

# Non-cross-linking gold nanoparticle aggregation as a detection method for single-base substitutions

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## ABSTRACT

**Aggregation of DNA-modified gold nanoparticles in a non-cross-linking configuration has extraordinary selectivity against terminal mismatch of the surface-bound duplex. In this paper, we demonstrate the utility of this selectivity for detection of single-base substitutions. The samples were prepared through standard protocols: DNA extraction, PCR amplification and single-base primer extension. Oligonucleotide-modified nanoparticles correctly responded to the unpurified products from the primer extension: aggregation for the full match and dispersion for all the mismatches. Applicability of this method to genomic DNA was tested with five human tumor cell lines, and verified by conventional technologies: mass spectrometry and direct sequencing. Unlike the existing methods for single-base substitution analysis, this method does not need specialized equipments, and opens up a new possibility of point-of-care diagnosis for single-nucleotide polymorphisms.**

## INTRODUCTION

Detection of single-base substitutions such as single-nucleotide polymorphisms (SNPs) and point mutations is clinically important for diagnostics, prognostics, risk assessment and disease prevention (1). For this purpose, several detection methods have been established, e.g. single-strand conformation polymorphisms (2), Taqman assay (3), Invader assay (4) and single-base primer extension assay (5–7). However, these methods usually include fluorometry or mass spectrometry; both require bulky and expensive instruments and technical expertise. This limitation hinders the utility of the above methods from point-of-care genetic diagnosis in small hospitals or developing countries.

As an easy and inexpensive alternative, colorimetric detection using gold nanoparticles (GNPs) has been attracting

considerable interests, because GNP aggregation accompanied by the surface plasmon shift can be clearly recognized with the naked eye. Aggregation of probe DNA-modified GNPs and its application to DNA detection were first reported by Mirkin and co-workers (8,9). Their method is basically a sandwich assay in which a target DNA molecule cross-links two GNPs by hybridization. Recently, we discovered that GNPs also aggregate in a non-cross-linking (NCL) configuration (10,11); formation of fully complementary duplexes on GNP surfaces induces the aggregation at relatively high salt concentration. The NCL aggregation exhibits extraordinary selectivity against terminal mismatches; single-base mismatches at the free ends of the duplexes make very stable dispersions. Unlike conventional hybridization-based assays, this system can detect the terminal mismatches without precise temperature control. In this paper, we demonstrate that the terminal selectivity of the NCL aggregation is applicable to detection of single-base substitutions in genomic DNA. Oligonucleotide-modified GNPs correctly responded to unpurified solutions of single-base primer extension products.

## MATERIALS AND METHODS

### GNP preparation

A colloidal solution containing  $1.4 \times 10^{12} \text{ ml}^{-1}$  ( $= 2.3 \text{ nM}$ ) GNPs with diameter of 15 nm was purchased from BBInternational. Single-stranded thiol modified-oligonucleotides, i.e. probe (5'-CAG CTC CAA CTA CCA C-3'-(CH<sub>2</sub>)<sub>3</sub>SH) and anti-tag (HS(CH<sub>2</sub>)<sub>6</sub>-5'-CAG GAC AGG CAC AAA CAC-3'), were obtained from Espec Oligo Service and Takara Bio, respectively, and were immobilized to the GNP surfaces as described previously (10). Briefly, 5 nmol of the probe or the anti-tag was incubated with 1 ml of the GNP solution at 50°C for 16 h. The solution was changed into 10 mM phosphate buffer (pH 7) with 0.1 M NaCl by addition of the necessary salts, and was kept at 50°C for 40 h. To remove unreacted oligonucleotide, the solution was centrifuged at 14 000 r.p.m. for 25 min with a TOMY centrifuge, ARO 15-24, and the supernatant was replaced by 1 ml of 10 mM phosphate buffer

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(pH 7) with 0.1 M NaCl and 0.01% Tween-20. After another centrifugation under the same condition, the precipitate was re-dispersed into 0.25 ml of the same buffer to make a stock solution containing 9.2 nM GNPs.

