (or amplicons) were amplified from genomic DNA samples, and were labeled with Cy5 dyes as previously described.²⁸ The central sequences of the sense strand of perfect matched PCR amplicons are complementary to the sequences of probe molecules, while the mismatched amplicons have one base-pair difference from that of the perfect matched ones.

C. Depositing GNP layers on glass surface using microfluidic method

In the first method of our work, a GNP submonolayer was coated on the glass surface before probe immobilization. Here, the microchip with aminated glass substrate was used. GNP solutions (20 nm in average diameter, Sigma life science) at different volumes were flowed through the microchannels. GNPs thus deposited onto the aminated glass surface and formed striplike layers along the microchannels. The uncovered amine groups on the glass surface were capped by acetyl groups by filling through the microchannels with acetic anhydride.²⁹ After washing the microchannels with 95% ethanol followed with citrate buffer solutions, the microchip was dried out for subsequent probe immobilization.

D. Preparation of DNA-GNPs conjugates

In the second method of our work, sample DNA molecules were bound to GNPs to form DNA-GNP conjugates before hybridizations. GNP solutions (5 nm in average diameter, Sigma life science) were added into the DNA samples (oligonucleotides or PCR products) in water. In the case of PCR products, the mixtures were incubated at 95 °C to denature and uncoil the DNA chains and so ss-DNA molecules were produced for binding to GNPs noncovalently. The DNA-GNP conjugates were snap cooled in an ice-water bath before the hybridization experiments. All the conjugate solutions were diluted to the desired concentration before hybridization.

E. Probe line printing, sample hybridization, and result read-out by fluorescent scanning

The procedures of probe DNA printing, target hybridization, and signal read-out are similar to our previous work.²⁶ Briefly, 1 μ l probe DNA solutions (in 1.0M NaCl+0.15M NaHCO₃) were added into the inlet reservoirs and filled through the microchannels by vacuum suction. The solution was incubated at room temperature for 1 h to anchor the probe molecules on the glass surface. After washing the microchannels, the PDMS channel plate was then peeled off and the glass slide was then rinsed and dried. All procedures were conducted at room temperature.

For DNA target hybridization, the glass chip with probe line arrays was sealed against the second PDMS channel plate. The straight channels were orthogonal to the printed probe lines on the slide. The DNA samples (oligonucleotides or PCR products) were prepared in the hybridization buffer (1X SSC+0.2% SDS). 1.0 μ l DNA targets were added to the inlet reservoirs. Sample solutions in different reservoirs were then pumped into the channels by vacuum suction simultaneously applied at the 16 channel outlets. Hybridizations were achieved at the intersections between complementary DNA targets in solution and probe lines, showing the hybridization patches of 300×300 μ m². The microchannels were rinsed immediately with 2 μ l hybridization buffer following hybridization.

Following the hybridization and washing procedures, the glass slide was scanned on a confocal laser fluorescent scanner (Typhoon 9410, Molecular Dynamics, Amersham Biosystems) at 25 μ m resolution.^{30,31} The excitation and emission wavelengths are 633 and 670 nm, respectively. The photomultiplier tube voltage was set to 600 V. The scanned image was analyzed by IMAGEQUANT 5.2 software. The average fluorescent signals were measured in relative fluorescent unit.

III. RESULTS AND DISCUSSION

A. GNP modified surface and its application to microarray DNA hybridization

In microarray technology, the effect of surface coverage on probe orientation and sample hybridization signals has been studied by many groups.^{32–35} It was found that probe density is a governing factor for the amount of hybridization as well as for the kinetics of the target hybridization. In order to maximize hybridization signals, the probe density should be high enough. However, at a very high probe density, the steric hindrance by the adjacent DNA probe molecules reduces the target capture efficiency and hybridization kinetics. On the other hand, at a lower

