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Luminescent Lanthanide Nanoparticles as Labels in DNA Microarrays for Quantification of Methyl Tertiary Butyl Ether Degrading Bacteria

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We report application of lanthanide nanoparticles for DNA quantification in a microarray platform as a substitute for conventional organic fluorophores. A non-PCR based DNA microarray assay for quantifying bacteria capable of biodegrading methyl tertiary-butyl ether (MTBE) was demonstrated. Probe DNA was immobilized on a glass surface, hybridized with biotinylated target DNA and subsequently incubated with Neutravidin-biofunctionalized nanoparticles. The fluorescence spot intensities, measured by a commercial laser scanner, show a linear relationship ($R^2 = 0.98$) with bacterial 16S rDNA over a range of target DNA concentrations, while the background fluorescence remained low. In addition, nanoparticles fluorescence shows a stronger intensity than Quasar570 (Cy3). Present sensitivity of the assay is 10 pM of target DNA. The selectivity of the DNA-nanoparticle-probes to discriminate a non-target DNA with two base pairs mismatch in the 16S rDNA gene sequence was shown. The use of Eu:Gd_2O_3 nanoparticles as biolabels provides a relatively non-toxic, inexpensive, rapid and sensitive alternative to the materials currently used in DNA microarrays.

Keywords: Nanoparticles, Lanthanide Europium Oxide, DNA Microarrays, MTBE, *Methylibium petroleiphilum* PM1.

More rapid and sensitive methods for detection of DNA are needed to advance the growing field of monitoring microbial populations involved in biodegradation of environmental pollutants. Microarrays have been demonstrated to provide a high throughput and convenient platform for gene screening and target DNA identification.[1] Current microarray imaging and analyses based on fluorescent organic dyes are often hindered, however, by problems such as photobleaching, spectral overlaps, and poor photostability.

Newly developed alternatives to conventional organic fluorophores in DNA-labeling include the use of nanomaterials as labels. Conventional fluorophores have been encapsulated into silica nanoparticles for more sensitive DNA detection in microarray applications.[2–4] Mirkin and co-workers demonstrated the application of gold nanoparticles for DNA microarrays.[5–10] Semiconductor quantum dots (QDs) were used for DNA detection in microarrays[11] and Fluorescent *In Situ* Hybridization (FISH).[12–14] QDs have narrow emission bands, negligible photobleaching, and are brighter than currently used labels. Their limitations include photoblinking, high production cost, toxic constituents, and luminescent dependence on particle size.

We recently demonstrated the use of luminescent lanthanide oxide nanoparticles as biolabels in immunoassays[15–17] and DNA hybridization in solution.[18] The Eu:Gd_2O_3 nanoparticles (NPs) used in this study have large Stokes shifts, narrow emission bandwidths, inherent photostability, and a long fluorescence lifetime (about 1–2 ms). A major advantage of these NPs is their low production cost by spray pyrolysis. Spray pyrolysis was shown to be an excellent method for efficient doping of Eu^{3+} ions into Gd_2O_3 nanoparticles thus achieving characteristic crystallographic structure and luminescent properties.[19] Figure 1 illustrates NPs' size and shape as determined by TEM (Transmission Electron Microscopy) and the fluorescence spectra of Eu:Gd_2O_3 nanoparticles and Quasar 570 organic dye. The average representative size of synthesized NPs is around 100 nm, ranging from 50 to 200 nm and the morphology is nearly spherical as shown in Figure 1(a). For subsequent experiments, the NPs were sized by centrifugation to collect particles