#### Estimation of the amount of the immobilized probe

To release the 3'-immobilized probe, DTT was added to the probe-GNP solution. The final concentrations were 10 mM for DTT and 4.6 nM for GNPs. The solution was incubated at room temperature for 16 h. After removal of the GNPs by centrifugation at 14 000 r.p.m. for 25 min, the released probe in the supernatant was quantified using OliGreen ssDNA Quantitation Kit (Molecular Probes).

#### Estimation of the hybridization efficiency

Four sample oligonucleotides, i.e. complementary strand (5'-GTG GTA GTT GGA GCT G-3'), typing primer (5'-GTG GTA GTT GGA GCT-3'), terminal mismatched strand (5'-GTG GTA GTT GGA GCT A-3') and random strand (5'-GAG GGC GTG GCT GAT-3'), were obtained from Sigma Genosys. The probe-GNP solution was concentrated to 20 nM through centrifugation at 14 000 r.p.m. for 25 min, removal of the supernatant and re-dispersion. Each DNA sample (10  $\mu$ M, 24  $\mu$ l) was mixed with 72  $\mu$ l of the concentrated probe-GNP solution. After 10 min incubation at room temperature, 24  $\mu$ l of 5 M NaCl was added. The mixture was cooled on ice for 1 h, and was centrifuged at 14 000 r.p.m. for 25 min to remove the GNPs. Absorbance at 260 nm of the supernatant was measured with a Cary 50 UV-Vis spectrometer (Varian). The absorbance value was compared with that of a negative control mixture without GNPs to calculate the amount of the hybridized sample.

#### Titration experiments

Sample solutions containing various concentrations of the complementary strand and the typing primer were prepared. Each sample solution (65  $\mu$ l), the probe-GNP stock solution (9.2 nM, 30  $\mu$ l), and Tween-20 (1%, 1  $\mu$ l) were mixed and incubated at room temperature for 10 min. After addition of 24  $\mu$ l of 5 M NaCl, the mixture was cooled on ice for 1 h. Precipitate of aggregated GNPs was tightened by a personal micro-centrifuge for 15 s, and the supernatant dispersion was transferred to a cuvette for extinction measurement at 530 nm with the UV-Vis spectrometer. Experiment was duplicated for each sample composition.

#### DNA isolation and PCR amplification

Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN) from colorectal adenocarcinoma cell lines (HCT-15, WiDr, SW480, DLD-1 and COLO205; Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University). *K-ras* codons 1–37 of the genomic DNA and templates (Takara Bio *ras* Mutant Set, c-Ki-*ras* codon 12) were amplified by PCR in 100  $\mu$ l scale. Each reaction mixture was composed of 250 ng of genomic DNA or 1 ng of template, 1  $\mu$ M of each primer (forward primer: 5'-GAC TGA ATA TAA ACT TGT GG-3' and reverse primer: 5'-CTA TTG TTG GAT CAT ATT CG-3'), 200  $\mu$ M of each dNTP, 2.5 U of Pyrobest DNA polymerase (Takara Bio), and Pyrobest Reaction Buffer (Takara Bio). Twenty-five

thermal cycles were performed using steps of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. After the PCR, the amplicons were purified using MagExtractor DNA Purification Kit (TOYOBO).

#### Single-base primer extension

Primer extension reactions were performed in 20  $\mu$ l scale. Each reaction mixture was composed of 0.4–0.7  $\mu$ M of the purified amplicon, 1  $\mu$ M of the typing primer, 25  $\mu$ M of each ddNTP, 4 U of ThermoSequenase (Amersham Life Science) and ThermoSequenase Buffer (Amersham Life Science). Forty-five thermal cycles were performed using steps of 94°C for 30 s, 37°C for 30 s and 72°C for 30 s.

#### Aggregation assay

For the use of immobilized probe, 5  $\mu$ l of the probe-GNP stock solution (9.2 nM) and 1  $\mu$ l of 1% Tween-20 were mixed with 10  $\mu$ l of the unpurified primer extension product. After 10 min incubation at room temperature, 4  $\mu$ l of 5 M NaCl was added to the mixture. After another 10 min incubation at room temperature, 2  $\mu$ l of the mixture was spotted onto a C18 reversed-phase TLC plate (Merck). The remaining 18  $\mu$ l of the mixture was allowed to cool on ice for 1 h.