FIG. 2. Schematic of the formation of the nanoscale-controlled spacing between oligonucleotide probes using GNPs. (a) Protonated amine groups on the APTES-treated glass surface. (b) GNPs, with adsorbed citrate ions, are then deposited to the aminated glass surface and form a submonolayer. (c) The remaining nonreacted amine groups are deprotonated using a pH 11 buffer and masked by acetic anhydride (Ac_2O). (d) When oligonucleotides probe DNA solutions flow through the submonolayer, the amine groups at the 5'-end of the molecules will bind only to GNPs which are well spaced out on the glass surface.

probe density, probes are more accessible to targets and hybridization kinetics is faster.³³ Based on this finding, different approaches have been proposed to achieve a controllable probe density by adjusting the spacing of surface functional groups (such as $-\text{CHO}$ or $-\text{NH}_2$) used for probe immobilization. For instance, nanoscale distance was created by either dendrimer coating or self-assembled dendrimers on glass surface.^{24,25} By covalent binding between modified DNA probes and the pendant functional groups on the dendrimer molecules, steric effect was reduced and a higher detection sensitivity was achieved in hybridizations.

In this paper, we made use of a submonolayer of GNPs on glass surface to achieve a nanoscale-controlled spacing for subsequent probe immobilization. As depicted in Fig. 2, GNPs deposited on the aminated glass surface are bound electrostatically between the positive amine surface and negative GNP surface. Due to the interparticle repulsion from the surface negative charges of GNPs, a self-assembled submonolayer forms on the glass surface.^{36,37} The “saturated” coverage was measured at $\sim 30\%$ by different groups and the spacing has been calculated at around 12.5 nm between GNPs.^{38,39} This spacing is comparable to the length of 21-mer oligonucleotides (~ 12 nm).⁴⁰ Since probe DNA molecules are to be immobilized onto the GNPs, the DNA strands will also be separated in nanoscale spacing.

We used a microfluidic method to deposit GNP submonolayer on glass surface on a 16-microchannel PDMS chip (Fig. 3). The confinement of solutions in microchannels resulted in striplike GNP layers on the glass surface. The inset in Fig. 3(b)(ii) shows pink-colored strips after flowing through 10 μl GNP solutions. The pink color of the layer reflects an unaggregated status of gold nanoparticles on the surface because closely contacted GNPs will induce a shift of the surface plasmon band and a visual color change from red to blue.³⁶ Grabar *et al.*³⁸ achieved different particle coverage by incubating with GNP solutions for different times. Unlike the bulk solution method, microfluidic method offered flexibility to immobilize different amount of GNPs on the same glass slide and the deposition was very fast due to the high surface-to-volume ratio in microchannels. The more solutions flowed through the channels, the more nanoparticles were deposited on the surface and resulted in a deeper color. From Fig. 3(b)(i), it can be seen that the strips from applying 2 μl solutions is barely visible. It was found that the GNP binding on glass could withstand the later acylation and washing steps.

The effectiveness of GNP submonolayer coated surface was evaluated with the hybridization of oligonucleotides samples. With the PDMS-glass microchip, probe DNA solutions were flowed through microchannels (vertically oriented) and incubated with the GNP strips on the glass slide. DNA probe molecules that are conjugated with amine groups at 5'-end were introduced for

FIG. 3. Different modified surfaces for hybridization experiments. (a) Image of an APTES modified glass slide with immobilized GNP strips. (b) Magnified image of the glass slide with insets showing (i) flow through 2 μl GNP solutions and (ii) flow through 10 μl GNP solutions. The glass slide was first treated with APTES solution to create an aminated surface. Then a PDMS channel plate with 16 microchannels was sealed against the glass slide. GNP solutions in different amounts were filled through 8 microchannels to give the GNP-coated surface. The deposition of GNPs onto the aminated surface resulted in the pink strips. The rest of the microchannels were left empty to give the aminated-only surface. The images were taken after washing the microchannels and peeling off the PDMS plate.

immobilization to GNPs.³⁶ After probe immobilization, the PDMS channel plate was peeled off and the second PDMS plate with horizontally oriented microchannel was sealed against with the glass slide for sample hybridization at the intersection between the horizontal microchannel and vertical probe strips. This orthogonal or intersection microfluidic method has been used in our previous work and many other groups.^{26–28,41–43} The resulting hybridization patches from oligonucleotides—complementary perfect match (PM) and one-base mismatch (MM) are shown in Fig. 4. For comparison, sample hybridization with probes immobilized on aminated glass surface without GNPs was also shown. It can be seen that the hybridization signals on GNP modified surface are higher than those on aminated surface, and more GNP coverage obtained by using more GNP solutions has resulted in even higher signals.

