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Colorimetric SNP analysis using oligonucleotide-modified nanoparticles†

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A point mutation in the p53 gene has been detected by means of fluorescence microscopy and fluorescent resonance energy transfer (FRET) through sequence selective aggregation of DNA-modified nanoparticles, in which fluorescent dyes were impregnated.

Complementary base pairing in nucleic acids would be useful in the construction of building blocks for making structures with mesoscopic dimensions (nanoscale, typically 1 to 1000 nm), because their programmability allows design of predetermined structures. We and several other research groups have been studying the design of structures using DNA conjugates,[1] DNA-modified particles,[2] and DNA itself.[3] Some of the structures have been applied to the methods for gene analysis.

We now present a novel method for colorimetric gene detection using the aggregation (networking) of oligonucleotide (ODN)-modified nanoparticles. ODNs were covalently immobilized onto the organic nanospheres impregnated with fluorescent dyes. By adding the single-stranded DNAs that are complementary to the modified ODNs, the spheres gathered to produce aggregates by cross-linked networking though specific base pairing (Fig. 1). The colors of the aggregates, which depended on the added DNA sequences, were observed using an ordinary fluorescence microscope. Fluorescent resonance energy transfer (FRET) between the particles also provided information about point mutations on added DNAs.

For the preliminary studies, 5′-amino-terminated thymidylic acid 20-mer (dT_{20}) was immobilized onto the carboxylate-modified polystyrene beads with 44 nm diameters, in which red dyes (BODIPY derivative (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene), excitation/emission maxima = 580/605 nm) were pre-loaded. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was used as a condensation reagent under the typical conditions. Aggregation of the spheres was observed using a fluorescence microscope equipped with appropriate optical filters and a CCD camera. These spheres showed aggregation in the presence of a complementary RNA homopolymer, polyA

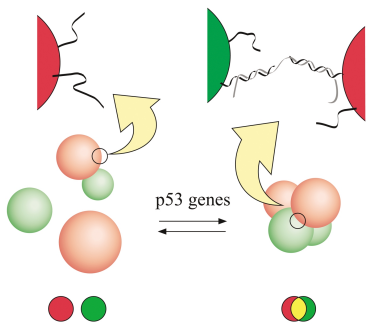

Fig. 1 Schematic illustration of the gene detecting system using the aggregation of ODN-modified nanospheres. Differently colored (red and green) spheres gather through the hybridization with single-stranded complementary DNA (p53 gene) to give the yellow aggregates under appropriate conditions.

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under the appropriate conditions (1.0 M NaCl, 40 mM Hepes (pH 7.0)) (ESI†). On the other hand, the spheres were fully dispersed in the presence of polyC and polyU under the same conditions. In addition, the aggregation with polyA was entirely suppressed by the addition of 5 M urea. The aggregation was reversible upon varying salt concentration and temperature; this is the feature of hybridization of nucleic acids.

We have applied this base selective aggregation of ODN-modified spheres to the detecting system of the mixed bases sequence, SNP (single nucleotide polymorphism) analysis. Mutations in the p53 gene are turning out to be the most common genetic alterations in human cancer.[4] It is also related to other many very important events in cells such as apoptosis, DNA repair, regulation of the DNA replication, cell cycle, and differentiation induction.[5] All ODNs used for the p53 detection were chemically synthesized. **WT45^{AGG}** (d(5′ATGTGTAACAGTTCCTGCATGGGCGGCATGAACCGG**AGG**CCCATC3′)) and **MT45^{AGT}** (d(5′ATGTGTAACAGTTCCTGCATGGGCGGCATGAACCGG**AGT**CCCATC3′)) are the 45-mer sequence of the p53 gene containing one of the hot spots. The former is the sequence of the wild type and the latter is that of the mutant, which contains mutated T instead of G (the mutation from Arg^{249} to Ser^{249}).[6] The 5′- and 3′-amino-terminated ODNs, **5cWT15** (d($H_2N(CH_2)_6$-TTTTTTTTTTGATGGGCCTCCGGTT3′)) and **3cWT15** (d(5′GGAACTGTTACACATTTTTTTTTTT-$(CH_2)_6NH_2$), are the 25-mer sequences consisting of the dT_{10} linker and the 15-mer sequences complementary to both ends of **WT45^{AGG}**.