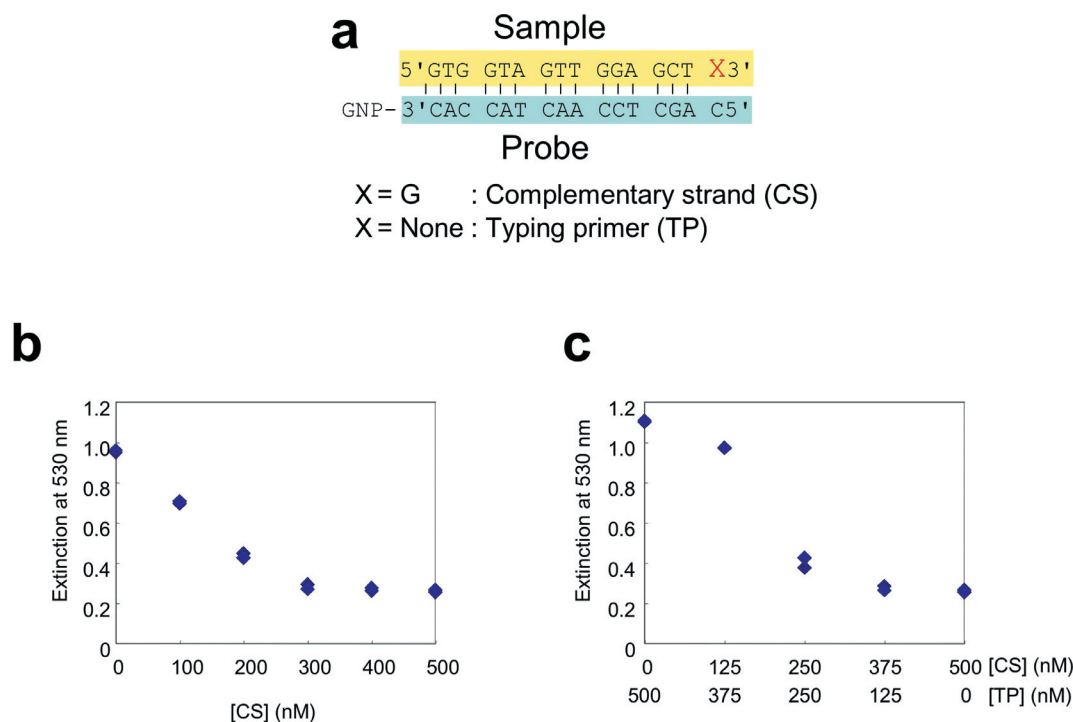
For the use of free probe with tag sequence, 1  $\mu$ l of 10  $\mu$ M probe-tag DNA and 1  $\mu$ l of 1% Tween-20 were mixed with 10  $\mu$ l of the unpurified primer extension product. After 10 min incubation at room temperature, 5  $\mu$ l of the anti-tag-GNP stock solution (9.2 nM) was added to the mixture. The rest of the procedure is the same as described above.

## RESULTS AND DISCUSSION

#### Characterization of the probe-GNPs

The amount of the 3'-immobilized probe was calculated as  $162 \pm 5$  probe molecules per GNP (mean  $\pm$  SD of triplicate measurements in a single experiment). This value is close to those in the literature (12–14). The hybridization efficiencies on the GNP surfaces were estimated from UV absorbance values of the DNA sample solutions. Because of the low sensitivity of the measurements, this set of experiments was carried out with higher concentrations of the samples and the probe (2  $\mu$ M for each) than in the rest of this paper. The hybridization lowered the sample concentrations in the supernatants by 0.60  $\mu$ M for the complementary strand, 0.55  $\mu$ M for the typing primer, 0.54  $\mu$ M for the terminal mismatched strand, and no significant decrease for the random strand. The differences among the first three values are considered to be within experimental error. These results indicate that the single-base differences hardly affect the hybridization efficiency, and only partial (~30%) hybridization of the complementary strand can induce the NCL aggregation.

