Polymerase Chain Reaction-Free Variable-Number Tandem Repeat Typing Using Gold Nanoparticle–DNA Monoconjugates

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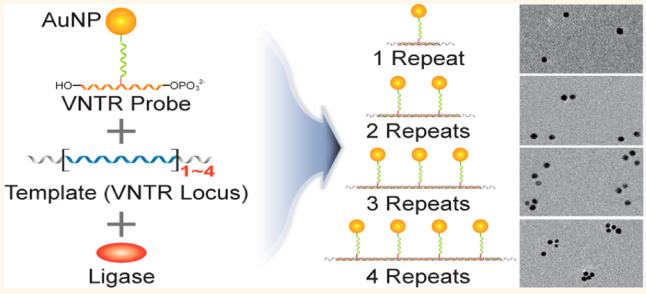
ABSTRACT In this work, we report a novel polymerase chain reaction (PCR)-free variable-number tandem repeat (VNTR) typing method using a T-shaped gold nanoparticle–DNA monoconjugate, called the "watching-gene assay". The T-shaped DNA probe was synthesized by "click" chemistry and linked with the gold nanoparticle to form the gold nanoparticle–DNA monoconjugate (a VNTR probe). Through a simple annealing and ligation reaction of the VNTR probe on a synthetic DNA template mimicking the human D1S80 VNTR locus, the number of tandem repeat units could be deciphered by counting the self-assembled gold nanoparticles. The number of tandem repeat units could be identified with more than 50% yield if the repeat number was less than four. In the case of the real human genomic DNA, the 18 repeat unit number could be successfully revealed by observing the 18-gold-nanoparticle cluster, which exactly corresponded to the number of tandem repeats of the real sample. Our "watching-gene assay" is rapid, simple, and direct for data interpretation, thereby providing an advanced PCR-free genetic polymorphism analysis platform.



KEYWORDS: polymerase chain reaction-free · gold nanoparticle–DNA conjugate · variable-number tandem repeat · polymorphism · genetic analysis

The polymerase chain reaction (PCR) has been considered the gold standard method of nucleic acid analysis, which is widely used in the fields of clinical diagnosis, microbiological detection, and forensics.[1−5] Due to its characteristics of high specificity, sensitivity, and multiplicity, PCR is an indispensable methodology for genetic variation analysis such as single-nucleotide polymorphisms (SNPs),[6] variable-number tandem repeats (VNTRs),[7] and copy number variations (CNVs).[8] The PCR process, however, holds disadvantages in terms of time/cost-consuming and labor-intensive procedures and needs stringent thermal cycling conditions.[9−11] To overcome such limitations, many efforts were dedicated to the development of a mutant polymerase that shows high processivity,[12] a miniaturized PCR microdevice enabling fast thermal cycling with low reagent consumption,[13,14] and an isothermal amplification method facilitating gene amplification without using a thermal cycling step.[15,16] Furthermore, to eliminate even the PCR process, PCR-free genetic analytical techniques have been proposed. The electrochemical branched-DNA assay and a nanogap-based sensor array were used for PCR-free mRNA quantification and expression profiling.[10,17] A fluorescent polymeric hybridization transducer and an electrochemical nanostructured microelectrode were also developed for directly sensing a target capture by measuring the change of the fluorescence and electrical signal.[18,19] More recently, a PCR-free electrochemical telomerase assay was reported based on chronocoulometry coupled with hexaammineruthenium chloride.[20] Thus, to date, the majority of the PCR-free methods depend on probe–target DNA hybridization and sensitive electrochemical sensing. Except for the direct target gene detection by the hybridization reaction,