Two types of carboxylate-modified polystyrene beads with 44 nm diameters were used as the nanosphere bases, in which yellow–green or red dyes (BODIPY derivatives, excitation/emission maxima = 505/515 nm for yellow–green and 580/605 nm for red) were pre-loaded. **5cWT15** and **3cWT15** were anchored onto the yellow–green and red spheres, respectively, through EDAC method. From the amount of recovered unreacted **5cWT15** or **3cWT15**, the surface coverage was estimated to be *ca.* 2.3×10^3 Å^2 per ODN (*ca.* 220 ODNs on a sphere). The ODN-modified spheres impregnated with red and yellow–green dyes are termed sphere **R** and **G**, respectively.

Fig. 2(a) shows photographs of a mixed dispersed solution of sphere **R** and sphere **G** in buffer solution (40 mM Hepes (pH 7.0)). Differently colored points of emission, red and green, separately dispersed, and fine Brownian motion were observed for each of the points. With the addition of $MgCl_2$ and **WT45^{AGG}** into this dispersed solution (10 mM $MgCl_2$, 40 mM Hepes (pH 7.0)), aggregates were produced from the solution (Fig. 2(c)). One can see the emitted aggregates with dimensions ranging from hundreds of nm to tens of μm. Although **MT45^{AGT}** induced the aggregation to some extent, a considerable number of the spheres remained dispersed (Fig. 2(b)).

In addition, it should be noted that the distributions of each type of sphere are different between the both systems. The differently colored red and green points were considerably separately distributed in the aggregates obtained in the presence of the mutant, **MT45^{AGT}** (Fig. 2(b)). On the other hand, the distributions of the two colored points of the aggregates obtained by the addition of the target, **WT45^{AGG}**, were perfectly superimposable (Fig. 2(c)). As a result of the

superposition of the red and green, the aggregates emitted yellow light. That is, while **WT45^{AGG}** efficiently connects between sphere **R** and **G** to give a well mixed sphere network, a non-specific interaction takes part in the aggregation with **MT45^{AGT}**. The area percentage of false positive (yellow area) in the aggregates with **MT45^{AGT}** was *ca*. 8% and that of false negative (red and green area) for **WT45^{AGG}** was less than 1%. 1 μL of the solutions containing the sub-picomole samples was sufficient for this detecting method.

The miscibility of the spheres **R** and **G** in the aggregates generated in the presence of the samples (**WT45^{AGG}** or **MT45^{AGT}**) should be confirmed by the FRET observations, because the efficiency of FRET is very sensitive to the distance between the constituents.[7] The fluorescence emission spectra of diluted solutions of samples used in the microscopic study (Fig. 2) are shown in Fig. 3. All spectra were obtained by excitation at 470 nm where only spheres **G** could be excited. A significant emission from spheres **R** (maximum of the emission: 605 nm) was observed for the solution with **WT45^{AGG}**, indicating that a substantial excitation energy transfer proceeded from spheres **G** (donors) to spheres **R** (acceptors). On the other hand, only a slight emission from spheres **R** was observed for the solution with **MT45^{AGT}**. The FRET observed here showed the difference in the distance between the spheres in the solution with **WT45^{AGG}** and those with **MT45^{AGT}**. The average distance between the fluorescent dyes in a sphere is *ca*. 36 Å while the Förster radius, R_0, of the two dyes (in **R** and **G**) used in this study is *ca*. 50 Å.[7] This means that effective FRET takes place only between the two spheres that directly contact with each other. Both types of spheres, therefore, were better mixed in the aggregates with **WT45^{AGG}** through the specific cross-linking between spheres **R** and **G**.

