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Using Luminescent Nanoparticles as Staining Probes for Affymetrix GeneChips®

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Abstract

Microarray technology provides efficient access to genetic information using miniaturized, high-density arrays of DNA probes. We investigated the application of luminescent nanoparticles as probes for Affymetrix GeneChips® detection without the need for signal amplification. Our goal is to investigate the feasibility of using luminescent nanoparticles as probes in a commercial microarray system without changing its configurations. With the present imaging modality and existing optical excitation and detection systems of the Affymetrix GeneChips®, our early results indicates that nanoparticles not only can be used for GeneChip® labeling but also are superior to the traditional fluorescent protein SAPE. The advantage of the particles lies in a simplified staining procedure, higher photobleaching threshold and enhanced luminescence signal. The nanoparticles can be used for detection of low-abundance targets without any amplification step. A concentration detection limit of 50 fM has been achieved. This work demonstrates the feasibility of using luminescent nanoparticles as probes for commercial microarray systems making them less costly, more reproducible and potentially quantitative.

DNA and oligonucleotide microarrays have revolutionized gene expression profiling by allowing highly parallel and quantitative monitoring of thousands, and in some cases all, of the gene transcripts from an organism (1). This greatly increased scale with DNA microarrays has made it possible to tackle qualitatively different questions in biology and medicine. However, despite their extensive profiling and genotyping capability, using microarrays for the assessment of transcripts at low abundance levels remains elusive due to the insufficient detection sensitivity. This can be improved by significant pre-amplification of the starting material (2-3) which is most often done using a two-round T7/aRNA method. This method, however, has increased noise over the more conventional one-round amplification procedures (4-5).

Alternatively, microarray sensitivity can potentially be improved by increasing the fluorescence signal of the gene target. Unfortunately, although the new generation of fluorophores such as the Alexa series generate greater signal, their limited sensitivity for microarrays as well as their photostability need further improvement. The rapidly evolving field of bionanotechnology has opened up a promising era in new probe development (6-8). Gold nanoparticle (NP)-promoted reduction of silver was reported to detect target DNA at a concentration of 50 fM (6). The detection however, relied on coating the gold NP with silver; a complicated process which caused reduced reproducibility. Two-color microarray-based

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Supporting Information Available: Reagents, instruments, and experimental details are available free of charge via the Internet at <http://pubs.acs.org>.

detection has also been demonstrated by using Cy3- and Cy5-doped gold-core/silica-shell NPs in a sandwich hybridization (7). Each NP was embedded with ~ 100 dye molecules, and the study revealed a detection limit of 1 pM for target DNA. Quantum dots have similarly been used for DNA microarray and achieved a detection limit of 2 nM for target DNA (8). So far however, NP probes have been tested only in proof-of-concept experiments where allele-specific surface-immobilized capture probes and gene-specific oligonucleotide-functionalized NP probes are hybridized to the target sequentially. Under these conditions only a few genetic markers can be simultaneously detected. Thus far, the use of NPs to analyze genetic information has not yet been demonstrated in a real commercial system.

Herein we report the first study using dye-doped silica NPs to visualize the hybridization signal of target RNA binding to probes on Affymetrix GeneChips®. Due to their extensive genetic content and the fact that they are supplied ready for use, Affymetrix GeneChip® arrays are quickly gaining acceptance as the optimal method for determining transcription profiles. An integral component of the Affymetrix GeneChip® technology is probe redundancy. Multiple probes homologous to different regions of the target RNA are designed. This improves the signal-to-noise ratio, increases the dynamic range of detection, as well as minimizes cross-hybridization effects. An additional level of redundancy comes from the use of mismatch (MM) probes identical to the perfect match (PM) probes except in a single base located in a central position. In the conventional GeneChip® labeling strategy (Figure 1), the biotinylated cRNA is fragmented and hybridized to the GeneChip® array. After hybridization the arrays are washed and stained with streptavidin-phycoerythrin (SAPE). The signal is then amplified by first washing with a solution containing biotinylated anti-streptavidin (goat) antibody, and then a final staining with SAPE. Comparisons of gene expression levels are generated based on the detection of the fluorescence signal emitted from each element on the array. This signal amplification strategy, however, is time-consuming, may generate amplification bias and disturbs the abundance levels of the original transcripts.

