

Rapid Naked-Eye Discrimination of Cytochrome P450 Genetic Polymorphism through Non-Crosslinking Aggregation of DNA-Functionalized Gold Nanoparticles

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Involvement of single-nucleotide polymorphism (SNP) genotyping in healthcare should allow for more effective use of pharmacogenomics. However, user-friendly assays without the requirement of a special instrument still remain unavailable. This study describes naked-eye SNP discrimination in exon 5 of the human cytochrome P450 2C19 monooxygenase gene, *CYP2C19*1* (the wild-type allele) and *CYP2C19*2* (the variant allele with G681A point mutation). The present assay is composed of allele-specific single-base primer extension and salt-induced aggregation of DNA-modified gold nanoparticles (DNA-AuNPs). Genetic samples extracted from human hair roots are subjected to this assay. The results are verified by direct sequencing. This study should promise the prospective use of DNA-AuNPs in gene diagnosis.

1. Introduction

Single-nucleotide polymorphisms (SNPs) are highly abundant genetic variations originating from single-base substitution in genomes. According to public databases, over 5 million SNPs have been validated in the human genome.^[1] Some categories of them are associated with medical issues, including drug metabolism.^[2] Involvement of SNP typing in healthcare could allow for more effective use of pharmacogenomics. Various excellent SNP detection methods have been developed, such as the invader assay and the TaqMan assay.^[3] Among them, the

assays based on allele-specific single-base primer extension have great advantages in theoretical simplicity and practical accuracy.^[4] To discriminate SNPs, extended and unextended primers are separated with HPLC,^[5] capillary electrophoresis,^[6] or flow cytometry.^[7] Alternatively, these mixtures are directly subjected to SNP typing with mass spectrometry^[8] or microarray-based analysis.^[9] However, more user-friendly methods that do not require expensive equipment and intricate data processing are still needed to discriminate SNPs easily and rapidly in medical services.

This paper describes naked-eye discrimination of the human cytochrome P450 genetic polymorphism by using aggregation/dispersion of gold nanoparticles (AuNPs). The aggregation of AuNPs yields significant red shifting and broadening in the surface plasmon band. This colloidal behavior changes the solution color from red to blue, owing to the interparticle plasmon coupling. Rothberg developed a colorimetric SNP detection method by utilizing physical adsorption of DNA strands onto a bare AuNP surface.^[10] This concept was successfully extended by other research groups.^[11] Besides, Mirkin and co-workers first prepared DNA-functionalized AuNPs, which consisted of an AuNP core surrounded by a dense shell of single-stranded DNA (ssDNA-AuNPs).^[12] Using crosslinking aggregation of ssDNA-AuNPs with an ssDNA crosslinker, they detected single-base differences in the base sequence of the crosslinker.^[13] The relevant methods were also devised by combining the crosslinking aggregation of the particles with various functions of nucleic acids, such as strand exchange^[14] and molecular recognition by aptamers.^[15] In addition, other excellent assays were also developed by a combination of the crosslinking aggregation and various enzymatic/chemical reactions, including ligation,^[16] elongation,^[17] and cleavage.^[18]

A dozen years ago, our research group identified an alternative aggregation mode.^[19] The ssDNA-AuNPs remain stably dispersed in a medium of high ionic strength, owing to an interparticle electrostatic and steric repulsion. By contrast, AuNPs modified with double-stranded DNA (dsDNA-AuNPs) are spontaneously aggregated under the same conditions, accompanying the solution color change from red to blue. Notably, this spontaneous aggregation of dsDNA-AuNPs is strongly inhibited when the dsDNA on the AuNP surface (surface dsDNA) has a single-nucleotide overhang at the distal end, located at the interface between the surface dsDNA layer and the disperse medium (Figure 1).^[20] These unique colloidal behaviors arise from physicochemical properties of the surface dsDNA layer, because these colloidal behaviors are observed irrespective of