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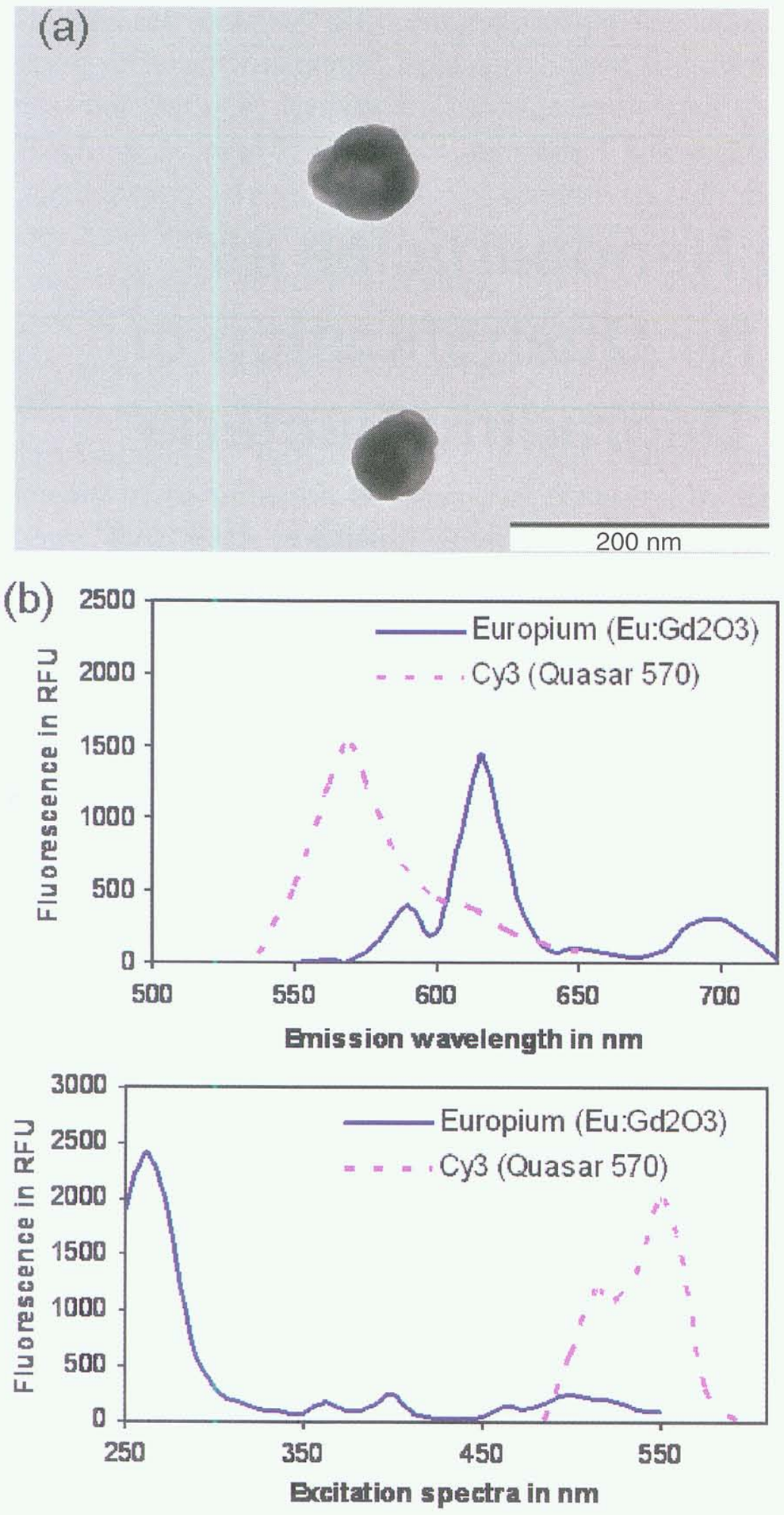


Fig. 1. (a) TEM image of $Eu:Gd_2O_3$ nanoparticles, representing their spherical shape and size (~100 nm), (b) fluorescence spectra of NPs and Quasar570 organic dye: emission spectra of $Eu:Gd_2O_3$ and Quasar570 were obtained in a plate reader excited at 260 nm and 550 nm, respectively. Excitation spectra were obtained at 616 nm ($Eu:Gd_2O_3$) and 570 nm (Quasar 570) of emission endpoints.