GNP-modified surface offers advantages of nanoscale spacing in DNA probe immobilization, and resulted in higher hybridization signals, and our results have confirmed this. However, the method currently can only be applied to oligonucleotide targets that have a similar sequence length with the probes, but not to longer DNA targets. As shown in Fig. 4(b), when 50-mer targets were applied, the hybridization signals (the middle image) are much lower than those from aminated surface only (the top image). This is because the dangling end of DNA strand with the fluorescent labels could drop on the GNPs and the fluorescence signals are thus quenched.⁵ The bottom image in Fig. 4(b) also shows the hybridization results on GNP modified surface but without acetic anhydride treatment. Because GNPs formed a submonolayer and cannot cover all the amine groups on the glass surface, the probe DNA molecules can still bind to the aminated surface

FIG. 4. Fluorescent images of the hybridization results from (a) 21-mer and (b) 50-mer target oligonucleotides at different modified surfaces. (a) Hybridization of Cy5 labeled 21-mer targets. The probe DNA lines were printed microfluidically on aminated glass surface, on surface modified by flowing through 2 μl GNP solutions, and on surface modified by flowing through 10 μl GNP solutions. (b) Hybridization of Cy5 labeled 50-mer targets. The probe DNA lines were printed microfluidically on aminated glass surface, on GNP modified surface with Ac_2O treatment to cap remaining amine groups, and on GNP modified surface without Ac_2O treatment.

FIG. 5. (a) GNP-DNA conjugates from the incubation of target DNAs with gold nanoparticles. (b) Perfectly matched target DNAs desorbed from GNPs and hybridized to the surface immobilized probes. (c) Mismatched DNAs remained bound to GNPs and were washed away through the microfluidic method.

noncovalently and hybridize with the targets later.^{44,45} These target molecules were less quenched by GNPs and can thus be detected, although with a weaker signal than obtained from the aminated surface (top image).

B. GNP-DNA conjugates and its application to single base-pair discrimination

The use of nanoscale spacing surface for DNA probe immobilization enhanced hybridization signals. However, the discrimination ratio between perfect matched and mismatched DNA was not improved, and longer oligonucleotide samples could not be detected efficiently through the use of the GNP modified surface. To resolve this problem, a different method of using GNPs was employed in the second part of our work.

The principle of GNP-assisted DNA discrimination was illustrated in Fig. 5. This is based on noncovalent binding between GNPs and targets, in competition with that between targets and immobilized probes. This noncovalent binding is thought to act between GNPs and nitrogen bases on the DNA chains, and is resulted from both hydrophobic interaction and electrostatic adsorption.⁴⁶ Here, target DNA labeled with fluorescent molecules were first incubated with GNPs solutions at high temperature in solution. The solution of GNP-DNA conjugates were then applied to DNA probes preprinted microfluidically on the glass slide through the microfluidic method. Because the base-pair interaction between matched DNA chains is strong, sample DNA molecules could desorb from GNPs and were hybridized with the immobilized probes. The read-out of the hybridization signals were achieved read-out through the fluorescent labels on the sample DNA molecules. On the contrary, mismatched DNA shows much less binding energy with the probes and thus still bind with GNPs. The conjugates would be washed away in the microfluidic flow and discrimination was made.

The proposed GNP-assisted discrimination method was first verified with two 50-mer oligonucleotides with one-base difference in center. The samples hybridized with the same probe molecules to produce two types of duplexes, namely, the PM duplex and the MM duplex. As shown in the images in Fig. 6(a), without preincubation with GNPs, the hybridization signals from mismatched targets are very close to those obtained from complementary targets. The discrimination ratio, which is the hybridization signal ratio of PM duplexes over MM duplexes, is around 1.4. With the use of GNP conjugates instead of free DNA molecules, the discrimination ratio was raised up to 6.8. A clear discrimination between two oligonucleotides was observed from the images in Fig. 6(a).

