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Ultrasensitive DNA Detection Using Highly Fluorescent Bioconjugated Nanoparticles

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We have developed an ultrasensitive DNA assay to detect gene products down to subfemtomolar concentrations using a highly fluorescent and photostable bioconjugated dye-doped silica nanoparticle. Sensitive DNA detection is extremely important in clinical diagnostics, gene therapy, and a variety of biomedical studies. Recently, great efforts have been made to develop new biotechnologies to improve the sensitivity and selectivity for gene analysis.[1−3] Among them, DNA hybridization, offering excellent selectivity by the DNA base pair coupled with optical detection, is one of the most widely used methods.[4,5] Usually, a fluorescent dye molecule is utilized to signal the hybridization. In ultratrace gene analysis, signal amplification is the most critical issue and is typically achieved by coupling fluorophores, such as organic dyes,[4,5] to the DNA probes. However, two major difficulties limit the sensitivity when these fluorophores are used. The first is the relatively low signal amplification. Because one DNA probe can only be labeled with one or a few fluorophores, the fluorescence signal is too weak to be detected when the target concentration is low. The second challenge is the poor photostability of many fluorophores. Most organic dyes suffer serious photobleaching, resulting in irreproducible signals for ultratrace bioanalysis.

To achieve strong and photostable fluorescence signals, several types of nanomaterials, such as quantum dots, silica, and metal nanoparticles,[6−13] have been explored as signaling probes for bioanalysis. Among them, a novel assay based on gold nanoparticle-promoted reduction of silver was reported to detect target DNA down to a concentration of 50 fM.[11] One gold nanoparticle, not one fluorophore, was used for signal amplification when one DNA strand hybridized its target. The signal was greatly amplified, and an excellent detection limit was achieved. However, the detection relied on a silver coating on the gold nanoparticle, a complicated process which also causes reduced reproducibility. To obtain better sensitivity and reproducibility, the hybridization should be signaled directly by a large amount of fluorophores which are attached to a single DNA probe. Recently, we have developed dye-doped silica nanoparticles (NPs) for signaling in bioanalysis.[12] The NP, sized between 2 and 100 nm, has a silica matrix that entraps a large number of fluorophores. There are three major advantages for the NP as a fluorescence probe in bioanalysis. First, a large number of fluorophores are encapsulated inside a single NP, which produces a strong fluorescence signal when it is excited properly. Therefore, when one probe DNA is labeled with one NP, the signal is greatly amplified as compared to one fluorophore, making it possible for ultrasensitive detection of DNA without any preamplification of the targets. Second, the NP is highly photostable, benefiting from the silica matrix shielding effect.[12] The fluorophores are well protected from environmental oxygen when they are inside the silica network, enabling the fluorescence to be constant and thus giving an accurate measurement for bioanalysis. Third, the silica surface serves as a universal biocompatible and versatile substrate for the immobilization of biomolecules. Biochemically modified NPs have been developed for various applications using known immobilization protocols based on the silica surface.[12−14]

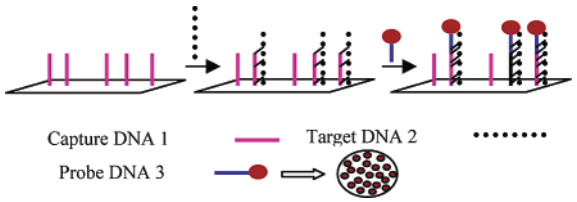

Figure 1. Schematic of a sandwich DNA assay based on NP.

In this work, we developed a new approach for the synthesis of organic dye-doped NPs using a reverse microemulsion consisting of a Triton X-100/cyclohexane/*n*-hexanol/hydrochloric acid system. Organic fluorophores usually have high quantum yields and are widely available with a large selection of spectroscopic properties. In our previous work on the synthesis of inorganic dye-doped NPs,[12] the reverse microemulsion method produced uniform-size NPs. However, most organic dye molecules such as tetramethylrhodamine (TMR) are hydrophobic and cannot be dissolved in water droplets in the reverse microemulsion. To address this problem, we converted TMR to a hydrophilic molecule by linking a dextran molecule to the TMR. Keeping the TMR-dextran inside the silica matrix was achieved by making the water droplets acidic (pH 1.5−2.0). With all of these modifications, uniform and highly fluorescent NPs (10−100 nm) were synthesized without leakage when immersed inside water.