Performance of the NCL GNP aggregation assay was evaluated by titration experiments (Figure 1). First, sample solutions containing various amounts of the complementary strand were mixed with aliquots of the probe-GNP solution. As shown in Figure 1b, 100 nM of the complementary strand was detectable with the spectrometer. However, reliable detection with the naked eye required a sample concentration higher than 200 nM. Second, mixtures of the complementary strand



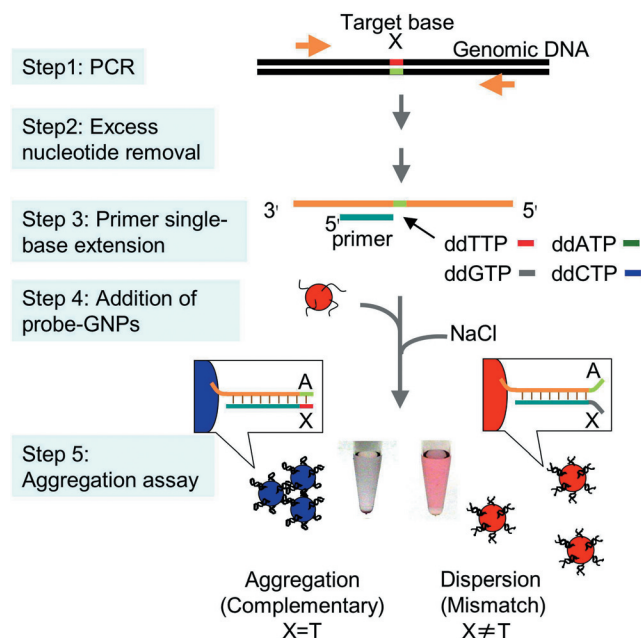
**Figure 1.** Titration experiments. (a) Sequences of the probe and the two samples: complementary strand (CS) and typing primer (TP). (b and c) Titration plots. The abscissas express the final concentrations. (b) The complementary strand. (c) Mixture of the two samples.

and the typing primer were tested in the same way (Figure 1c). The total concentration of the two components was kept 500 nM. The pure sample of the typing primer did not bring about aggregation. The dangling end of the surface-bound duplex is considered to make a similar effect to terminal mismatches on colloidal stability of GNPs. However, its interference with the complementary duplex was not very serious; the GNPs satisfactorily responded to the 50:50 mixture. This system seems robust enough for detection of samples produced by imperfect primer extension reactions.

Scientific implication of the above results has not yet been resolved. The NCL aggregation turned out to be possible with an unexpectedly low surface coverage of the complementary duplex. The hybridization efficiencies and the result for the 50:50 mixture lead to the conclusion that the coverage can be as low as 15% of the total DNA on the GNP surfaces. This behavior is difficult to explain only by the cancellation of colloidal repulsion forces (electrostatic and steric). We speculate that there exists a specific interaction between two complementary duplexes. Direct force measurements using an atomic force microscope (15) might provide effective information to resolve this problem.

### Responses of the GNPs to primer extension products

Figure 2 shows steps for the detection of single-base substitution using NCL GNP aggregation. The suspected substitution site is denoted by X. The sample is amplified by PCR (Step 1). After removing the residual dNTPs and PCR primers from the amplicon (Step 2), single-base extension reaction is performed with a typing primer and a set of ddNTPs (Step 3). Probe DNA-modified GNPs and NaCl are sequentially added to