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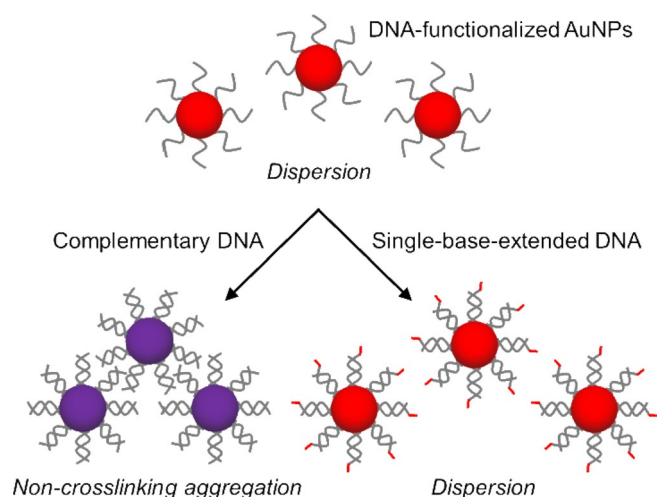


Figure 1. Schematic illustration of the colloidal behaviors of DNA-functionalized gold nanoparticles in a medium of high ionic strength.

the composition,^[21] size,^[22] and shape^[23] of the particle core. One possible explanation is that the π - π stacking interaction between the terminal base pairs of different particles dominates the interparticle attractive forces, although direct evi-

dence has not yet been provided. We have thus far devised a colorimetric SNP genotyping method by combining the non-crosslinking aggregation of dsDNA–AuNPs with allele-specific single-base primer extension. The proof-of-concept study was conducted by using chemically synthesized DNA duplexes as a gene model.^[20]

In the present study, we further demonstrate the validity of our SNP detection method by using genetic samples obtained from human hair roots. Quite recently, we have compared the rapidity of solution color change between the crosslinking and non-crosslinking aggregation modes. The results showed that the non-crosslinking aggregation of ssDNA–AuNPs induced by an addition of a large excess number of complementary ssDNAs compared to the surface ssDNAs can exhibit more rapid color change than the crosslinking counterpart.^[24] In general, a typing primer used for single-base extension is a chemically synthesized oligonucleotide. There are no technical and cost issues to prepare these compounds in large amounts, allowing one to easily perform rapid non-crosslinking aggregation of dsDNA–AuNPs. Thus, this aggregation mode is fully compatible with the single-base extension.

Figure 2a shows the workflow of the present colorimetric SNP genotyping. We attempted to identify the SNP in exon 5 of the human cytochrome P450 2C19 monooxygenase gene,