The effect of the molar ratio between GNPs and target oligonucleotides on hybridization signals and discrimination ratios was also investigated. The molar concentration of GNPs can be calculated from the total gold concentration as well as the size of the GNPs.⁴⁷ For 5 nm diameter

FIG. 6. (a) Images of hybridized patches of PM and MM target oligonucleotides in triplicate. Here, the oligonucleotides were preincubated with GNPs (5 nm) at different ratios. (b) Discrimination ratios between PM and MM duplexes. The discrimination ratios were calculated by dividing the signal of PM DNAs with that of MM DNAs (the higher ratio, the better). (c) The fluorescent hybridization signals from the images in (a), and the results at oligo/GNPs=1:1 are expanded and shown in the right inset.

GNPs in our study, the particle molar concentration is around 86 nM. Conjugates of different GNP/DNA ratios were thus prepared in this manner. Figure 6(c) compares different hybridization intensities from conjugates of different oligo/GNP ratios (2:1 and 1:1, respectively). It was found that the more GNPs were incubated with DNA samples, the weaker were the hybridization signals. The ratio at oligo/GNP=1:2 even resulted in nondetectable hybridizations (data not shown). This observation could be explained by the relatively strong binding between GNPs and DNA as well as the slow kinetics of desorption. Despite the reduction in the fluorescent intensities, GNPs do enhance the discrimination of single base-pair mismatch, and the hybridization signals are still adequate as shown in both the images and the inset graph in Fig. 6(c).

The proposed nanoparticle-assisted microfluidic method was applied to the room-temperature discrimination of two related *Botrytis* subspecies, *B. cinerea* and *B. squamosa*.⁴⁸ The two PCR amplicons differ in only one base pair in the middle of the 264 bp long sequence.²⁸ Although these two targets were already discriminated in our previous work,²⁸ thermal stringency up to 62 °C had to be applied to the microchip for washing away mismatched duplexes. In this work, amplicons were first incubated at 95 °C with GNPs. This incubation serves for two purposes: one is to denature double-strand amplicons as the usual procedures and another is to promote the subsequent binding of ss-DNA to GNPs. The later snap chilling procedures (at 4 °C) prevented the renaturation of ss-DNA molecules. Although both of the two complementary strands were bound to GNPs and coexisted in the same solutions, they can be still used as samples for later microarray hybridization because the renaturation between long ss-DNA of high complexity is much slower than that between long ss-DNA with short oligonucleotide probes.⁴⁰ The discrimination ratio without the use of GNPs is ~3.6 while it goes up to ~27.7 with the assistance of nanoparticles (Fig. 7). Compared with the discrimination ratio of ~6.7 using temperature stringency at 50 °C (results not shown), the GNP-assisted method has not only improved discrimination, but also

FIG. 7. (a) Images of hybridized patches of PM and MM PCR products in triplicate. Here, the amplicons were preincubated with GNPs (5 nm) at different ratios. (b) Discrimination ratios between PM and MM amplicons. The discrimination ratios were calculated by dividing the signal of PM DNAs with that of MM DNAs (the higher the ratio, the better).

alleviated the need of high temperature and related heating devices in the microfluidic chip applications.

IV. CONCLUSION

In this work, two GNP-related methods were integrated with microfluidic technology for DNA discrimination analysis. The microfluidic chip offers flexibility in fast coating of a submonolayer of gold nanoparticles on glass surface. The submonolayer can then be used to create nanoscale spacing between probes. The hybridization efficiency of sample DNA was improved on the modified glass surface. However, such advantage was not observed with longer oligonucleotides, let alone PCR products. On the other hand, a recently discovered ss-DNA binding property of gold nanoparticles was applied to the discrimination of two PCR products with single base-pair mismatch. Because no thermal stringency was needed to melt mismatched duplexes, room-temperature discrimination was achieved and sample usage was also reduced due to the integration of the microfluidic technology.

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