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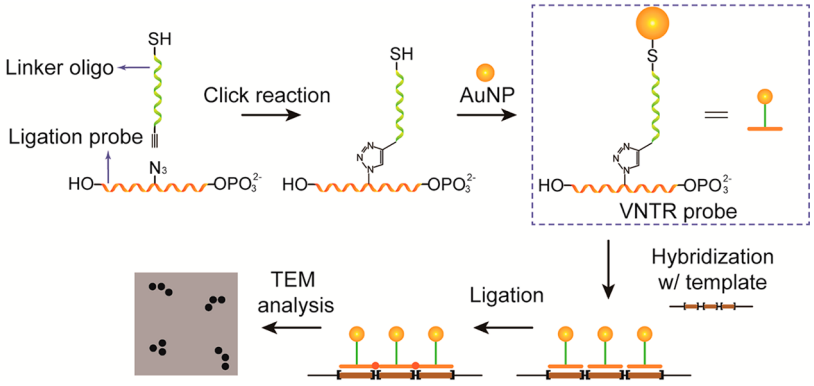

Scheme 1. Schematics for the PCR-free VNTR typing. A T-shaped VNTR probe (blue box) consisting of a linker oligonucleotide, a ligation probe, and a gold nanoparticle was synthesized by "click" chemistry and thiol-mediated gold conjugation. The VNTR probe was annealed and ligated on the template, which contains tandem repeat units. The ligated product was analyzed by TEM, and the number of gold nanoparticles was counted to identify the number of tandem repeat units.

a PCR-free genetic variation typing such as SNP and VNTR analysis is rarely performed.

On the other hand, there has been significant advances in the field of DNA–nanocrystal conjugates, which can be applied for nanoscale sensors, photonic circuits, and medical diagnostics.[21–23] In particular, the gold nanoparticle has been extensively explored for building DNA-based plasmonic nanostructures owing to its unique plasmonic property and efficient attachment chemistry.[23] To control well-defined nanoparticle–DNA structures, it is of great importance to obtain gold nanocrystals functionalized with discrete numbers of DNA strands. Claridge *et al*. successfully separated discrete nanoparticle–DNA conjugates by an anion-exchange purification method, and multinanoparticle building blocks were self-assembled through a DNA hybridization and enzymatic ligation reaction.[24,25] Besides plasmonic applications, we notice that discrete gold nanoparticle conjugates can serve as a nanoprobe for PCR-free genetic variation analysis due to the specific hybridization capability of the attached DNA strands and facile imaging of gold nanoparticles in the electron microscope.

Among genetic variations, the VNTR marker has played an important role in human population studies,[26] paternity tests,[27] forensic human identification,[28] and molecular typing of microorganisms.[29] The VNTR consists of 10 to 100 bases in one repeat length, and the repeat number is variable between individuals.[5] For example, the forensic DNA VNTR marker D1S80 has a 16-bp repeat unit and contains alleles spanning the range of 16–41 repeat units. The conventional method for VNTR typing is based on the PCR amplification of the VNTR locus followed by an electrophoretic separation together with size makers or allelic ladders.[11,30]

In this work, we report a novel PCR-free VNTR typing method. Using a T-shaped gold nanoparticle–DNA monoconjugate, we performed a direct hybridization and ligation reaction on the VNTR template, and then the number of tandem repeat units was deciphered by counting the number of self-assembled gold nanoparticles by using a transmission electron microscope (TEM). Seeing the tandem repeat number directly, we refer to our methodology as the "watching-gene assay".

RESULTS AND DISCUSSION

To realize the watching-gene assay, we first synthesized a T-shaped gold nanoparticle–DNA monoconjugate (called VNTR probe) (Scheme 1). The VNTR probe consists of three functional units: a ligation probe (16 bp) for specific hybridization on a tandem repeated template, a linker oligonucleotide (30 bp) for connection between a gold nanoparticle and the ligation probe, and a 5 nm diameter gold nanoparticle as a TEM tag. The ligation probe and the linker oligonucleotide were conjugated through the copper-catalyzed azide–alkyne Huisgen–Meldal–Sharpless cycloaddition "click" reaction (CuAAC).[31] The resultant triazole ring structure bridges between the linker oligonucleotide and the ligation probe. The linker oligonucleotide is positioned at the center of the ligation probe in order to prevent any possible ligation inhibition caused by the triazole moiety and the gold nanoparticles in the downstream reaction. The T-shaped "click" product was then attached to the surface of a 5 nm gold nanoparticle through gold–thiol chemistry. Mono- or polyconjugated gold nanoparticle–DNA products were produced, and only monoconjugates could be isolated from the mixture by using anion exchange high-performance liquid chromatography (HPLC).[24] The synthesized VNTR probe was hybridized with a synthetic DNA template mimicking the human D1S80 VNTR locus in which the repeat number ranged from one to four. Typing of three tandem repeat units is described in Scheme 1 as a representative. After the annealing process, the VNTR probe can be correctly hybridized with the repeat unit on the template, and the number of annealed VNTR probes is matched with the repeat number of the VNTR locus. The adjacent