Our efforts focused on replacing the conventional SAPE with dye-doped silica NPs (9) in attempt to increase the fluorescence signal and thereby the sensitivity. Each of our dye-doped silica NPs contains a large number of dye molecules, emitting a fluorescence signal more than 10,000 times that of a fluorophore (10). This extreme brightness facilitates ultrasensitive determination of trace analytes that are undetectable with existing fluorescence labeling techniques. We aim to investigate if this new NP reporter technology enhances the commercial microarray performance without either adding additional amplification bias or requiring a change in the configuration of the commercial system.

The NPs were spherically uniform with an average diameter of 60 ± 3 nm (RSD = 5%, n = 200). Fluorescence intensity of RuBpy NPs was measured and the effective fluorescence intensity of each particle is equivalent to that of 30,000 RuBpy dye molecules (Supporting Information). Moreover, the silica matrix of the NPs serves as a protective shell or dye isolator; it limits the effect of the outside environment on dye molecules which are otherwise susceptible to photobleaching due to their inherent molecular nature. To compare the photobleaching thresholds of SAPE and NPs, photostability tests were performed on both RuBpy NPs and the SAPE solutions. Within one hour of continuous Xenon lamp excitation, no noticeable photobleaching was observed for NPs. However, significant decrease in fluorescence intensity was observed for the PE solution during the same time period (Figure 2). This observation suggests that the silica matrix isolates the dye molecules from the outside environment and thereby prevents photo-oxidation by oxygen (11). Unlike SAPE, which should be kept in the dark either wrapped in foil or stored in an amber tube, the dye-doped NPs can be exposed to room light without interfering with their signaling properties; they are more robust tools for microarray staining. Indeed, after scanning NP-stained microarray ten times successively, only negligible signal variation was observed. More importantly, the remarkably high

photochemical stability of dye-doped NPs allows them to withstand numerous illumination cycles and permits several microarray measurements to be taken at different times. This enables optimization of the measurements or for verification of the measurements by different researchers. Furthermore, this improved photostability would possibly allow for extended integration time, improved signal-to-noise ratios, enhanced sensitivity and quantitation resulting in overall increased assay reliability.

In order to integrate the dye-doped NPs into the microarray system, our initial idea was to replace the SAPE with streptavidin-labeled NPs. However, considering the effects of strong steric hindrance between the rigid NP and the solid-phase surface, different NP labeling strategies were designed. NHS-PEG-biotin molecules were immobilized onto amine-modified NPs (13). These PEG linkers allowed the conjugated biotin molecules to extend out from the NP surface in order to increase binding efficiency. PEG linkers are also well-known for their ability to prevent nonspecific interactions and act as hydrophilic spacers, minimizing any detrimental interactions between the attached biomolecule and the solid surface. To demonstrate the successful immobilization of biotin molecules on the NPs, streptavidin-coated microspheres and biotin-PEG-labeled NPs were mixed (12-13). The confocal images of the resultant microsphere-NP complexes proves the successful biotinylation of the NPs (Supporting Information).

The Affymetrix GeneChip® Test3 arrays (T3 array) were used to compare the performance of different NP detection strategies. This array contains a limited number of probe sets representing genes from various organisms including mammals, plants, and eubacteria. The array also has two inbuilt controls—the B2 oligo which serve as a positive hybridization control as well as four bacterial genes involved in biotin metabolism (bioB, bioC, bioD, creX). These four genes are spiked into the hybridization cocktail in varying molar ratios and are used to evaluate hybridization efficiency. Biotin-labeled cRNA samples from a human lung cancer cell line (A549) were hybridized on the arrays and stained with NPs using three different strategies (Figure 3). The first array was stained with streptavidin-directly-labeled NPs (NP-SA); the second array was stained with PEG-biotin-streptavidin-labeled NPs (NP-PEG-biotin-SA); and the third array was first incubated with 1 mg/mL streptavidin, then washed and stained with PEG-biotin-labeled NPs (SA+NP-PEG-biotin) (Figure 4). In this last case, the streptavidin is first bound with biotin tags on the cRNA. Because each streptavidin has four biotin binding sites, it can further bind to biotin tags on the NPs. We found the binding efficiency of the three types of particles as follows: NP-SA < NP-PEG-biotin-SA < SA + NP-PEG-biotin.