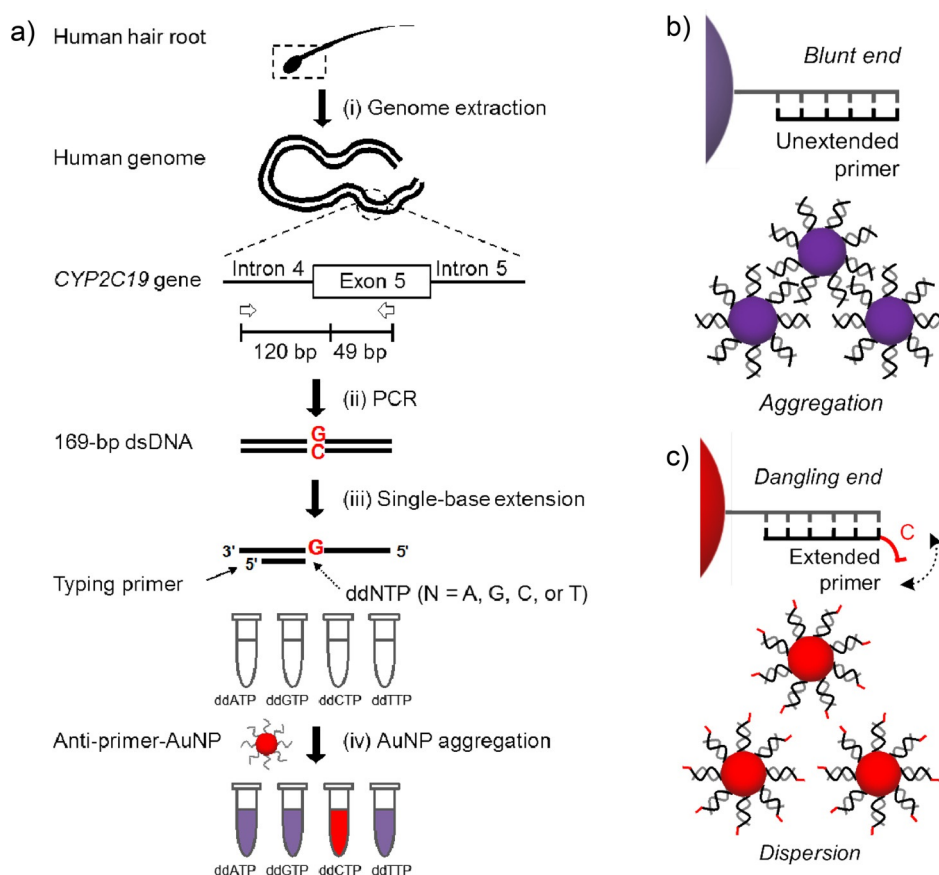


Figure 2. a) Workflow of the colorimetric SNP typing of a genetic sample containing guanine (G) at the SNP site as an example (the wild-type allele). The process is composed of i) genome extraction with an isohair kit, ii) conventional PCR amplification, iii) single-base primer extension, and iv) non-crosslinking aggregation of the DNA–AuNPs. b) The AuNP surface after an addition of the reaction solutions with ddATP, ddGTP, or ddTTP. The particles undergo the non-crosslinking aggregation. c) The AuNP surface after an addition of the reaction solutions with ddCTP. The particles undergo the stable dispersion.

*CYP2C19*1* (the wild-type allele) and *CYP2C19*2* (the variant allele with G681A point mutation).^[25] This single-base substitution leads to premature termination of the protein synthesis. As this enzyme is involved in the metabolism of several frequently prescribed drugs, the production of such inactive enzymes is responsible for the poor drug metabolism. Therefore, the SNP discrimination of the *CYP2C19* gene before dosing could provide significant therapeutic benefits to patients.

Initially, we isolated genomic DNA from human hair roots by using a commercially available kit. This process took 10 min or less. We then amplified a fragment of the *CYP2C19* gene (169 base pairs) by PCR.^[26] After purification of the PCR amplicons by using magnetic beads, we confirmed the sufficiently high purity with gel electrophoresis (Figure 3). We next carried out the single-base primer extension by using the obtained PCR amplicons for a template. A set of four reaction mixtures was prepared for one analyte; each mixture contained ddATP, ddGTP, ddCTP, or ddTTP together with the PCR amplicon, the typing primer, and the DNA polymerase. In Figure 2a, the genetic sample has guanine (G) at the SNP site as an example (the wild-type allele). Accordingly, the reaction mixture involving ddATP, ddGTP, or ddTTP yields the unextended typing primer, whereas the mixture with ddCTP produces the single-nucleotide-extended primer.

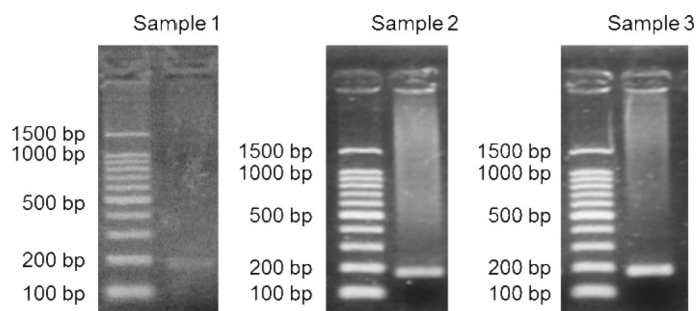


Figure 3. Electrophoresis of the PCR amplicons (the 169 bp fragments of the *CYP2C19* gene) obtained from three individuals on 2% agarose gel (100 V, 30 min).

Subsequently, we added the unpurified reaction products to a dispersion of ssDNA–AuNPs at high ionic strength. The ssDNA–AuNPs used in this study were synthesized according to a previously reported procedure.^[20] The nominal diameter of the AuNP core was 15 nm. The obtained ssDNA–AuNPs had, on average, 90 surface ssDNAs per particle. The base sequence of the 16-nucleotide ssDNA on the AuNP surface was designed to be complementary to the unextended typing primer (Figure 4). As a result, the final solution containing ddATP, ddGTP, or ddTTP exhibited the red-to-blue color change, because the DNA duplexes formed on the AuNP surface had the blunt end to trigger the non-crosslinking aggregation (Figure 2b). The distinct color change required an incubation time of 10 min or less at room temperature. In contrast, the final solution containing ddCTP remained red in color, because the extended typing primer produced a single-nucleotide (dideoxycytidine) overhang at the outermost surface; the resulting

