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A Doubly Signal-Amplified DNA Detection Method Based on Pre-Complexed $[Ru(bpy)_3]^{2+}$-Doped Silica Nanoparticles

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Extensive research has been carried out to develop sensitive and selective detection methods for oligonucleotides because they have many applications in therapeutics, diagnostics, and basic research.[1,2] Many important technological advances involving the use of various readout systems, mainly fluorescence readout systems, have been reported.[3–7] Fluorescent readouts enable rapid, simple, and specific detection of targets. However, the current research is focused on increasing the sensitivity of the techniques to detect extremely low amounts of analytes. For this purpose, it is necessary to amplify the targets by processes such as polymerase chain reaction (PCR); these amplification processes, however, increase the cost, duration, and complexity of the analysis. Various nanomaterials have been proposed as effective alternatives to organic fluorescent dyes. These nanomaterials increase the signal intensity by grafting neighboring chemistry; some of these nanomaterials are DNA-modified gold nanoparticles with silver decomposition,[8–10] cationic polymers with high intrinsic fluorescence,[11–14] and fluorescent bioconjugated nanoparticles.[15–17] The use of fluorescent-dye-doped silica nanoparticles to detect target molecules has already been investigated.[18–21] A single silica nanoparticle contains a large number of fluorophores inside the silica matrix. Hence, unlike a single organic fluorescent dye, a fluorescent-dye-doped silica nanoparticle produces amplified fluorescent signals when excited properly.[22] In addition, the silica nanoparticle can undergo surface modification to incorporate biomolecules onto its surface. Recently, our group reported a electrogenerated chemiluminescence (ECL) assay of oligonucleotides utilizing tris(2,2-bipyridyl)-dichlororuthenium(II) hexahydrate $[Ru(bpy)_3]^{2+}$-doped silica nano*p*articles (RSNP). The amplified ECL signal on the gold electrode achieved through dendritic amplification showed a good relationship with the concentration of the target DNA over a wide linear range (10 fM–10 pM).[23] In this study, we demonstrated doubly amplified signal detection for DNA using pre-complexed RSNP (see Scheme 1),[24,25] which show ultrahigh sensitivity to target DNAs and can be used to detect target DNAs in the range of 10–100 aM. It is therefore advantageous to use $[Ru(bpy)_3]^{2+}$ instead of conventional organic dyes, such as rhodamine and fluorescein, as a dopant, also because of its manifold applications as an excellent probe in biological detection systems.[26–30]

Our strategy to detect target DNA is based on the sandwich-like assembly of three oligonucleotides which is commonly used in the detection of non-labeled target DNAs: half of the target DNA strand is hybridized (i.e., forms a duplex) with capture DNA on the chip and the other half strand of the target DNA is hybridized with probe DNA on the nanoparticle.

Scheme 1 illustrates our strategy: a doubly signal amplified detection method using pre-complexed RSNP. Target DNA (red) is captured on the biochip that contains capture DNA (blue), which is complementary to 5′-end of the target DNA. Two types of DNA-coated RSNP—$probe_{1,2}$RSNP and $probe_{2c}$RSNP—are used. $Probe_{1,2}$RSNP is coated with poly(A) $probe_2$DNA and $probe_1$DNA (yellow), which is complementary to 3′-end of the target DNA. $Probe_{2c}$RSNP is coated with poly(T) $probe_{2c}$DNA, which is hybridized with poly(A) $probe_2$DNA present on $probe_{1,2}$RSNP. Single signal amplification is accomplished by the interaction of $probe_1$DNA on the $probe_{1,2}$RSNP with the target DNA captured by the immobilized capture DNA on the biochip

