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An Atto-molar Detectable Colorimetric DNA Biosensor based on Enzyme Amplification Method

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Abstract

In this study, we report on the rational design of a nano-complex that consists of silica nanoparticles (SiNP), probe DNA and horseradish peroxidase (HRP) for the detection of target DNA. SiNPs were modified to probe DNA and streptavidin for the subsequent binding of biotinylated HRP enzymes. The localized binding of multiple HRP enzymes to the SiNP was used to amplify the signal of the target probe. Afterwards, magnetic microparticles (MMP) were functionalized with capture DNA and used in a sandwich reaction with the target DNA. The MMPs containing the target DNA were then bound to SiNPs via DNA hybridization. The interaction forces that resulted from the DNA hybridization between the functionalized MMP containing target DNA and SiNPs was use to separate unbound MMP; a magnetic field was used to effectively remove unbound MMP. For detection of DNA, we exploited the reaction of HRP enzyme immobilized on silica probes with tetramethylbenzidine (TMB) that is a substrate of HRP, followed by stopping the reaction with 2 M H_2SO_4. The resulting end products were analyzed by UV-vis spectroscopy. Using this method, we could detect down to 500 aM of target DNA a short time. This method is very simple and highly sensitive relative to established DNA biosensors and can be used to detect specific DNA markers associated with tumors, bacterial infections or other disease.

Keywords: Silica nanoparticle, Magnetic microparticle, Enzyme reaction, Horseradish peroxidase, DNA biosensor, Nanobiochip, Enzyme amplification

Introduction

Recent studies that have examined different methods of sensing biomarkers using nanomaterials has led to the development of gold nanoparticles[1-4], silica nanoparticles[5,6], quantum dot[7-9], and functionalized carbon nanotube[10,11]. Indeed, these nanomaterials have significantly improved the sensing performance of a variety of optical and electronic biosensing system, and have found wide applications in areas including molecular diagnostics, environmental monitoring[12], and anti-terrorism[13-19]. All of the nanomaterials including silica nanoparticles (SiNPs) coupled with biomolecules have become increasingly important biological nanoprobes[20,21]. SiNPs have special properties in comparison to other nanomaterials including excellent biodegradability and the ability to be readily functionalized. Especially, biodegradability and biocompatibility are of critical importance for their use in biosensors and bioapplication fields.

Highly sensitivity assays for DNA detection are of central importance in many applications, including medical diagnostics, treatment of genetic disease, pharmaceutical development, warning against bio-warfare agents and genomics research[22-24]. All organisms can be defined and identified by their genomes, which contain millions of nucleotide base pairs of chromosomal DNA. For microbes, such as bacteria, strains are considered genomic species if they share a DNA-DNA rehybridization rate of $\geq$70%. This leaves a large amount of other genomic sequences available for both detecting and differentiating among most pathogenic bacteria and viruses. Therefore, sequence-specific detection of DNA targets associated with either genetic or pathogenic disease has become increasingly important in molecular diagnostics. For example, the anthrax lethal factor (5′GGATTATTGTTAAAT---ATTGATAAGGAT 3′) was chosen as a sensing target. This sequence is important for bioterrorism and biowarfare applications, and has been extensively studied in the literature[25,26].

Such biodetection commonly relies on hybridization of DNA, and requires proper transducers to achieve ultrasensitive measurements. Some of the most promising biosensing devices are based on novel probe materials. Although many of the advances in probe technologies have initiated the development of novel forms