This result demonstrates that the PEG linkers improved biomolecule recognition efficiency and reduced NP non-specific binding. The superiority of the third strategy over the second may be due to better stability and smaller size of biotin-labeled NPs than protein-attached NPs, and the additional washing step. The third strategy was used in all further studies. Using NPs as the staining probe, we found the percentage of genes present on the T3 array to be 21.8%, which is within the range obtained with the normal double SAPE staining (16-25%). The background noise using NP staining probe is at a similar level to the SAPE staining, illustrating minimal nonspecific binding of NPs on the microarrays.

To demonstrate whether bioconjugated NPs could improve the Affymetrix gene expression analysis by increasing its signal and thereby achieving higher sensitivity, we diluted the four bacterial hybridization controls genes (bioB, bioC, bioD, creX) by 100 fold. Thus only the concentrations of the commercial stock solutions of these four genes are available while those in the biological sample (cRNA samples from the cell line) are unknown. An aliquot of three hybridization cocktails was hybridized on three arrays in parallel and then stained with different methods. The first was single stained with SAPE; the second was stained with SAPE, followed by antibody amplification and second SAPE staining; the third was incubated with streptavidin

followed by NP-PEG-biotin staining. After the washing and scanning steps, we found the detection limit of gene expression with one SAPE staining was 1 pM, with two SAPE stainings was 50 fM, and with one RuBpy NP staining was 50 fM (Table 1). The NP single staining probe exhibited sensitivity which is 20X higher than the SAPE single staining probe, and comparable to two SAPE stainings.

It should be noted that all the Affymetrix chip signals were scanned on the Affymetrix GC3000 scanner using the optimal excitation/emission wavelengths for PE (Ex 532 nm; Em 570 nm) but not for RuBpy (Ex 460 nm; Em 600 nm). By comparison, the emission signal of RuBpy NPs excited at 532 nm is only 5% as intense as when excited at 460 nm. Even under such unfavorable conditions, the NPs were still more sensitive than one SAPE staining and comparable to two SAPE stainings. Changing the laser excitation diode and scanner filters or synthesizing dye-doped NPs matching the configuration of the scanner would possibly lead to a major enhancement in the signal from the NP stained microarrays. Another noteworthy issue is the steric crowding effects due to the physical size of the NPs. Each feature (size: 11 μ m) of the array consists of more than 1 million copies of oligonucleotide probes. Therefore, each probe occupies a square of 11 nm on each side. The NP is around 60 nm in diameter (several times larger than streptavidin-phycoerythrin). This large and bulky size may block some biological binding sites in close proximity. Therefore some biomolecules were not signaled, leading to lower sensitivity. Reduction of the particle size may address this problem. However, this is not a critical issue at a low target concentration because the distance between hybridized targets will be nearly as large or larger than the diameter of a NP. In that case, the ratio of target molecule to NP will be close to 1.

In conclusion, we used luminescent NPs as staining probes for Affymetrix Genechips® and found that they simplified the staining procedure as well as showed enhanced detection sensitivity and photostability when compared to SAPE. As NPs can potentially be used for detection of low-abundance targets without the amplification step, the method is potentially less costly, more quantitative, and the NP probe itself is less photolabile. In order to further improve the performance of dye-doped NPs for gene chips, a few modifications need to be implemented. The single-wavelength laser diodes and the Axon scanner optics need to be optimized for our luminescent NPs or we need to synthesize dye-doped NPs matching the Ex/Em wavelength of PE. The NP size also needs to be optimized to reduce the steric crowding effect. If dye-doped NPs and the optical setup are optimized in the Affymetrix GeneChip® array, NP probe could be expected to be more sensitive, less costly, and potentially more reproducible and quantitative, and thereby greatly increases the scope for precise molecular diagnosis in single gene and genetically complex diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Signal amplification scheme in the Affymetrix system.

Figure 2. Photostability test of RuBpy-doped silica NPs (top) and PE (bottom) in solution phase under the exposure of a 150 W xenon lamp.

Figure 3.
Test3 array images using different NP staining strategies.

Figure 4.
Strategy of NP-based labeling for microarray technology.

Table 1

Control gene expression on Test3 arrays after 100-fold dilution (A= absent. P = present).

Final concentrations of control cRNAs	bioB (15 fM)	bioC (50 fM)	bioD (0.25 pM)	Cre (1 pM)
SAPE (one staining)	A	A	A	P
SAPE (two staining)	A	P	P	P
NP (one staining)	A	P	P	P