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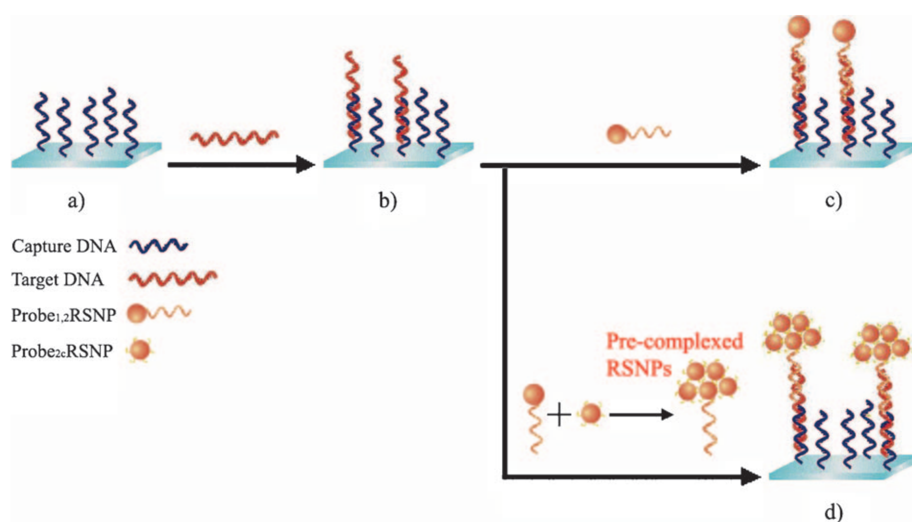
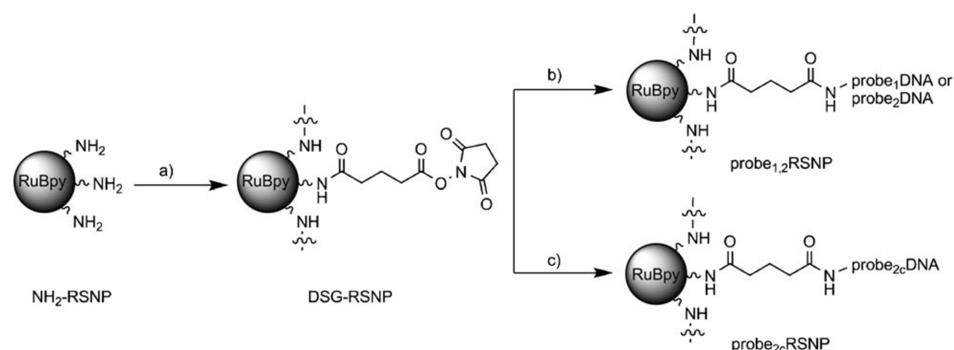
(Scheme 1c). Double signal amplification is realized as follows: probe$_{1,2}$RSNP is hybridized with probe$_{2c}$RSNP by base pairings between probe$_2$DNA of probe$_{1,2}$RSNP and probe$_{2c}$DNA of probe$_{2c}$RSNP (to obtain pre-complexed probe$_{1,2}$RSNP and probe$_{2c}$RSNP complex). Then, probe$_1$DNA of probe$_{1,2}$RSNP in the pre-complexed probe$_{1,2}$RSNP and probe$_{2c}$RSNP is hybridized with the target DNA (Scheme 1 d).



Scheme 1. Schematic illustration of a doubly signal amplified detection method using pre-complexed RSNP. a) Capture DNA (blue) is immobilized on a glass slide. b) Target DNA (red) is hybridized with capture DNA. c) Probe$_1$DNA (yellow) of probe$_{1,2}$RSNP is hybridized with the target DNA (method involving single signal amplification). d) Probe$_{1,2}$RSNP is hybridized with probe$_{2c}$RSNP by base pairings between probe$_2$DNA of probe$_{1,2}$RSNP and probe$_{2c}$DNA of probe$_{2c}$RSNP. Then, probe$_1$DNA of probe$_{1,2}$RSNP of pre-complexed probe$_{1,2}$RSNP and probe$_{2c}$RSNP is hybridized with the target DNA (method involving double signal amplification).