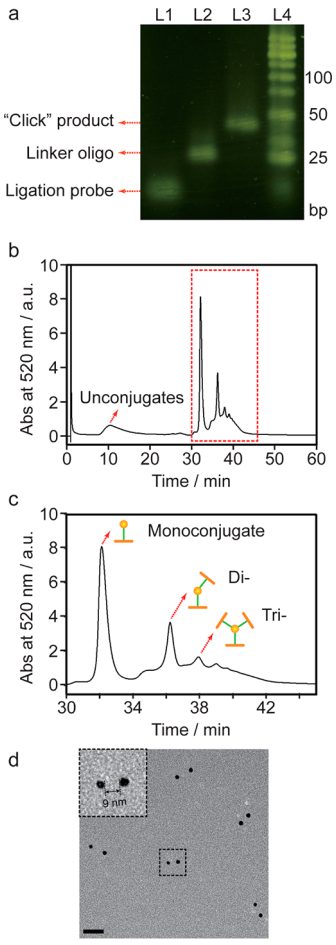

Figure 1. (a) Slab gel electrophoretic analysis of the "click" products purified by HPLC. (b) Anion exchange HPLC chromatogram for separation of a mixture produced by conjugation between gold nanoparticles and "click" products. (c) Enlarged image of the dotted rectangular region of (b), showing mono-, di-, and triconjugates. (d) TEM image showing a dimer structure generated by hybridization of gold nanoparticle−cDNA monoconjugates (scale bar: 30 nm).

VNTR probes were linked to each other by ligation reaction. The reason for ligation is that there is a possibility for the VNTR probe to be detached from the template due to the low melting temperature of the ligation probe (16 bp, *ca.* 45 °C), which would result in miscounting the number of tandem repeat units in the end. Finally, the ligated product was analyzed by TEM, and the number of assembled gold nanoparticles was counted. The number of gold nanoparticles should correspond to the repeat number of the VNTR unit, thereby enabling us to visualize the genetic length polymorphism without PCR.

The "click" products formed by conjugation between the ligation probe and linker oligonucleotides were purified by HPLC and confirmed by gel electrophoresis. Lane 3 in Figure 1a shows a clear single product band, whose size is 46 bp. In HPLC purification, three fractions were collected, and the mass of each fraction was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure S1). The peak of the "click" product appeared as a majority, and the mass value of *m/z* 15 204 was closely matched to the theoretical value (*m/z* 15 198). The yield of the "click" reaction between the ligation probe and the linker oligonucleotide was ~67%. The yield was relatively lower than other CuAAC reactions probably due to the steric hindrance derived from the internal position of an azide functional group.[31] Separation of the gold nanoparticle−DNA monoconjugates from the gold nanoparticle−DNA polyconjugates is crucial for accurate counting of tandem repeat units. According to the previous literature, 20 nm gold nanoparticles conjugated with a single DNA strand could be effectively isolated by anion exchange HPLC when the DNA length was longer than 30 bp.[24] In our case, the "click" product has a T-shape consisting of a 16-bp ligation probe and a 30-bp linker oligonucleotide, and the gold nanoparticle was 5 nm in diameter. As expected, anion exchange HPLC successfully separated nonconjugates, monoconjugates, and multiconjugates, as shown in Figure 1b and c. Figure 1c (an enlarged box image of Figure 1b) shows three distinct peaks, each of which corresponds to mono-, di-, and triconjugates, at an elution time of 32.1, 36.2, and 38.0 min, and the peak of the monoconjugate was dominant. Under the identical HPLC conditions with those above, we prepared two monoconjugates (gold nanoparticle−cDNA1 and gold nanoparticle−cDNA2), and the pattern in the LC chromatogram (Figure S2) is quite similar to that of Figure 1b. Since the sequence of the cDNA1 and cDNA2 is complementary, the hybridization of the two monoconjugates resulted in the dimer structure with 9 nm distance (Figure 1d), confirming that the major peak in the HPLC purification is the gold nanoparticle−DNA monoconjugates.