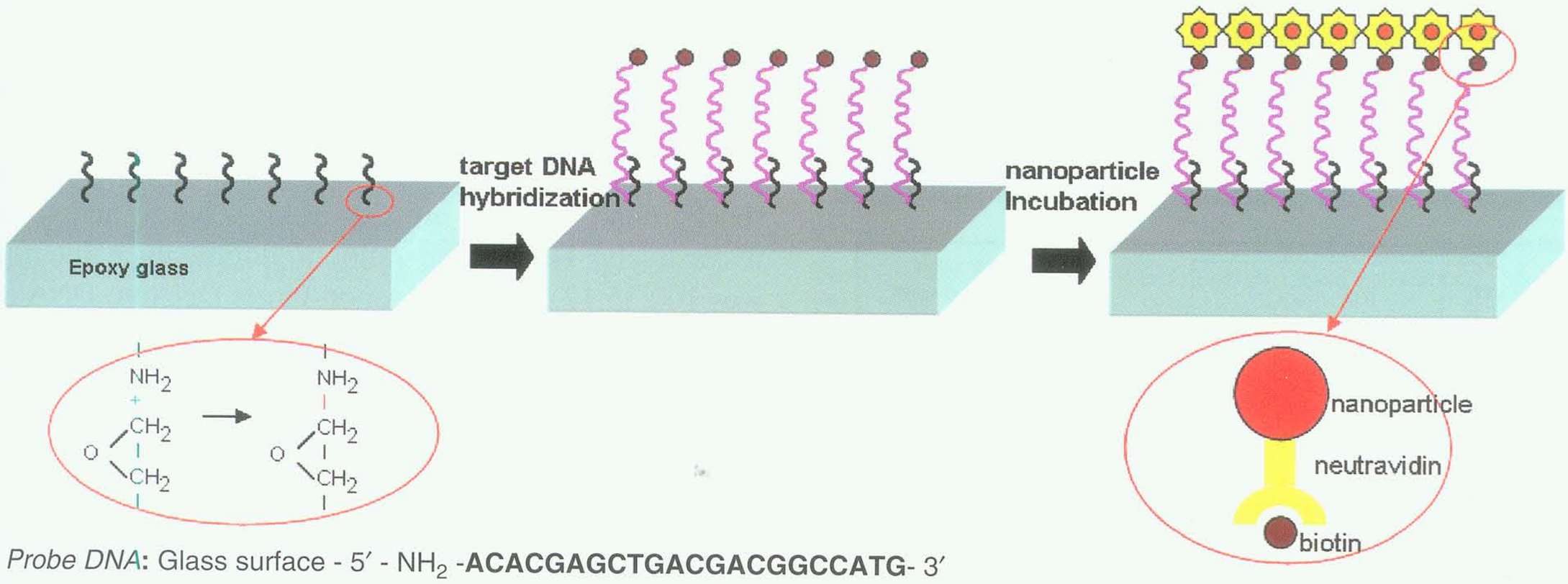
with diameters from 5 to 60 nm.[20] The $Eu:Gd_2O_3$ NPs have strong and narrow red-shifted emission at 616 nm and broad excitation spectra at 260 nm, 395 nm, and 500–520 nm (Fig. 1(b)).

We report in this paper the application of biofunctionalized $Eu:Gd_2O_3$ NP labels to quantify DNA in a high-throughput microarray format. A rapid, sensitive, and a non-PCR based DNA microarray assay was developed for quantifying bacteria capable of biodegrading methyl tert-butyl ether (MTBE). MTBE is currently a widespread groundwater contaminant as a result of its use as a fuel additive between 1980s and 2003, until a MTBE ban was enacted in US.[21] The population density of *Methylibium petroleiphilum* PM1 bacteria, naturally present at MTBE contaminated aquifers, has been linked to MTBE biodegradation.[22, 23]