RSNP was synthesized by a reverse microemulsion method according to the reported procedure.[23,31] The resulting amine-modified RSNP (2 mg in 1.5 mL of phosphate buffered saline (PBS) at pH 7.4) were treated with an amine-reactive homobifunctional crosslinker, disuccinimidyl glutarate (DSG), to obtain *N*-hydroxysuccinimidyl (NHS) ester-presenting RSNP. The conjugation of the amine-modified oligonucleotides, probe$_{1,2}$DNA (1.45×10^{-5} M in PBS at pH 7.4) and probe$_{2c}$DNA (1.45×10^{-5} M in PBS at pH 7.4), to NHS ester-presenting RSNP afforded probe$_{1,2}$RSNP and probe$_{2c}$RSNP, respectively (Scheme 2).



Scheme 2. Synthetic procedure for preparing DNA conjugated RSNPs: a) disuccinimidyl glutarate (DSG), 2 h, 40 °C; b) probe$_1$DNA, probe$_2$DNA, 3 h, 40 °C; c) probe$_{2c}$DNA, 3 h, 40 °C.

The transmission electron microscopy (TEM) image of RSNP shows that the particles are spherical and monodisperse and have an average diameter of 60 nm (Figure 1 a).

The DNA-conjugated RSNP retained their spherical shape and monodispersity even after surface modification as shown in Figure 1 b (probe$_{1,2}$RSNP) and c (probe$_{2c}$RSNP). The photoluminescence spectrum of RSNP with an excitation at 458 nm shows a strong emission at 580 nm (Figure S1).

To verify our approach to ultrahigh sensitive detections by the doubly signal amplified method, a 42-mer oligonucleotide was used as a target DNA (for the sequences of oligonucleotides used in this experiment, see Table 1, Experiment 1). We immobilized capture DNA on microarrays on the di(*N*-succinimidyl)carbonate (DSC)-activated dendron-modified glass slides by following the standard procedure, as described in the user manual of NSB POSTECH (www.nsbpostech.com). The immobilized capture DNA on microarrays were then exposed to various concentrations of the target DNA (from 1 nM to 10 aM) in a frame-seal incubation chamber for 1 h at 40 °C, washed with PBS and deionized water at 25 °C, and then dried with compressed N_2 gas. For the first amplification, probe$_{1,2}$RSNPs (25 μL of 100 μg mL^{-1}, dispersed in 10 mM PBS) were deposited onto the microarrays; for the second signal amplification, pre-

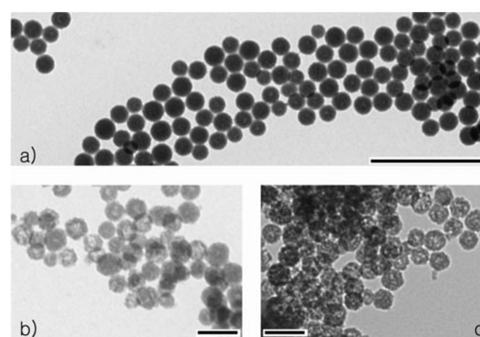


Figure 1. TEM images of a) RSNP (scale bar = 500 nm); b) probe$_{1,2}$RSNP (scale bar = 100 nm); c) probe$_{2c}$RSNP (scale bar = 100 nm).

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complexed $probe_{1,2}$RSNP (12.5 μL of 100 μg mL^{-1}, dispersed in 10 mM PBS) and $probe_{2c}$RSNP (12.5 μL of 100 μg mL^{-1}, dispersed in 10 mM PBS) were deposited onto microarrays, respectively. The microarrays were incubated for 1 h at 40 °C, washed with PBS and deionized water, and then dried with compressed N_2 gas. Figure S2 shows the representative fluorescence-scan images of the microarrays. By using the doubly signal amplified detection method, we obtained a subfemtomolar detection limit (Figure S2, right column); on the other hand, by using single signal amplification, we obtained a picomolar detection limit (Figure S2, left column). As a control, we carried out the above-mentioned experiments in the absence of the target DNA. The fluorescence-scan images of the microarrays showed only a trace of fluorescence (Figure S2, control). This result indicates that the doubly signal amplified detection method using the pre-complexed RSNP greatly enhances the sensitivity of detecting DNA in the range of 10–100 aM by a factor of ~10^6.