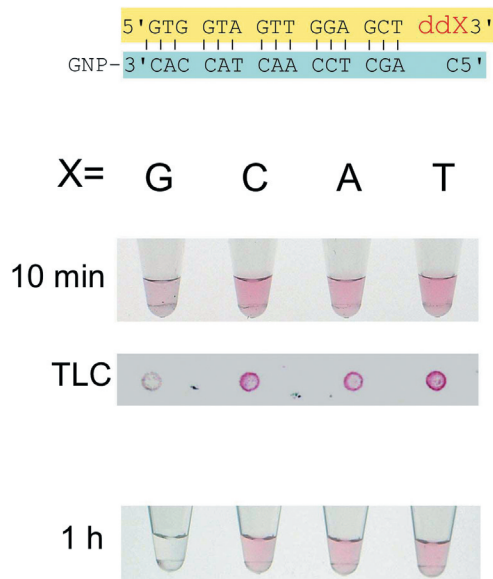
**Figure 2.** Schematic diagram for the detection of single-base substitution at target base X using NCL GNP aggregation assay. In Step 3, the same (not the complementary) kind of ddNTP as X is attached to the primer. This notation is used throughout this paper.

the extension product (Step 4). The probe sequence is fully complementary to one of the four possible extension products. Other three products make single-base mismatches at the free ends of the duplexes. Figure 2 illustrates an example of adenine-ended probe. This system informs us whether X is



thymine or not; the GNPs specifically aggregate together only if ddTTP is attached to the primer.

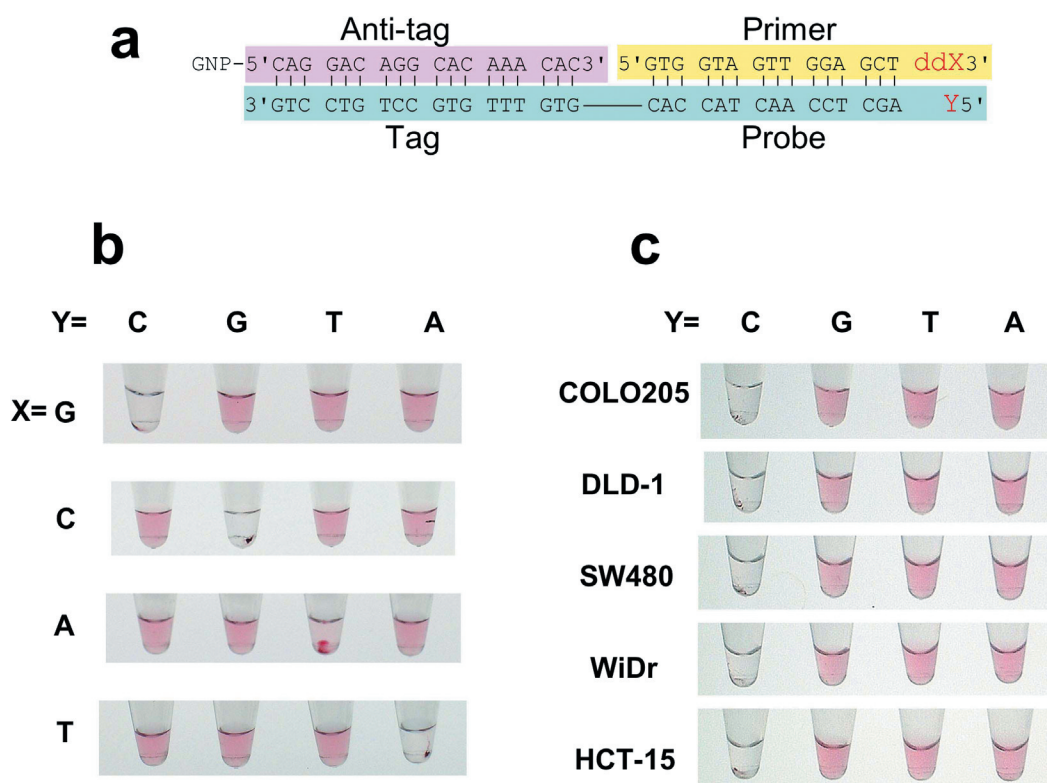
To examine the specificity of the NCL aggregation, we adopted *K-ras* oncogene as a model sequence. Although we