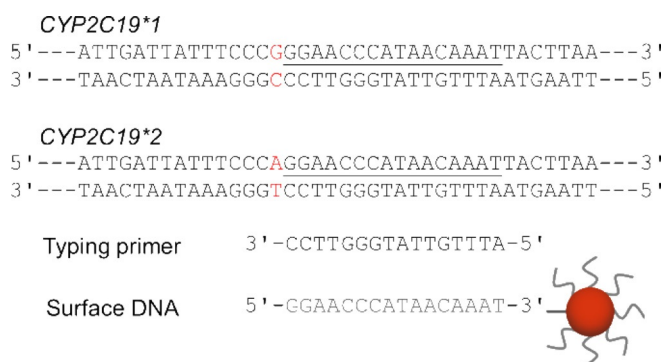


Figure 4. Base sequences of the human cytochrome P450 2C19 monooxygenase genes (in part), *CYP2C19*1* (the wild-type allele) and *CYP2C19*2* (the variant allele with G681A point mutation), as well as the typing primer and the surface DNA. The SNP site is highlighted in red. The typing-primer binding site is underlined.

dsDNA–AuNPs with the dangling end were stably dispersed under the same conditions (Figure 2c). It should be noted that the amount of typing primer was not lower than the total amount of the surface ssDNA in order to make sure that the solution color change took place rapidly.^[24]

Figure 5a shows the results of the colorimetric SNP genotyping of three individuals (sample 1–3). For sample 1 and sample 2, the addition of the single-base extension product to the colloidal dispersion of ssDNA–AuNPs using ddCTP made the solution red in color. On the other hand, the addition of the reaction product using ddATP, ddGTP, or ddTTP induced the solution color change from red to blue. Taken together, only ddCTP was incorporated into the 3'-end of the typing primer; namely, both sample 1 and sample 2 contained homozygous *CYP2C19*1* (the wild-type allele), which has G (comple-

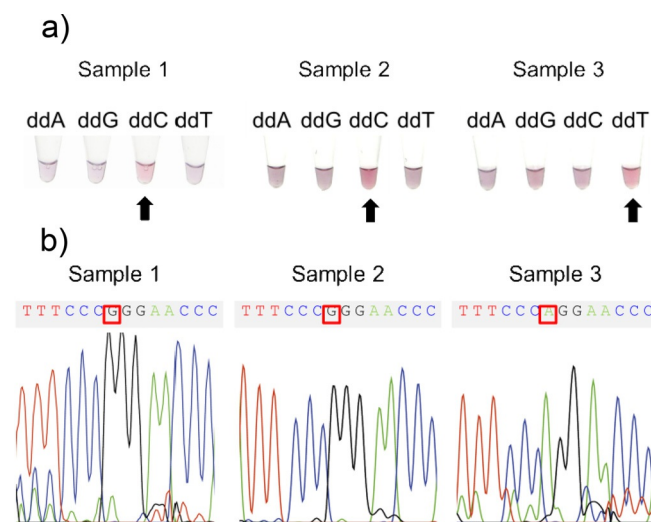


Figure 5. a) The colorimetric SNP typing of the *CYP2C19* gene. The samples were obtained from three individuals. The photographs were taken at room temperature. Arrows indicate the red color caused by the dispersion of the dsDNA–AuNPs with the dangling end. b) The direct sequencing of the PCR products using the forward primer. The SNP site is highlighted with a red rectangular frame.

mentary to ddCTP) at the SNP site. Similarly, for sample 3, the colloidal dispersion of the ssDNA–AuNPs with the single-base extension product using ddTTP exhibited a red color, whereas the other tubes were blue in color. The results mean that sample 3 contained homozygous *CYP2C19**2 (the point-mutated allele), which has A (complementary to ddTTP) at the SNP site. All results of the colorimetric SNP genotyping were verified by direct sequencing of the PCR amplicons (Figure 5b). The heterozygous *CYP2C19* gene was not found within the real samples tested in this study, owing to a limited number of samples. The allele frequency of *CYP2C19**2 was reported to be 22–32% in Asians and 6–15% in Caucasians.^[27]

In conclusion, we demonstrated the rapid colorimetric SNP typing of a human gene by combining the non-crosslinking aggregation of dsDNA–AuNPs and the single-base primer extension. The current method has three merits compared to pre-existing SNP detection methods. First, the simple and straightforward principle eliminates the need for complicated data processing. Second, only fundamental techniques in biochemical experiments are required; neither labor-intensive reagent synthesis nor instrumental operations are necessary to identify alleles. Finally, the experimental procedure is accomplished in a short duration time, even though the reaction conditions have not yet been fully optimized. This study reinforces the promise for the prospective use of non-crosslinking aggregation of dsDNA–AuNPs in future personal medicine.