Linear DNA oligoprobes were designed based on the *M. petroleiphilum* PM1 16S rDNA gene sequence[23] and were commercially synthesized (Biosource-Invitrogen, Carlsbad, CA). A schematic diagram of our DNA assay is illustrated in Figure 2. Aminated probe DNA was diluted in 1× Nexterion™ spot solution (Schott, Elmsford, NY). 20 μM of probe DNA was spotted on the Nexterion™ HiSense E Epoxysilane coated glass by Lucidea Microarrayer (GE, Piscataway, NJ). Humidity was maintained at 70%. After stabilization, the non-spot area was passivated with a solution containing $NaBH_4$, SSC, and SDS for 20 minutes at 42 °C and washed twice with 1× and 0.2× SSC. Hybridization with bacterial target DNA was carried out in a hybridization cassette (Corning Inc., Corning, NY). To prevent non-specific binding of NPs onto the glass slide, 30 μL of pre-hybridization (5× SSPE/6M Urea/0.5% Tween20/10× Denhardts) buffer was added and incubated for 1 hour at 42 °C. Different amounts of target biotinylated DNA were diluted in DIG Easy Hyb buffer (Roche Diagnostic, Basel, Switzerland) and denatured by heating at 95 °C for 5 minutes before hybridization. Hybridization between the target and probe DNA was carried out for 8 hours at 42 °C followed by subsequent washing with 2× SSC/0.1% SDS; 1× SSC; 0.5× SSC each at ambient temperature and drying in nitrogen stream.

After size separation by centrifugation, 0.5 mg of NPs were biofunctionalized with 200 μg of Neutravidin™ (Pierce, Rockford, IL) dissolved in phosphate buffer (PB, 25 mM, pH = 7.5). Passive adsorption of Neutravidin onto particles' surface was achieved in a rotating mill for 6 hours at ambient temperature. Twice-washed NP-Neutravidin complexes were suspended in 100 μL incubation buffer (0.05% Tween-20/0.1% BSA in 1× PBS) and incubated on the glass slide, with already hybridized biotinylated target DNA, for 1 hour at ambient temperature. For comparison, Quasar570 (Cy3 replacement) labeled target DNA was hybridized in parallel spots on the microarrays. Fluorescence images of NPs and Quasar570 were obtained with an Axon microarray laser scanner Genepix 4000B (Molecular Devices, Sunnyvale, CA) at 650 levels of photomultiplier (PMT) and 5 μm scanning steps. The fluorescence signal of each spot was measured by Axio-vision software (Zeiss, Thornwood, NY).

Our functionalized $Eu:Gd_2O_3$ NPs were used as labels in comparison with Quasar570 to quantify *M. petroleiphilum* PM1 16S rDNA gene. PM1 target DNA was hybridized with complementary probe DNA immobilized on the glass substrate and post-incubated with functionalized $Eu:Gd_2O_3$ nanoparticles. The fluorescence of NP labels was compared to the fluorescence of conventional organic dye, Quasar570 (Cy3) by using Axon microarray scanner. When a hybridization reaction with 100 pM of target DNA was visualized with NPs and a conventional organic dye in parallel, the fluorescence spot intensities



Probe DNA: Glass surface - 5′ - NH_2 -**ACACGAGCTGACGACGGCCATG**- 3′

Target DNA: 3′ - TTGTAGAGTGC**TGTGCTCGACTGCTGCCGGTAC**GTCGTGGACACAAGA - biotin - 5′ - particle

Fig. 2. Schematic illustration of DNA microarray hybridization using Neutravidin-functionalized Eu:Gd_2O_3 NPs and bacterial strain PM1 16S rDNA sequences for probe and target DNA. Nucleotide sequences in bold corresponds to the complementary region between probe DNA and target DNA.

show Eu:Gd_2O_3 labels have a stronger fluorescence than Quasar570 (Fig. 3). Average relative fluorescence intensities are 138 ± 7 and 205 ± 40 RFU for Quasar570 and Eu:Gd_2O_3 NPs, respectively. The excitation of Axon laser scanner used in this study is optimized for Cy3 (Quasar570, Ex. 530–550 nm) and Cy5 (Quasar670, Ex. 630–650 nm) dyes. Europium oxide NPs can be excited at 500–530 nm (Fig. 1), although their optimum excitation is in the UV light.[17] Considering the optimum excitation of Eu:Gd_2O_3 NPs cannot be fully assessed with our experimental setup, this result (Fig. 3) demonstrates that Eu:Gd_2O_3 labels provide an excellent alternative to conventional organic dyes. The use of UV laser excitation source would result in much higher sensitivity of the detection system. However, the heterogeneity of the nanoparticles' fluorescence signal, reflected in the standard deviation of nine spots (SD of nanoparticles = 40; SD of Quasar570 = 14), illustrates the drawback of NPs aggregation. Further study of better buffer formulations, such as addition of surfactant (Triton) and/or polymer (PEG), is in progress to improve colloidal stability, thus to minimize particle aggregation.