**Figure 3.** Specificity tests for primer extension products from four templates with different bases at *K-ras* codon 12 using probe-immobilized GNPs. The wild-type (X = G) and the mutants (others) can be discriminated by colors of the tubes (20  $\mu$ l) or the spots on the TLC plate (2  $\mu$ l).

consider that our method is especially suitable for SNPs typing, this non-SNP sequence was convenient for the first evaluation, because PCR templates of various well-defined mutants are readily available. Four templates with A, T, G and C at their first bases of codon 12 were amplified with PCR. For each amplicon, single-base extension was carried out using the typing primer with the same sequence as codons 7–11. The cytosine-ended probe–GNPs and NaCl were added in this order to the unpurified products (Figure 3). After 10 min incubation at room temperature, purple color of sample G became distinguishable from other three. Since only sample G made an extension product complementary to the probe, this sample brought about aggregation of GNPs, whereas other samples did not. ‘Spot tests’ using a C18 reversed-phase TLC plate (9) made the difference more obvious (Figure 3). Furthermore, this technique reduced sample volume from 20 to 2  $\mu$ l. In this case, the signal was amplified by concentration effect due to absorption of water into the TLC plate. Another way to clarify the difference is simply waiting for the precipitation of the aggregates. We found that cooling on ice accelerates the aggregation. One hour was sufficient to observe the clear supernatant only for sample G (Figure 3).

Probe sequences do not need to be immobilized to GNPs in advance. Use of free probes with a common tag sequence enabled a single type of GNPs to respond to multiple samples (Figure 4a). In this case, 5' end of an 18mer anti-tag sequence, unrelated to the sample sequence, was immobilized to GNP surfaces with thiol–gold interaction. Four types of 34mer



**Figure 4.** (a) Use of free probes with a common tag sequence, which is complementary to the anti-tag sequence immobilized to the GNPs. Four probes with different 5' terminal bases (Y = C, G, T and A) were prepared. (b) Combinatorial specificity tests using the four probes and the same samples as shown in Figure 3. (c) Results of NCL aggregation assay for samples from genomic DNA in human tumor cell lines.

probe-tag DNA (Figure 4a, Y = C, G, T and A) were prepared and hybridized with the same extension products described above in a combinatorial fashion. To the 16 mixtures, the anti-tag-GNPs and NaCl solutions were added in this order. Figure 4b shows the photographs of the tubes left on ice for 1 h. As expected, GNPs of four complementary pairs precipitated, whereas other twelve pairs kept in dispersion.

To confirm the practicability of this method for genomic DNA, we carried out a series of experiments consisting of DNA extraction from human tumor cell lines, PCR amplification of *K-ras* gene, primer extension of codon 12 and NCL aggregation assay. The free probes and the anti-tag-GNPs illustrated in Figure 4a were used for this purpose. Figure 4c shows the results for colorectal carcinoma cell lines (HCT-15, WiDr, SW480, DLD-1 and COLO205). These results indicate that all the cells analyzed here have guanine as their first bases of *K-ras* codon 12. This conclusion was verified with two conventional technologies: MALDI-TOF mass spectrometry for the primer extension products (Supplementary Figure S1 and Table S1) and direct sequencing for the PCR amplicons (Supplementary Figure S2). Results of the verification, summarized in Supplementary Table S1, are perfectly consistent. In addition, the mass spectra revealed that about halves of the primers were not extended. The aggregation assay was robust enough to respond such mixtures, as expected from the titration experiment.

## CONCLUSIONS

We demonstrated application of the NCL GNP aggregation to detection of single-base substitutions in genomic DNA. We believe that our method is currently the easiest technique to determine single-base primer extension products, because the manual operation is only mixing, and the results can be clearly seen with the naked eye. The use of tag sequence turned out to be effective for economy of labor and expense for GNP preparation. Optimized tag design would realize a universal type of GNPs applicable to a large number of target sequences. We will establish the reliability of NCL aggregation assay by accumulating the results for various SNP sequences. For application to heterozygotes, a slightly modified procedure—split primer extensions for different probes—is under investigation (Supplementary Figure S3). A preliminary experiment with a model sample (a mixture of PCR products) was successful.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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