To show the excellent utility of the NPs in ultrasensitive DNA/mRNA analysis, we employed a sandwich assay,[11] shown in Figure 1. There are three DNA strands in this assay: capture DNA1 (5′TAA CAA TAA TCC T-biotin 3′); probe DNA3 (5′ biotin-T ATC CTT ATC AAT ATT 3′) labeled with a NP (60 nm) to form a NP-DNA3 conjugate; and target DNA2 (5′GGA TTA TTG TTA AAT ATT GAT AAG GAT 3′). The combined sequences of DNA1 and DNA3 are complementary to that of the target DNA2. The biotinylated DNA1 is first immobilized on an avidin-coated glass substrate. DNA2 and NP-DNA3 are then added for hybridization. The detection of the DNA2 is done by monitoring fluorescence signals of the NP-DNA3 conjugates left on the glass surface after thorough washing steps and with proper excitation.

Subfemtomolar concentration detection limits in DNA analysis have been achieved using the NP-based assay. The rationale of the NP's high signal amplification is that one NP-DNA3 conjugate hybridizes to one target DNA2 sequence. Therefore, one target DNA2 brings one NP to the surface, giving approximately 10^4 effective dye molecules to signal a single hybridization event taking place on the glass surface. This strategy was confirmed by SEM and fluorescence images (Figure 2). After the hybridization of NP-DNA3 to DNA2 following thorough washing, the fluorescence images of different [DNA2] were taken as shown in Figure 2A−C. As [DNA2] was decreased, the number of NPs on the surface was decreased. To further verify the results, we took SEM images

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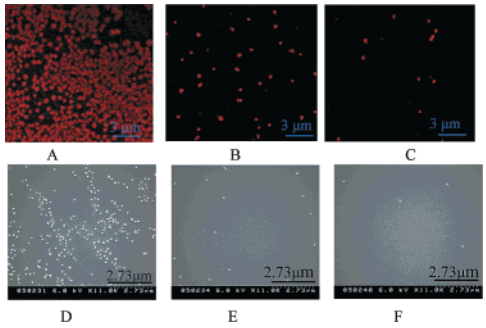

Figure 2. (A−C) Fluorescence images of different concentrations of the target DNA: (A) 3.0×10^{-12} M, (B) 5.0×10^{-14} M, (C) 1.0×10^{-14} M. (D−F) SEM images corresponding to the same concentrations in (A−C). The strict control experiments and washing were carried out to exclude potential physical binding of the NP on the glass surface.

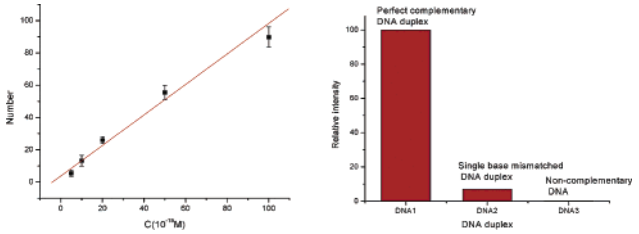

Figure 3. (Left) The number of fluorescent NPs on the glass surface versus [DNA2] based on fluorescence image counting. Five fluorescence images were counted for each sample, and there are four parallel samples for each target concentration. Fluorescence images were taken using a 1024 ES confocal scanning microscope (Bio-Rad). (Right) Sequence selectivity of the assay using NP-DNA probes. The [DNA2] is 1.0×10^{-11} M. The ratio of relative intensities of three DNA2 targets is 100:7:0.2 (complementary DNA vs single base mismatched DNA vs random DNA).