Experimental Section

General

All chemical reagents were obtained from Wako Pure Chemical Industries unless otherwise noted. Citric-acid-coated AuNPs (15 nm in diameter) were purchased from BBI Solutions. Tris(2-carboxyethyl)phosphine hydrochloride was obtained from Thermo Fisher Scientific. Ultrapure water (>18 M Ω cm) was prepared by using a Milli-Q instrument (Millipore) and was sterilized for all experiments. Chemically synthesized DNA was purchased from Tsukuba Oligo Service or Eurofins Genomics. The DNA concentration was determined by measuring the extinction at 260 nm with a UV-2550 spectrophotometer (Shimadzu). The 3'-mercaptopropyl DNA was densely grafted onto the particle surface through Au–S bond formation by following a reported procedure.^[19] The number of immobilized DNA molecules per particle was evaluated with the reported method.^[19b]

Preparation of Genetic Samples

PCR amplicons obtained from genomic DNA of consenting adults were used as a genetic sample. The study was performed in accordance with the principles stated in the Declaration of Helsinki using the protocol approved by the RIKEN ethics committee. Isolation of genomic DNA from hair roots was performed by using an Isohair Easy kit (Nippon Gene) according to the manufacturer's protocol. A 169 bp fragment of the *CYP2C19* gene was amplified by PCR on a 100 μ L scale.^[26] Each reaction mixture involved the genomic DNA, 0.5 μ M of the forward primer (5'-AATTACAACCA GAGCTTGGC-3'), 0.5 μ M of the reverse primer (5'-TATCACTTC CATAAAAGCAAG-3'), 200 μ M of each dNTP, and 2.5 U of Ex Taq DNA polymerase. After the initial activation of the polymerase at

94 °C for 20 s, 35 cycles were performed using steps of 98 °C for 10 s, 53 °C for 30 s, and 72 °C for 60 s. The thermal cycling was carried out with an iCycler (Bio-Rad Laboratories). The PCR amplicons were purified by using MagExtractor PCR (Toyobo) for a sample of the colorimetric assay and a Gel Clean-Up kit (Toyobo) for a sample of the DNA sequencing. The PCR amplicons, thus purified, were assessed by electrophoresis on a 2% agarose gel (100 V, 30 min). The gel was stained by SYBR Gold Nucleic Acid Gel Stain (Invitrogen) for 60 min at room temperature. The gel images were captured by using a FAS-III system (Toyobo).

Primer Extension

The single-base primer extension was performed with Thermo Sequenase DNA polymerase (GE Healthcare) under the conditions recommended by the manufacturer. Each reaction mixture (40 μ L) contained the PCR amplicon, the typing primer (1 μ M), each of ddNTP (25 μ M), and the DNA polymerase (16 U). After the initial activation of the polymerase at 95 °C for 3 min, 90 cycles were performed using steps of 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 30 s. The thermal cycling was carried out with the iCycler.

Colorimetric SNP Typing

The unpurified primer extension product (1 μ M, 10 μ L) was directly mixed with an ssDNA–AuNP dispersion (20 nM, 5 μ L) and an aqueous Tween 20 solution (1 w/v%, 1 μ L). After incubation at room temperature for 10 min, an aqueous NaCl solution (2.5 M, 4 μ L) was further added to the mixture. The final concentrations of the ssDNA–AuNPs, the unpurified extension product, and NaCl were 5 nM, 0.5 μ M, and 0.5 M, respectively. After another incubation at room temperature for 10 min, the samples were photographed with an IXY 600F PC1676 digital camera (Canon).

DNA Sequencing

The results of colorimetric SNP genotyping were verified with direct sequencing of the PCR amplicons by using a 3730xl DNA analyzer (Applied Biosystems). The temperature profile was as follows: after the initial activation of the polymerase at 96 °C for 1 min, 25 cycles were performed using steps of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, followed by the final inactivation at 4 °C.

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