In order to examine the quantitative potential of Eu:Gd_2O_3 NP labels in DNA microarrays, a series of target DNA was hybridized with probe DNA as described above. The fluorescence intensity of Eu:Gd_2O_3 labels for each target DNA hybridization was also measured by Axon microarray scanner. Eu:Gd_2O_3 NP spot intensities show a linear relationship ($R^2 = 0.98$) with PM1 16S rDNA over a dynamic range of target DNA concentrations, while the background fluorescence remained low at a constant 30–40 RFU (Fig. 4). The fluorescence signal of 1 pM of target DNA was clearly visualized with signal-to-noise ratio (S/N) of ~2 while 10 pM of target DNA had higher S/N of ~4.5. In comparison to reported detection limit of other microarray technologies, such as 4 nM target DNA with S/N of ~2 for QDs;[11] 10–50 pM for cyanine dye-based DNA microarray;[24] ~5 pM for cyanide dye-doped silica nanoparticles DNA microarray;[3] ~1 pM for scanometric detection of gold-silver enhanced nanoparticles,[6] Eu:Gd_2O_3 NPs are competitive as DNA labels.

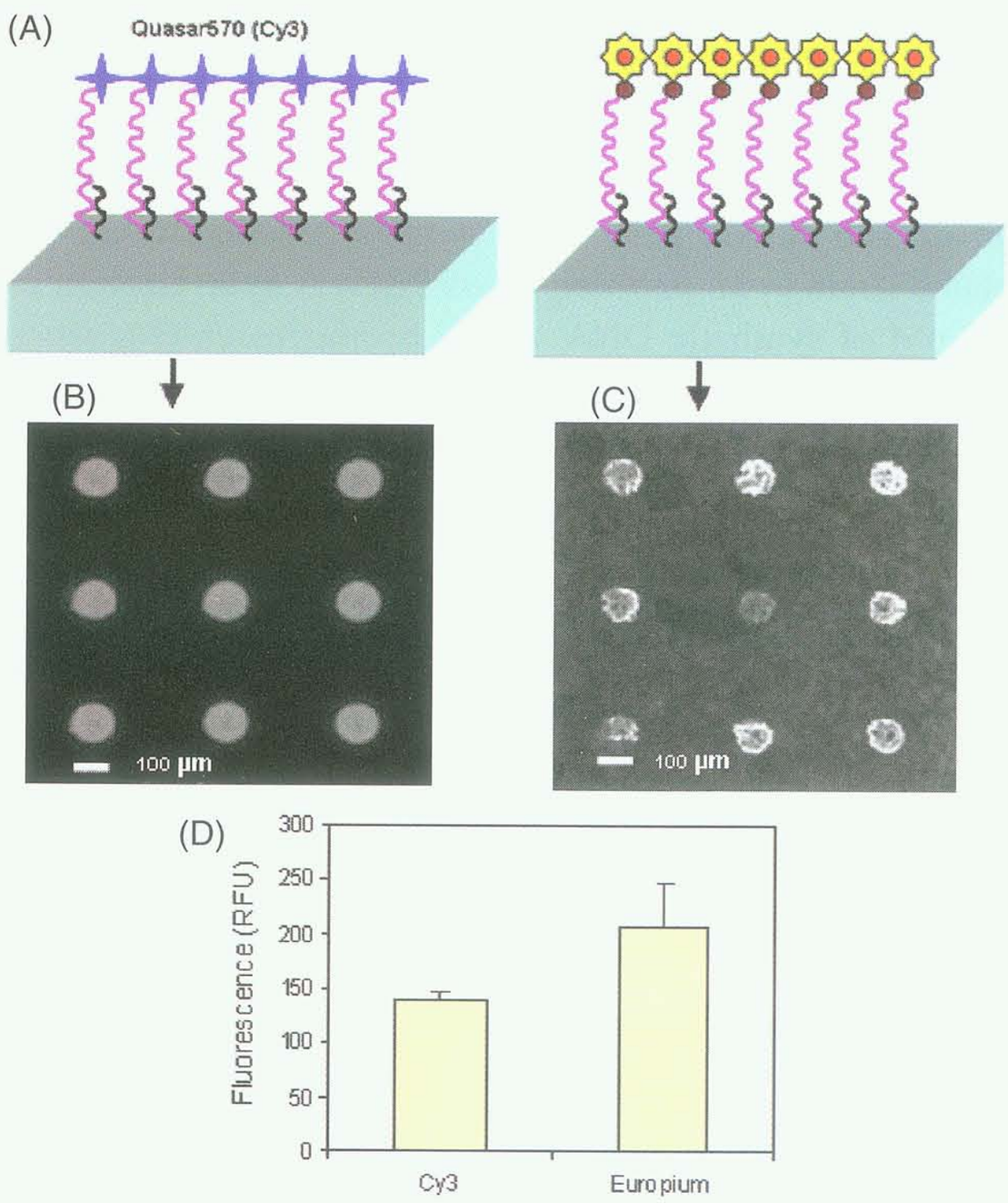


Fig. 3. Comparison of fluorescence signal between Quasar570 and europium NPs labeled target DNA. (A) Schematic illustration of DNA-label complex for Quasar570 and europium nanoparticles. Fluorescence image of (B) Quasar570 and (C) europium NPs scanned by laser microarray scanner. (D) Fluorescence signal measured and analyzed for Quasar570 and europium NPs subsequent to hybridization with 100 pM target DNA. The diameter of the spots on the chip is 100 μm. The signal and error bars represent average and standard deviations based on measurements of fluorescence intensity of nine spots.