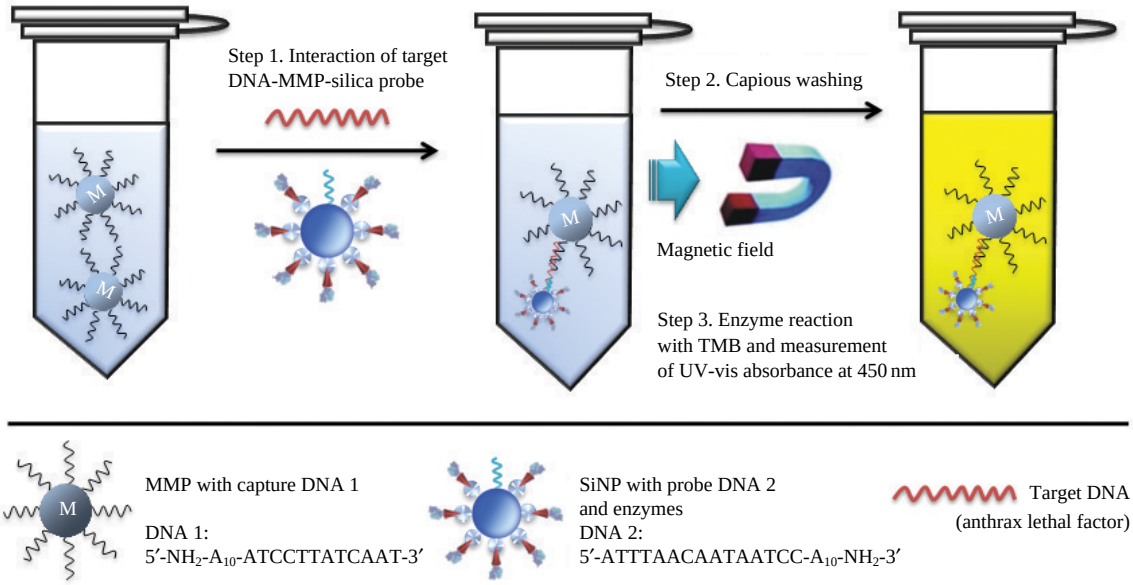

Figure 1. Schematic description of the colorimetric DNA biosensor using enzyme amplification method.

of detection, which have been based upon electrical, electrochemical, or Raman-based signal transduction scheme, colorimetric schemes are very attractive for such bioassays because of their high sensitivity, simplicity, miniaturization, and low cost.

The use of enzyme labels to produce colorimetric signals has been extremely useful in the development of ultrasensitive bioaffinity assays for detecting proteins and DNA. The horseradish peroxidase (HRP) enzyme is one of the most widely used biomaterials in biosensors and diagnostic applications. It is often used in conjugates to determine the presence of a molecular target. For example, an antibody conjugated to HRP may be used to detect a small amount of a specific protein in a western blot and enzyme-linked immunosorbent assay (ELISA). In addition, its optical properties can be easily enhanced by attaching high quantities of the enzyme to a mediate-material such as nanoparticles or a nanotubes.

In this works, we designed a multi-component SiNP-based nanoprobe for DNA detection that integrates DNA recognition (probe DNA), colorimetric signal amplification (HRP), and a mediated carrier (SiNP). Moreover, to separate unreacted multi-component SiNP from target DNA, we used capture-DNA functionalized magnetic microparticles (MMP). The research on the detection of anthrax lethal factor using multi-component SiNP-based nanoprobe consisting of probe DNA and HRP, in which an amplification of HRP via SiNPs lead to improve the sensitivity of DNA sensing, have not been reported in other publications. In this study, we found that the multi-component DNA-SiNP nanoprobe possessed a high hybridization specificity as well as other inherent advantages, while it concurrently eliminated the need for additional signal amplification steps.

Results and Discussion

The scheme used in this study was based upon the biocatalytic amplification of enzyme tags (Figure 1). The enzyme amplification based bioassay used in this study involved two types of particles, a MMP functionalized with a single-component DNA, and a SiNP functionalized with a single-component DNA and enzyme (HRP), which can act as the reporter group for the target DNA of interest. Enzyme conjugation to the SiNP was mediated via a streptavidin-biotin reaction. The MMP probes were then added to a solution containing the target DNA of interest. After the MMP probes have been given enough time to react with the target, the silica probes were added to form a sandwich structure with the MMP probes that have captured the DNA target. A magnetic field was then used to separate the sandwich complexes from the test solution, and the supernatant was discarded. After copious washing steps, the immobilized enzyme on the silica probes was reacted with tetramethylbenzidine (TMB), which is the substrate for HRP. The reaction was then stopped with 2 M H_2SO_4. The resulting end products were analyzed by UV-vis spectroscopy.