By using the T-shaped monoconjugates, we conducted the VNTR typing on the synthetic human D1S80 VNTR locus where the sequence of GAG GAC CAC CAG GAA G is repeated. Using the synthetic template, which contains one to four tandem repeat units, we followed the procedures in Scheme 1. Figure 2 shows TEM images of the ligated VNTR probes. If the template has only one repeat unit, the single gold nanoparticle was well dispersed without being assembled (Figure 2a). In the case of the repeat number of two, a well-assembled dimer structure of the gold nanoparticles was displayed (Figure 2b). Considering that the two adjacent gold nanoparticles are apart by 16 bp, the theoretical distance between them should be 5.4 nm. However, the interparticle distance was measured as 2.3 ± 0.4 nm, which is much shorter than expected because of the inward conformational change of the two single-stranded linker oligonucleotides. On the contrary, the outward stretching of the flexible linker DNA leads to the enlongated interparticle distance of

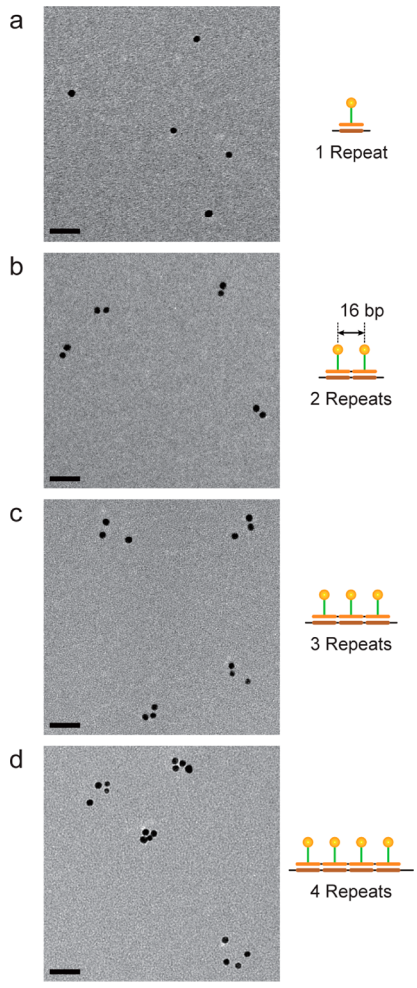

Figure 2. TEM images of a PCR-free VNTR typing assay. A synthetic template containing one, two, three, and four tandem repeat units produces (a) separated single gold nanoparticles, (b) a two-gold-nanoparticle assembly, (c) a three-gold-nanoparticle assembly, and (d) a four-gold-nanoparticle assembly, respectively (scale bar: 30 nm).

8.3 ± 4.0 nm (Figure S3a), showing a larger distance and a wider distance distribution than those of Figure 2b. It seems that such a conformational change of single-stranded DNAs (linker oligonucleotides) as well as the gold nanoparticle–DNA monoconjugate behavior in solution state prior to drying on the TEM grids contributes to the significant variation of the interparticle distance.[25]

Figure 2c shows the self-assembled three-gold-nanoparticle tags that were obtained by using the VNTR templates containing three repeat units. The number of gold nanoparticles was equal to that of the tandem repeat units. The gold nanoparticles were arranged typically with a bent conformation and partly with a linear or triangular shape (Figure S3b), depending on the geometry of the hybridized dsDNA or the direction and length variation of the linker oligonucleotides.