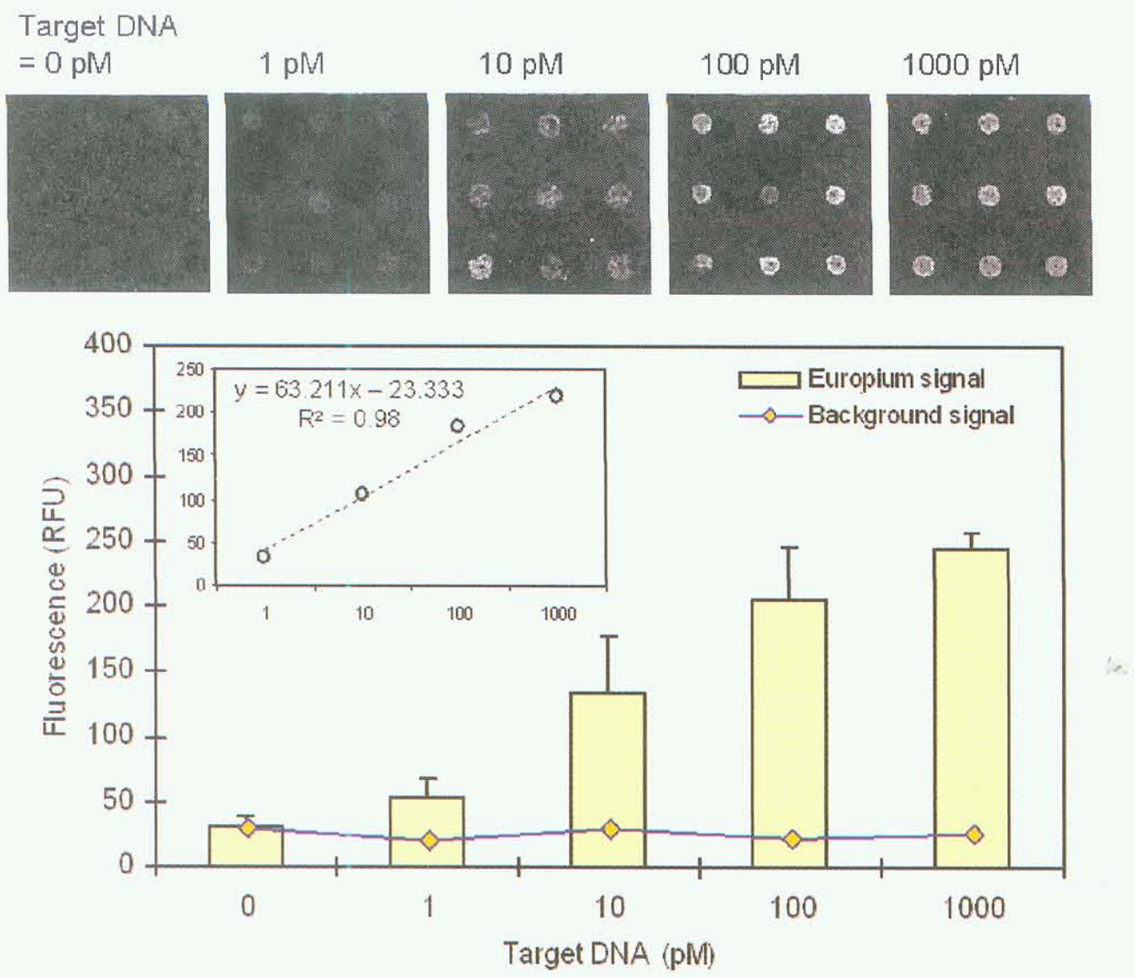


Fig. 4. Quantification of PM1 bacterial 16S rDNA in microarrays using europium NP labels. A linear relation ($R^2 = 0.98$) was observed between fluorescence values and the amount of 16S target rDNA (graph inserted). The signal and error bars represent average and standard deviations based on measurements of fluorescence intensity of nine spots.

To show the selectivity of the DNA-NP-probes, a comparison hybridization experiment with two base pairs mismatched DNA was performed in parallel to complementary target DNA. DNA with two base pairs mismatch in the target sequence was designed to represent an example of 16S rDNA gene from a phylogenetically closely related strain to *M. petroleiphilum* PM1. A hybridization of 100 pM of synthetic DNA with 2 bp mismatch (sequence shown in Fig. 5) was performed and followed by NPs incubation and assay readout in the same manner as described above. The image of microarray hybridization with non-target (2 bp mismatch) DNA showed a significantly lower fluorescence intensity in contrast to perfectly complementary target (Fig. 5). The fluorescence intensities of complementary DNA, non-target DNA, and no DNA template (background control) are 204 ± 17, 72 ± 5, and 25 ± 7, respectively. Hence, the ratio of fluorescence intensities was 100:35:12 (target DNA:non-target DNA:no DNA template). This result demonstrates the selectivity of our assay to discriminate a non-target DNA with only 2 bp mismatch in the 16S rDNA gene sequence.

The Eu:Gd_2O_3 NP labels are more resistant to photobleaching and offer more intense fluorescence than conventional organic fluorophores. In addition, the coupling of biotin-Neutravidin as a bridge between nanoparticles and DNA bypassed the need for a direct DNA labeling step. In contrast, direct DNA labeling with organic dye is complicated and susceptible to photobleaching. Therefore, the synthesis of nanoparticles by spray pyrolysis and the easy DNA coupling technique provide a low cost, simple platform for DNA analysis. We have shown the potential applicability of DNA-NP-probes for quantifying target DNA of bacteria that are responsible for the biordegradation of methyl tertiary-butyl ether (MTBE).

Our approach could easily be extended for use in a multiplexed quantification by employing a variety of fluorescent lanthanide nanoprobes for simultaneous detection of multiple pathogens, SNPs, or gene expression studies (work in progress) in a high-throughput microarray platform. Furthermore, this approach would be straightforwardly adopted as a high-throughput platform for rapid, sensitive, and simple DNA analyses during bioremediation cleanup of polluted groundwater and soil.