Figure 2 shows a plot of fluorescent intensity versus concentration for each measurement, illustrating averages of fluorescent intensities and standard deviations as error bars.[32] Next, we applied this strategy to detect 22-mer target DNA with a length comparable to that of miRNA.[33,34] We adopted the sequence of miR-16 for this experiment and used the same method and molar ratios as described above (for sequences of oligonucleotides used in this experiment, see Table 1, Experiment 2). Figure 3 shows the fluorescent image obtained when double signal amplification was used to detect 22-mer target DNA; a subpicomolar detection limit was obtained when this method was used.

In general, commercially available gene chips or oligonucleotide microarrays based on fluorescent tagging are used to detect targets at several nanomolar concentrations.[35] Several detection methods involving elaborate experimental designs have been reported for ultrahigh-sensitive DNA detection. However, to the best of our knowledge, only few of these designs can be used at femtomolar or subfemtomolar detection limits. Here, we have successfully demonstrated a new strategy for an ultrahigh-sensitive detection method involving the use of RSNP for the detection of oligonucleotides in the 10–100 aM range without additional amplification or target labeling. The study described in this paper involves the use of pre-complexed nanoparticle complex to obtain high sensitivity. We believe that this method will be a significant improvement over conventional microarray/fluorescence readout systems.

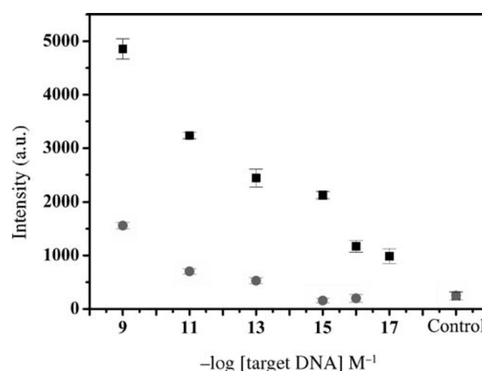

Figure 2. Quantitative mean fluorescent intensity from microarray images for target DNA length = 42-mer (black square: doubly amplified, gray circle: singly amplified, λ_{ex} = 458, λ_{em} = 600 nm).

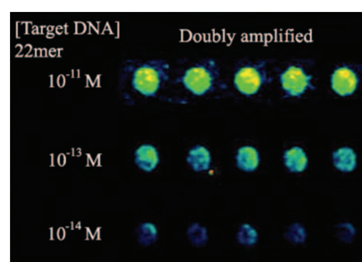

Figure 3. Microarray images obtained by fluorescence scanning (λ_{ex} = 458, λ_{em} = 600 nm). Target DNA length = 22-mer.

Experimental Section

Materials: All chemicals were reagent grade and used as received, unless mentioned. All types of DNAs used in this study were purchased from Bioneer Corporation (Daejeon, Korea). Triton X-100, cyclohexane, 1-hexanol, ethanol, TEOS, APTES, NH_4OH (25–30 wt %), and PBS (pH 7.4) were purchased from Sigma–Aldrich (Milwaukee, U.S.A.). DSG and DSC were purchased from Peirce (Rockford, U.S.A.). Acetonitrile (CH_3CN), dipropylethylamine (DIPEA), dimethylsulfoxide (DMSO) were purchased from Across (New Jersey, U.S.A.). $[Ru(bpy)_3]^{2+}$ was purchased from TCI (Tokyo, Japan). Dendron-modified glass slides were purchased from NSB Postech (Pohang, Korea). Frame-seal incubation chambers were purchased from Bio-Rad Laboratories (Hercules, U.S.A.).

Table 1. DNA sequences involved in the experiment.