Finally, Figure 2d represents the assembled gold nanoparticles after PCR-free VNTR typing with a template that has four tandem repeat units. As expected,

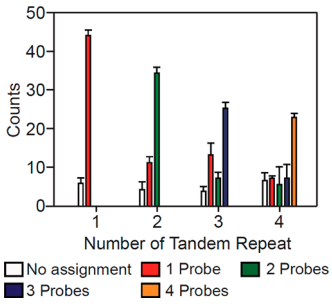

Figure 3. Statistical evaluation of the PCR-free VNTR typing assay. The number of gold nanoparticles (Au NPs) was matched with that of tandem repeats, but the efficiency was gradually reduced as the number of tandem repeats increased. The sampling number was 50 for each template, and the average count was obtained from triplicate experiments.

four-gold-nanoparticle clusters were observed, indicating that there are four repeat units on the template. Since the variation in the position and length of the VNTR probes increases proportionally with the number of repeat units, the arrangement of the gold nanoparticle assembly differed markedly. For example, in the case of the top and left gold nanoparticle clusters, three nanoparticles were aggregated while the other one remained relatively apart. The assembly at the center was similar to that of the bottom one by forming a rectangular aggregate, but they significantly differ in terms of spacing between the gold nanoparticles. Thus, the conformational degree of the freedom of the single- and double-stranded DNA entropically influences the shape of the gold nanoparticle cluster even when an identical template is used.

Our proposed "watching-gene assay" methodology was statistically evaluated. We counted the correct gold nanoparticle assemblies out of 50 cluster samples (Figure 3). In the case of one repeat unit, the produced TEM image revealed a single gold nanoparticle with 88% yield. With two repeat units, 34 samples showed a two-gold-nanoparticle assembly among 50 clusters. The template containing three repeat units resulted in a three-gold-nanoparticle assembly with a 50.6% yield. In the case of four repeat units, there were 23 four-nanoparticle assembled clusters among 50 (46% yield), whereas one-, two-, and three-nanoparticle assemblies were also observed at 14.6%, 11.3%, and 14.7%, respectively. As the repeat number increases, the efficiency of the perfect hybridization and ligation reaction of the VNTR probes on the template is gradually reduced due to the byproducts caused by partial hybridization and ligation. However, the number of gold nanoparticles produced by the byproducts does not exceed the designated number of tandem repeat units, meaning that the maximal number of gold nanoparticles can be a reliable indicator of the correct number of tandem repeat units.

Encouraged by the success of PCR-free VNTR typing using a synthetic template, we performed the same

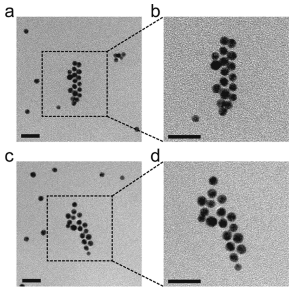

Figure 4. TEM images of the PCR-free VNTR typing assay using human genomic DNA templates. A D1S80 VNTR marker having 18 repeat units produces 18 assembled gold nanoparticle clusters in (a) and (c). (b and d) Enlarged images of (a) and (c), respectively (scale bar: 20 nm).

procedure for VNTR typing of a D1S80 locus with a human genomic template. Human genomic DNA was obtained by a buccal swab from a volunteer and extracted according to the manufacturer's protocol. The D1S80 locus was sequenced, revealing the repeat number of 18 (Figure S4). A 500 ng amount of genomic DNA was mixed with 1 pmol of the VNTR probes, then heated at 55 °C, and slowly cooled to 16 °C for annealing. After the ligation reaction, the assembled gold nanoparticles were observed by TEM (Figure 4). Both Figure 4a and c showed the 18-gold-nanoparticle cluster, which exactly corresponds to the number of tandem repeats of the real sample. Besides the 18-gold-nanoparticle cluster, an excess of the VNTR probe and other gold nanoparticle clusters of less than 18 were also detected. The correct 18-gold-nanoparticle clusters were found with a probability of 2 out of 10 clusters (approximately 20% yield). Interestingly, although the human genomic DNA has microvariants that have partially different sequences in the middle of a repeat unit, desirable complete hybridization and ligation in the repeating region could occur by using a low annealing temperature (16 °C). Further optimization of the probe design and the reaction conditions and a TEM preparation process could enhance the efficiency of our methodology.