The first step in this process was preparing silica nanoprobes that contained immobilized DNA and the

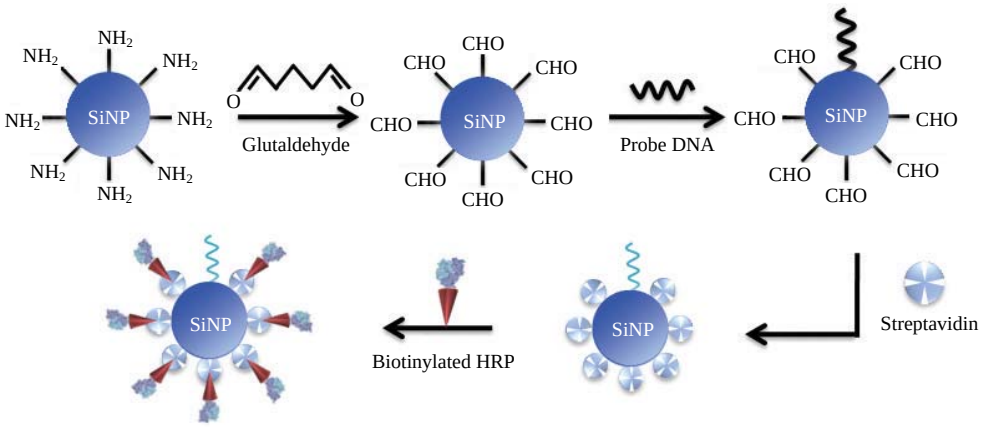

Figure 2. Schematic description of preparation of silica nanoparticles functionalized with DNA and HRP.

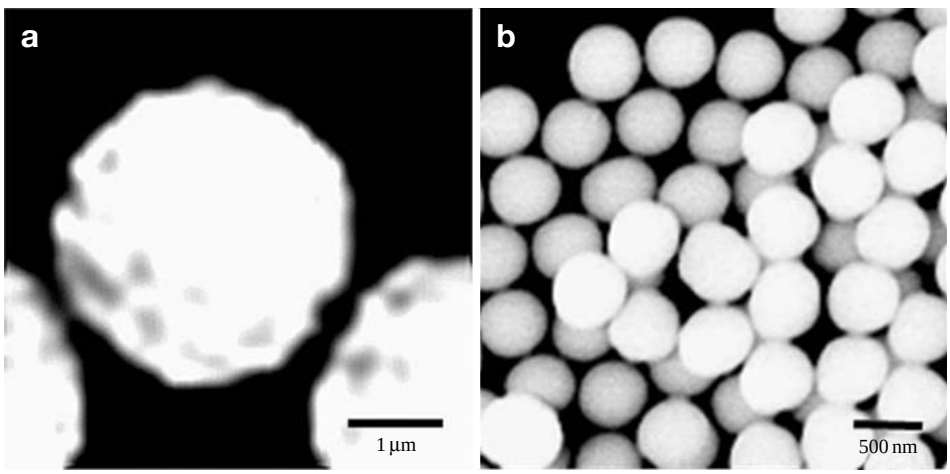

Figure 3. FESEM images of (a) magnetic microparticles and (b) silica nanoparticles.

HRP enzyme. Figure 2 outlines the scheme used to immobilize capture DNA, streptavidin and biotin labeled HRP on the silica nanoprobes. In order to conjugate amino-functionalized SiNP with 3′ Amino-functionalized DNAs (5′-ATTTAACAATAATCC-A_{10}-NH_2-3′), glutaraldehyde was used to bridge the amine groups. After this reaction was complete, streptavidin was attached to the surface of the reacted probe DNA through chemical adsorption in turn. Subsequently, ethanolamine and bovine serum albumin (BSA) were added to SiNPs suspension to passivate all unreacted glutaraldehyde sites on the SiNPs. When all of the glutaldehyde groups were passivated, biotin labeled HRP was added to the streptavidin modified SiNPs. Because of the high affinity between streptavidin and biotin, biotinylated-HPR readily bound to the immobilized streptavidin in large numbers.

To separate target DNA captured silica nanoprobes using only magnetic forces, MMPs were used as the capture material. To prepare the magnetic probe, 5′ Amino-functionalized DNAs (5′-NH_2-A_{10}-ATCCTT-ATCAAT-3′) were directly linked to tosylactivated MMPs using the procedure recommended by Dynal Biotech Inc. The capture DNA-MMP complexes after hybridization with the target DNA and the silica nanocomplex were separated from unreacted target DNA and silica nanoprobes using a magnetic field. Figure 3(a) and 3(b) are field emission scanning electron microscopy (FE-SEM) images of 2.8 μm tosylactivated MMPs and 500 nm amino-functionalized SiNPs. The MMPs and SiNPs were uniform in size with an average diameter of 2.8 μm and 500 nm, respectively.