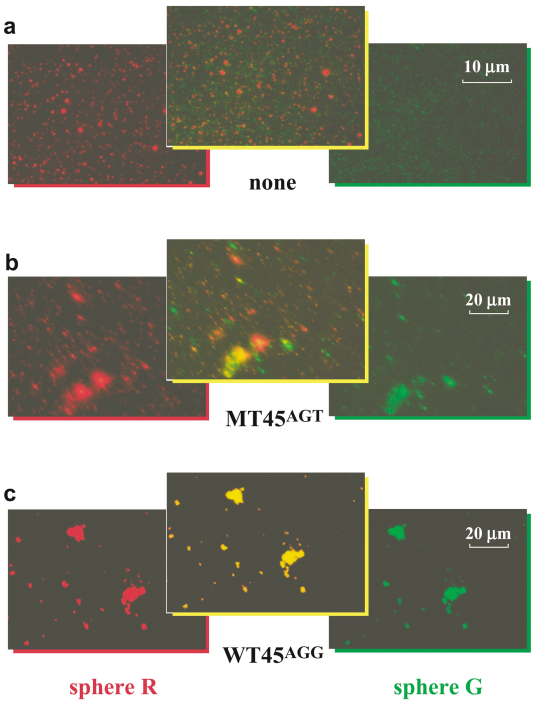

Fig. 2 Fluorescence microscopic images of the ODN-modified nanospheres. (a) 1 μL of dispersed mixed solution containing 5.7×10^8 of both spheres in 40 mM Hepes (pH 7.0). The spheres **R** and **G** are separately observed as green and red points. The sphere **R** were slightly aggregated even in the absence of ODN 45-mers and $MgCl_2$. (b) The solution consisting of 5.7×10^8 of both spheres with 210 fmol of **MT45^{AGT}** in the presence of 40 mM Hepes and 10 mM $MgCl_2$. Sphere **R** and **G** were mostly separately distributed even in the aggregates. Only part of them seemed to co-aggregate with each other, which showed a yellow emission. (c) The turbid solution of the aggregates consisting of 5.7×10^8 of both spheres with 210 fmol of **WT45^{AGG}** which contains 40 mM Hepes and 10 mM $MgCl_2$. The distribution of the each type of sphere was exactly superimposable. All of the aggregates emitted yellow light. Spheres **R** and **G** are connected *via* hybridization with **WT45^{AGG}** to give well-mixed aggregates.

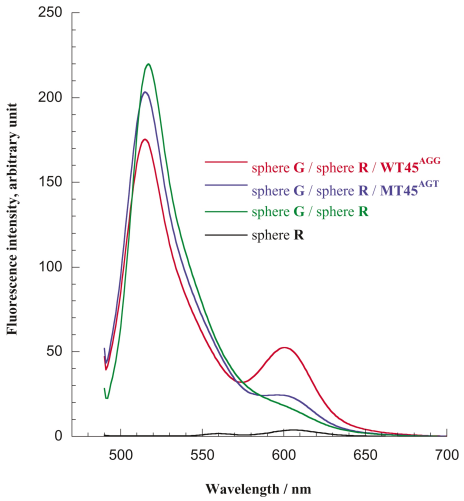

Fig. 3 Fluorescence emission spectra of the dispersed and aggregated spheres. All of the spectra were measured using 1.0 mL solutions containing 1.0×10^9 of each sphere and 40 mM Hepes (pH 7.0) by excitation at 470 nm at room temperature. black curve: only spheres **R**, green curve: spheres **G** and **R** in the absence of ODNs (sample of Fig. 2(a) diluted with buffer solution), blue curve: spheres **G** and **R** with 400 fmol of **MT45^{AGT}** and 10 mM $MgCl_2$ (sample of Fig. 2(b) diluted with buffer solution), red curve: spheres **G** and **R** with 400 fmol of **WT45^{AGG}** and 10 mM $MgCl_2$ (sample of Fig. 2(c) diluted with buffer solution).

The difference in the miscibility of both types of spheres observed by microscopy was also confirmed by this macroscopic method. To our knowledge, this is the first example to show the FRET regulation between the spheres. If a special combination of the donor and acceptor that have ideal energy levels is chosen, FRET itself would also be an excellent means of gene analysis in this system.

Significant differences in the aggregates colors and the FRET results are due to the point mutation, one base displacement out of 45 bases. The method presented here, therefore, would be a promising candidate as novel convenient colorimetry for SNP analysis.[8] The extension to the **RGB** (red, green, and blue) three components system should allow us to analyze the composition of gene mixtures (allele analysis). In addition, the intended superstructures which consist of various nanoparticles such as certain metals, semiconductors, or proteins might be constructed by the same strategy presented here.

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