CONCLUSIONS

In conclusions, we have successfully demonstrated PCR-free VNTR typing using gold nanoparticle–DNA monoconjugates, proving the feasibility of a novel "watching-gene assay" platform for genetic polymorphism analysis. The T-shaped VNTR probes synthesized by "click" chemistry could be annealed and ligated with the templates containing tandem repeat units, and the resultant gold nanoparticle assembly was analyzed by TEM. The number of self-assembled nanoparticles of the VNTR probes could be easily counted, so that the number of tandem repeat units could be visualized. Compared with a conventional PCR method, our "watching-gene assay" is rapid, simple, and direct for data interpretation by eliminating the PCR process, DNA separation, fluorescence detection, *etc*. We believe that our methodology can provide a novel PCR-free VNTR typing platform for genetic variation analysis and can be applicable for real samples with further optimization.

EXPERIMENTAL SECTION

Oligonucleotides. The ligation probe, the linker oligonucleotide, and the synthetic template were purchased from Integrated DNA Technologies (USA). The ligation probe had a phosphate group at the 5′ end for ligation and an azide moiety at the internal ninth thymine sequence for "click" reaction. The 5′ and 3′ ends of the linker oligonucleotide were modified with a hexynyl and a thiol group for "click" and gold–thiol chemistry, respectively. The repeat number of a synthetic template mimicking the human D1S80 VNTR locus ranges from one to four. To confirm the synthesis of gold nanoparticle–DNA monoconjugates, two 3′-end-thiolated oligonucleotides (cDNA1 and -2) complementary to each other were used, leading to a dimer structure by hybridization between the gold nanoparticle–cDNA1 and the gold nanoparticle–cDNA2 monoconjugate.

Ligation probe: 5′-CTT CCT GGT GGT CCT C-3′

Linker oligonucleotide: 5′-GTA GTG ACG CTA TGT GAT CGA GAT ATC GTA-3′

Synthetic template (D1S80): 5′-$(T)_{10}$(GAG GAC CAC CAG GAA G)$_n$$(T)_{10}$-3′ ($n$ = 1–4)

cDNA1: 5′-GTA GTG ACG CTA TGT GAT CGA GAT ATC GTA TTT TT-3′

cDNA2: 5′-TAC GAT ATC TCG ATC ACA TAG CGT CAC TAC TTT TT-3′

T-Shaped Linking between the Ligation Probe and the Linker Oligonucleotide. The ligation probe and the linker oligonucleotide were conjugated through the copper-catalyzed azide–alkyne Huisgen–Meldal–Sharpless cycloaddition "click" reaction. The reaction conditions were adopted from the previous literature with modification.[31] Briefly, to the linker oligonucleotide (12 μL of a 1 mM stock) and the ligation probe (9 μL of a 1 mM stock) solution were added a mixture of the $CuSO_4$–Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand complex (10 μL of a 20 mM stock solution in H_2O/dimethyl sulfoxide (DMSO)/*tert*-butanol, 4:3:1, for TBTA; 10 μL of a 20 mM stock solution in H_2O/DMSO/*tert*-butanol for $CuSO_4$), sodium ascorbate (10 μL of a 40 mM stock), sodium bicarbonate (5 μL of a 200 mM solution), and 7 μL of DMSO, and the mixture was vortexed at room temperature for 15 h. After centrifugation at 15 000 rpm for 15 min, the supernatant was collected and concentrated in a SpeedVac. Prior to manual injection to an HPLC, the reaction mixture was dissolved in 100 μL of water. The collected "click" products were desalted with a PD-10 column according to the manufacturer's protocol (GE Healthcare, USA). After concentration with a SpeedVac, the "click" products were dissolved in 50 μL of water and stored at −4 °C before use. The final concentration of the "click" products measured by absorbance at 260 nm was approximately 50 μM.