The activity of indirectly immobilized enzymes on the SiNP via streptavidin was compared with that of directly immobilized enzymes on SiNP via glutaraldehyde-amine coupling chemistry (Figure 4). The enzyme functionalized SiNP were reacted with TMB for 10 min, which was then stopped with 2 M H_2SO_4. The activity of the immobilized enzyme was quantified with UV-vis spectroscopy by measuring the absorb-

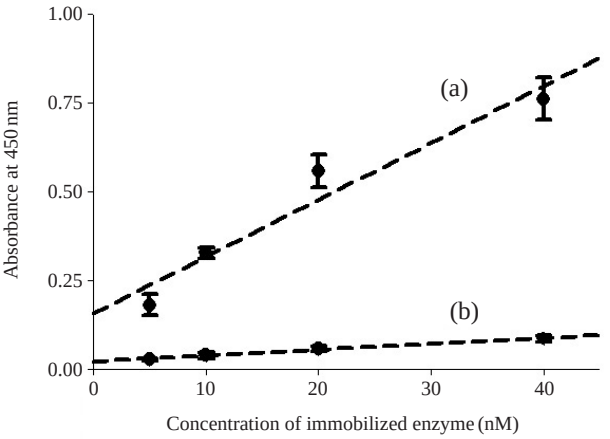

Figure 4. Comparison of the activity of immobilized enzymes on silica nanoparticle. (a) indirect immobilization of enzyme via streptavidin and (b) direct immobilization of enzyme via chemical linker on silica nanoparticles.

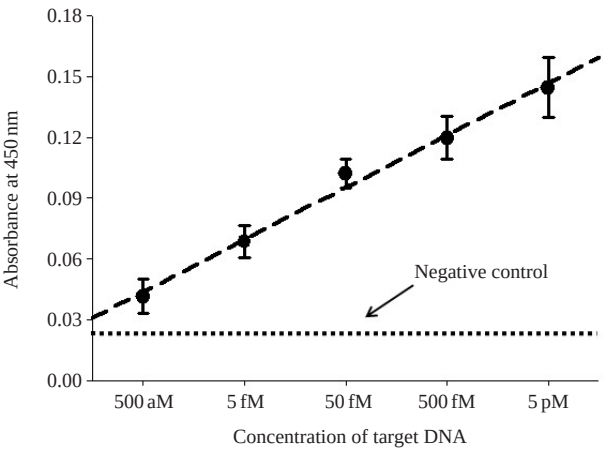

Figure 5. Colorimetric signal response of DNA biosensing system for target DNA as a function of concentration.

ance of the substrate at 450 nm. As shown in Figure 4, the activity of indirectly immobilized enzymes via streptavidin was higher than that of directly immobilized enzymes.

The number of surface immobilized DNA molecules on the probes was determined based on changes in UV-vis absorbance at 260 nm before and after the addition of DNA. Using this method, the number of DNAs on the silica probes and MMP probes was determined to be 125 DNAs (STD 10.8%) and 1.5×10^6 (STD 7.7%), respectively. The number of immobilized HRP enzyme molecules on the silica probe was determined to 2.35×10^4 (STD 6.8%) based on the change in UV-vis absorbance at 280 nm before and after the addition of enzyme.

The enzyme amplification based bioassay method (Figure 1) was tested with oligonucleotides specific to the anthrax lethal factor (5′-GGATTATTGTTAAAT-----ATTGATAA GGAT-3′) as the target in 0.15 M PBS buffer over a concentration range of 500 aM to 5 pM. All target concentrations with the 500 aM to 5 pM concentration range could easily be differentiated from the negative control (Figure 5). A linear relationship between target concentration (notice that the x-axis is a log scale) and absorbance intensity at 450 nm was observed over a four order of magnitude concentration range. Importantly, the total assay time for the detection of DNA using enzyme amplification method via SiNPs is within a hour, and the required steps and instruments for the entire assay were significantly simple with ultrahigh sensitivity compared to other typical bioassays.