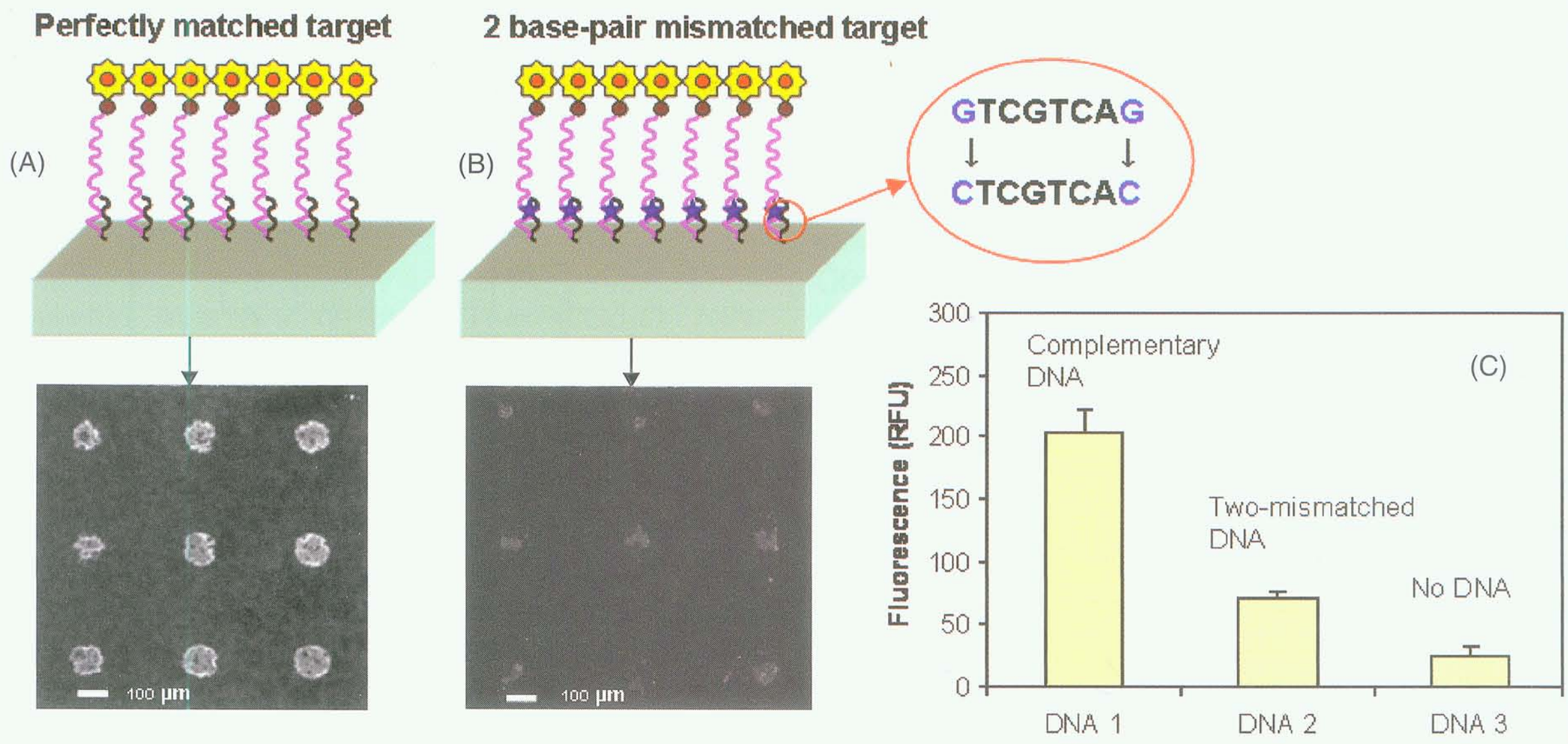


Fig. 5. Sequence selectivity of the assay using nanoparticle labels. Fluorescence images (A, B) and intensity (C) were compared. DNA (100 pM) with complementary sequence (A) and two-mismatches, non-target (B) were hybridized with probe DNA spotted on the glass. (C) The fluorescence intensities for complementary DNA, non-target DNA, and no DNA template (background control) are 204 ± 17, 72 ± 5, and 25 ± 7, respectively.

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