Characterization of the "Click" Products. The collected fraction from an HPLC was analyzed by MALDI-TOF MS (Bruker autoflex III, Bruker Daltonics, Germany) and agarose slab gel electrophoresis. For MALDI-TOF MS analysis, a sample volume of ~2 μL was desalted with a ZipTip (GE Healthcare, USA) following the instruction manual before spotting it with a precrystallized matrix. The matrix solution was prepared by dissolving 35 mg of 3-hydroxypicolinic acid (Aldrich, Milwaukee, WI, USA) and 6 mg of ammonium citrate (Aldrich) in 0.8 mL of 50% acetonitrile. A sample of 2 μL mixed with the matrix solution was spotted on a stainless steel sample plate, air-dried, and analyzed using a MALDI-TOF mass spectrometer. All measurements were

taken in a linear positive ion mode with a 25 kV accelerating voltage, a 79% grid voltage, and a 160 ns delay time. The mass spectra of each collected fraction are shown in Figure S1b–d. For electrophoresis, the collected sample was separated on a 3% agarose gel, stained with a cyanine dye (SYBR Gold, Invitrogen, USA), and observed under UV light (Figure 1a).

Synthesis of Gold Nanoparticle–DNA Monoconjugates. The procedure for the preparation of gold nanoparticle–DNA monoconjugates is as follows.[24] Citrate-coated, 5 nm gold nanoparticles (0.083 μM) were purchased from Ted Pella (USA). A 9 mg sample of bis(*p*-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP) was added to 15 mL of a citrated gold nanoparticle solution as a stabilizer, and the mixture was vortexed at room temperature for 12 h. NaCl was added until the color of the solution changed from red to cloudy purple. After centrifugation at 2000 rpm for 20 min, the supernatant was removed, and the pellet was resuspended in 100 μL of a BSPP solution (1 mg of BSPP in 1 mL of water). The final concentration of the gold nanoparticle solution quantified by measuring the absorbance at 520 nm was ~1 μM (extinction coefficients of 5 nm gold nanoparticles: $\varepsilon_{520} = 9.3 \times 10^6\ M^{-1}\ cm^{-1}$). To prepare gold nanoparticle–DNA conjugates, 4 nmol of the synthesized "click" products was treated with 0.3 M dithiothreitol (DTT) for reduction of disulfide bonds and purified with an NAP-5 column (GE Healthcare, USA) according to the manufacturer's protocol. After drying, the "click" products were mixed with 93 μL of a concentrated gold nanoparticle solution, 5 μL of a BSPP solution (10 mg of BSPP in 100 μL of water), and 2 μL of 100 mM phosphate-buffered saline (PBS). The mixture was vortexed gently at room temperature for 24 h, and then 0.5 μL of 450 mM sulfhydryl-terminated polyethylene glycol (methyl–PEG_4–thiol, Thermo Scientific, USA) was added to prevent nonspecific binding of DNA during VNTR typing and incubated for 2 h. For separation of the gold nanoparticle–DNA monoconjugates from multiconjugates, the resultant solution was injected directly into HPLC and collected at the designated elution time (*i.e.*, 32.1 min). The solution was centrifuged at 14 000 rpm for 80 min under 10 °C, and a pellet was collected. The final concentration of the monoconjugate solution was 0.2–0.4 μM.

Purification of "Click" Products and Separation of Gold Nanoparticle–DNA Conjugates Using HPLC. After "click" conjugation between ligation probes and linker oligonucleotides, the products were purified using a Dionex DNA-Pac PA100 anion exchange column (USA) on a Shimadzu (Japan) 20A HPLC with an in-line degasser, a manual injector, and a multiwavelength UV–vis detector.[24] For "click" product purification, 25 mM Tris (pH 8) was used as mobile phase "A", and 2 M NaCl in 25 mM Tris (pH 8) was employed as mobile phase "B". Then, 100 μL of a sample solution was injected. During 5 min at the initial stage, low ionic strength was maintained by flowing 97.5% mobile phase "A" at a flow rate of 1.5 mL/min, leading to strong binding of the sample to the column. After 5 min, the gradient of NaCl concentration was adjusted to 10 mM/min. Under these conditions, the ligation probe, the linker oligonucleotide, and the "click" product were eluted at a retention time of 49, 59, and 64 min, respectively (Figure S1a). Gold nanoparticle–DNA monoconjugates (the VNTR probes) were also separated from multiconjugates with a similar procedure to that above. Gold nanoparticle–DNA monoconjugates were separated using a 20 mM/min gradient of NaCl concentration, and the nonconjugates, the monoconjugates, and the diconjugates were eluted at retention times of 10, 32, and 36 min respectively, which was monitored by observing the absorbance at 520 nm of 5 nm gold nanoparticles (Figure 1b and c). The gold nanoparticle–cDNA1 and -2 separations were also carried out with the same procedure, and their LC chromatograms are shown in Figure S2.