Conclusions

In this study, we demonstrated a ultrahigh sensitive DNA detection method using nanomaterials. SiNPs were modified with a probe DNA and HRP (colorimetric signal molecules). The SiNPs played an important role in signal amplification through the adsorption of a significant number of HRP molecules. DNA hybridization is a key component of this sensor system, which creates a sandwich formation that connects the MMPs with SiNPs for the separation of the HRP colorimetric signal and excess unbound components. The amount of bound target DNA (anthrax lethal factor) was quantified by UV-vis spectrometer at 450 nm. Low to 500 aM of DNA was detected using this method. This novel ultrasensitive sensor system has an advantage in that detection can be confirmed with the naked eye due to the drastic change in color and it can be used to detect antibody-antigen interactions. Given the simplicity and high sensitivity of this method, it holds promising for use in performing DNA-based diagnostics where resources are limited, such as field detection. Furthermore, it can be used in detecting critical diseases, which have specific protein markers that are based on immune reactions such as antigen-antibody molecules.

Materials and Methods

Silica Probe and MMP Probe Preparation

To prepare the silica probes, 1 mL of an aqueous suspension of the amino-functionalized silica nanoparticles (1.95×10^{11} per mL) was centrifuged for 5 min at 10,000 rpm, and the supernatant was removed. The

particles were resuspended in 0.15 M PBS solution, and the centrifugation step was repeated once more. The resulting silica nanoparticle pellet was resuspended in 1 mL of 8% glutaraldehyde in 0.15 M PBS solution at pH 7.4. The solution was mixed for 5 hrs on a rocking shaker. The solution was then subjected to centrifugation for 5 min at 10,000 rpm, and the supernatant was discarded (this step was repeated twice). The resulting pellet was resuspended in a 0.15 M PBS solution, and 3′Amino-functionalized DNAs (1 mL at 15 μM; 5′-ATTTAACAATAATCC-A_{10}-NH_2-3′) was added to the solution. The solution was left on the shaker for 1 hr to link the DNAs to the activated silica particles. 1 mL of streptavidin (1 mg/mL, 0.15 M PBS) was then added to the DNA-modified silica particles, and the centrifugation step was repeated twice. The resulting pellet was resuspended in 1 mL of 0.2 M ethanolamine to passivate all unreacted glutaraldehyde sites on the silica nanoparticles for 30 min at room temperature. Centrifugation was performed again, and the supernatant was removed by decantation. Bovine serum albumin solution (10% BSA) was subsequently added to further passivate the biomolecule-inactive regions of the silica particle surface. The centrifugation step was repeated twice, and the supernatant was removed. The resulting pellet was resuspended in 2 mL of HRP solution (1 mg/mL, 0.15 M PBS, 0.1% Tween 20) to allow HRP to bind to the streptavidin-modified silica particles. The solution was left on a shaker for 3 hrs, and the particles were purified by centrifugation. For preparation of the magnetic probe, 5′Amino-functionalized DNAs (5′-NH_2-A_{10}-ATCCTTATCAAT-3′) were directly linked to tosylactivated MMPs using the procedure recommended by Dynal Biotech Inc.

Determining the Number of DNAs and Enzymes on the Probes

The number of surface immobilized DNA molecules on the probes was determined based on changes in the UV-vis absorbance at 260 nm before and after the addition of DNA. The number of surface immobilized enzyme molecules for the silica probe was determined based on changes in the UV-vis absorbance at 280 nm before and after the addition of enzyme.

The Enzyme Amplification Based Bioassay for DNA

The enzyme amplification based bioassay method (Figure 1) was tested with oligonucleotides that were specific to anthrax lethal factor (5′-GGATTATTGTTAAAT-------ATTGATAA GGAT-3′) as the target in 0.15 M PBS buffer over a concentration range of 500 aM to 5 pM. In a typical assay, a sample solution (50 μL) at a set target DNA concentration was added to 50 μL of the MMP probes functionalized with the capture DNA (2.5 mg/mL), and the solution was shaken on an orbital shaker at room temperature for 10 min. The silica probes were then added to the solution and allowed 40 min to hybridize. After hybridization, MMPs containing target-linked silica probes were easily separated from the supernatant by applying a magnetic field. During magnetic separation, a 1.5 mL tube containing the assay solution was placed in a Bio-Mag® microcentrifuge tube separator at room temperature. After 15 seconds, the unreacted solution components (DNA and silica particles) were washed away with PBS buffer. The immobilized enzymes on the silica probes were then reacted with TMB for 10 min. The reaction was stopped with 2 M H_2SO_4. The resultant end product was analyzed by UV-vis spectroscopy.

Acknowledgements

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