Exp	DNA Name	Sequence (5′ to 3′)	Nucleotide	Modification
1	capture DNA	GCTGCTAAGGCACTGCTGACGGGGGG	26	3′-amine
	target DNA	GTCAGCAGTGCCTTAGCAGCACGTAAATATTGGCGTTAAGAT	42	none
	$probe_1$ DNA	GGGGGGATCTTAACGCCAATATTTAC	26	5′-amine
	$probe_2$ DNA	AAAAAAAAAAAAAAAAAAAAAAAAAA	26	5′-amine
	$probe_{2c}$ DNA	TTTTTTTTTTTTTTTTTTTTTTTTTT	26	5′-amine
2	capture DNA	CGCCAATATTT	11	5′-amine
	target DNA	TAGCAGCACGTAAATATTGGCG	22	none
	$probe_1$ DNA	ACGTGCTGCTA	11	3′-amine
	$probe_2$ DNA	CAGTTGGAATG	11	3′-amine
	$probe_{2c}$ DNA	CATTCCAACTG	11	3′-amine

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Modifications of RSNP with oligonucleotides: DSG (35 mg) was added to RSNP (2 mg) dispersed in PBS (1.5 mL) at pH 7.4. After stirring for 2 h at 40 °C, the reaction mixture was washed with PBS (4×). The resulting DSG-modified RSNP were then treated with oligonucleotides (1.45×10^{-5} M in PBS at pH 7.4). After incubation for 3 h at 40 °C, RSNP were again washed with PBS (4×).

Preparation of microarrays: DSC-activated dendron-modified glass slides were prepared by following the standard procedure described in the user manual (www.nsbpostech.com). In this procedure, the glass slides were treated with DSC (900 mg) and DIPEA (200 µL) in CH_3CN (200 mL). After 4 h, the glass slides were washed and sonicated several times with absolute ethanol. The amine-modified capture DNA was printed on DSC-activated dendron-modified glass slides by using a microarrayer (QArray Mini, Genetix, Ltd.). A mixture of 10 µL of spotting buffer (10 mM $MgCl_2$ and 50 mM $NaHCO_3$ at pH 8.5), 2 µL of DMSO, 2 µL of capture DNA (200 µM), and 6 µL of deionized water was used as spotting mixture. The process was carried out at room temperature in a dust-free environment with a relative humidity of 50 to 80%.

Target DNA hybridization: Target DNA hybridization was performed for 1 h at 40 °C in the frame-seal incubation chamber containing 25 µL of PBS and various concentrations of target DNA. The slides were washed with PBS and deionized water at 25 °C and then dried with compressed N_2 gas.

Singly signal-amplified detection: $Probe_{1,2}$RSNP (25 µL, 100 µg mL^{-1}) dispersed in 10 mM PBS was deposited onto the microarray in the frame-seal incubation chamber and incubated for 1 h at 40 °C. The slide was then washed with PBS and deionized water at 25 °C and then dried with compressed N_2 gas.

Doubly signal-amplified detection: $Probe_{1,2}$RSNP (12.5 µL, 100 µg mL^{-1}) dispersed in 10 mM PBS and $probe_{2c}$RSNP (12.5 µL, 100 µg mL^{-1}) dispersed in PBS (10 mM) were mixed and incubated for 1 h at 25 °C. 25 µL of pre-complexed RSNP were deposited onto the microarray in the frame-seal incubation chamber and incubated for 1 h at 40 °C. The slide was then washed with PBS and deionized water at 25 °C and then dried with compressed N_2 gas.

Fluorescence scanning and signal quantification: Image acquisition and fluorescence intensity analysis were performed using a microarray scanner with $\lambda_{ex}=458$ nm, $\lambda_{em}=600$ nm (ScanArray Gx PLUS, PerkinElmer Life and Analytical Sciences) and software (ScanArray Express ver. 3.0.3.0002, PerkinElmer Life and Analytical Sciences) for quantitative microarray analysis.

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Keywords: biochips · DNA detection · microarrays · nanoparticles · signal amplification

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