Gold Nanoparticle Dimer Synthesis Using cDNA Hybridization. To demonstrate the synthesis of a gold nanoparticle–DNA monoconjugate, two 3′-end-thiolated complementary oligonucleotides (cDNA1 and -2) were used, resulting in a dimer structure by hybridization of each gold nanoparticle–cDNA monoconjugate. A gold nanoparticle–cDNA monoconjugate was prepared following the procedure of the VNTR probe synthesis. After mixing of each 5 μL of 0.2 mM gold nanoparticle–cDNA, the solution was incubated at 55 °C for 1 min, slowly cooled to 16 °C, and held for 90 min. The diluted solution was analyzed with a TEM (Figure 1d).

PCR-Free VNTR Typing Using Synthetic Templates. The commercial T4 ligase buffer contains DTT, so a modified buffer was needed to remove DTT.[25] Tris-HCl (5 mmol) and magnesium chloride (1 mmol) were mixed in 9 mL of water, and a concentrated NaOH solution was added to reach pH 7.5. The solution was stored frozen. Before VNTR typing, the solution was immediately thawed and mixed with 1/9 volume of 100 mM adenosine 5′-triphosphate (ATP) disodium salt hydrate. For annealing between a synthetic template and a VNTR probe (gold nanoparticle–DNA monoconjugate), 6–12 μL of a 0.2 μM probe and 0.5 μL of a 2 μM template stock solution were mixed in a final volume of 16.5 μL. The mixture was incubated at 45 °C for 1 min, slowly cooled to 16 °C, and held for 90 min. After adding 3 μL of the prepared buffer and 2 μL of 40 U/μL T4 ligase (New England BioLabs, USA), the solution was incubated for 45 min. Finally, the diluted solution was analyzed with a TEM.

PCR-Free VNTR Typing Using Human Genomic DNA. The human genomic DNA templates were extracted using a centrifugal spin-column method (QIAamp DNA Investigator kit, Qiagen, USA). After PCR amplification of the D1S80 VNTR locus, the sequence of the amplicons was analyzed (Solgent, Korea) (Figure S4). The used genomic template contained 18 repeating units in the D1S80 VNTR locus. For the VNTR typing assay, the genomic DNA (10 μL of 50 ng/μL) was mixed with a VNTR probe (6.5 μL of 0.1 μM). The mixture was incubated at 55 °C for 1 min, slowly cooled to 16 °C, and held for 90 min. After adding 3 μL of the ligation buffer and 2 μL of 40 U/μL T4 ligase (New England BioLabs, USA), the solution was incubated for 45 min. Finally the diluted solution was analyzed with a TEM.

TEM Analysis. Before spotting of a 0.5 μL diluted sample, the carbon-coated TEM grid (Ted Pella, USA) was treated with a UV cleaner (AH-1700, AHTECH, Korea) for 2 min for better hydrophilic adhesion. After 3 min, 0.7 μL of a 0.5× Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer was added on the sample spot, and excess moisture was removed by touching the grid edge with a filter paper. Prior to air-drying of the TEM grid, a buffer addition and removal process was carried out twice. Finally, TEM analysis was performed using a Phillips Tecnai G^2 F30 instrument.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: LC chromatogram and MALDI-TOF MS spectra for the separated "click" products and the gold nanoparticle–cDNA conjugates; TEM images of the PCR-free VNTR typing assay; sequence data of the D1S80 VNTR locus. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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