of the same glass slides, shown in Figure 2D−F (not the same area, but the same set of experiments with the same [DNA2] as those shown in Figure 2A−C, respectively). The SEM images clearly showed the same trend and also indicated individual NPs, not aggregates of particles, attached to the surface. Therefore, we were able to count the number of fluorescence spots on the fluorescence images for quantitative analysis. These numbers were proportional to the [DNA2] (Figure 3, left). On the basis of the control experiments with buffers, we calculated the detection limit of this assay to be 0.8 fM (minimal signal higher than 3 times the standard deviation). When the [DNA2] is higher than 1.0×10^{-12} M, counting the number of fluorescent spots is not reliable. Thus, measurement of fluorescence intensity should be used for high [DNA2].

To investigate the signal amplification by the NP when it was employed as a fluorescence probe as compared to a fluorophore, comparison experiments were carried out in which a probe DNA3 sequence was conjugated either with a NP or with a single TMR. Under the same conditions, these two probes were hybridized to the DNA2. The resultant fluorescence signals showed that the NP provided an approximately 10^4 times higher signal than that by the TMR in the same assay.

The NPs have also provided an excellent capability in discriminating two target DNA2 sequences that differ by only one base upon DNA hybridization (5′GGA TTA TTG TTA AAT ATT GAT AAG GAT3′ and 5′GGA TAA TTG TTA AAT ATT GAT AAG GAT3′). After hybridization, the samples were washed with the hybridization buffer for 2 min at 45 °C (optimum stringency temperature). NP-DNA3 dissociated from the surface for the one-base mismatched DNA2, while that for the perfect complementary DNA2 retained a detectable signal. The ratio of the background subtracted fluorescence intensities of the two sequences was 100:7 (Figure 3, right). To test the applicability of the NP-based assay in a mixture where other DNA species exist, a mixture of DNA2 with five random DNA sequences (5′ CGA TTA GGC3′, 5′ GGC AAG CCG ATA ACG GGA TTA 3′, 5′ GCG AGG ATT TGA CGA AAG CGC ACC TTA AAG3′, 5′ AAG CCA TGA AGC GGC TTA TGA TTC TTA CCG CCC ACT 3′, 5′ AAG GGA CGA TGA AAG CCG ATG AAG CCT CCT TTA CCT GGA3′) was studied. The results showed that fluorescence intensities of the pure DNA2 sample and that of the mixture were 59.92 ± 2.43 and 60.34 ± 1.97, respectively, showing little nonspecific binding of random DNA sequences.

The high photostability of the NPs made the assay reproducible and accurate. A comparison of the photostability of NP versus TMR was carried out in solution using an intense xenon lamp. During a 1200-s continuous irradiation, the fluorescence intensity of the NPs remained constant, while that of the pure TMR was reduced by 85%.

To achieve reproducible bioanalysis, we have taken specific measures to minimize NP agglomeration and nonspecific binding on the glass substrate. An efficient surface modification for both the NPs and the glass surface was needed. Because the NP silica surface is negatively charged, we have coated a positively charged avidin layer on it. The avidin coating helped to minimize nonspecific binding as the glass surface was also fully covered with an avidin layer. Reducing nonspecific binding is a crucial step in using nanomaterials to realize ultrasensitive bioanalysis. Based on our experiments, the NP charge, NP's surface functionality, and the substrate surface charge are the critical issues. However, it seems that there are no universal solutions to this difficult problem.

In summary, we have developed an ultrasensitive DNA analysis assay with a 0.8 fM detection limit using a bioconjugated NP-based sandwich assay. The organic dye-doped silica NPs are synthesized using a modified reverse microemulsion. The NPs are highly fluorescent and extremely photostable for bioanalysis. They exhibit an excellent signaling ability in the presence of a trace amount of DNA targets. With an effective surface modification, nonspecific binding and NP aggregation are all minimized. In addition, the NP-based DNA bioanalysis assay can effectively discriminate one-base mismatched DNA sequences. We expect this assay to be widely useful in a number of biomedical applications where accurate and ultrasensitive gene analysis is critical.

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