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COMMUNICATIONS

Published on Web 03/04/2003

Amplified DNA Sensing and Immunosensing by the Rotation of Functional Magnetic Particles

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The amplified detection of DNA or antigens/antibodies is a central challenge in bioelectronics.[1] Enzymes coupled to the nucleic acid/DNA or antibody/antigen recognition pairs were employed as biocatalytic labels for the biosensing processes. The biocatalyzed generation of electroactive products,[2] the biocatalyzed precipitation of insoluble products on transducers,[3] and bioelectrocatalyzed redox biotransformations[4] were extensively used to amplify DNA or antigen/antibody detection processes. The catalyzed deposition of metals was used for the amplified electrochemical[5] or microgravimetric quartz crystal microbalance[6] detection of DNA. Alternatively, the assembly of nanoarchitectures on surfaces with dendritic-type nucleic acids structures,[7] or the construction of nucleic acid-functionalized metal,[8] semiconductor,[9] or vesicle[10] particulate systems on surfaces, was employed for the amplified DNA sensing. In the past few years, our laboratory has been active in developing the subject of magnetobioelectronics. We were able to demonstrate the magnetic switching of the bioelectrocatalytic functions of redox-enzymes using relay-modified magnetite particles[11] and to highlight the magnetic switching of the biocatalyzed generation of electro-chemiluminescence using quinone-functionalized magnetite particles and the horseradish peroxidase (HRP)/luminol system.[12] Recently, we demonstrated that the rotation of magnetic particles on electrode surfaces by means of an external rotating magnet leads to amplified bioelectrocatalysis due to a convection-controlled (rather than diffusion-controlled) interaction between the substrate and the enzyme.[13] Here we wish to report on the amplified detection of DNA and antibodies by the application of rotating functional magnetic particles and by using electrogenerated chemiluminescence as the read-out signal. Several recent articles have addressed the collection of nucleic acid-functionalized magnetic particles and the electrochemical analysis of respective DNA units.[14]

Scheme 1 outlines the concept for the amplified detection of the target DNA **2**. Amine-functionalized borosilicate-based magnetic particles (5 μm, MPG Long Chain Alkylamine, CPG Inc.) were modified with **1** using the heterobifunctional cross-linker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester.[15] The coverage of the particles was estimated[16] to be ca. 52 000 oligonucleotide molecules·particle^{-1}. The probe **1** is complementary to a part of the target sequence **2**. The **1**-functionalized magnetic particles are hybridized in a single step with a mixture that includes (variable concentrations) the target **2** and the biotin-labeled nucleic acid **3**, that is complementary to the free segment of **2**. The three-component double-stranded DNA assembly **1**/**2**/**3** is then interacted with avidin-horseradish peroxidase (HRP) that acts as a biocatalytic label. The DNA/avidin-HRP-functionalized magnetite particles are subsequently mixed with magnetite particles modified with the naphthoquinone unit **4**. The mixture of the magnetic particles is then attracted to an electrode support by means of an external magnet. Electrochemical reduction of the naphthoquinone to the respective hydroquinone results in the catalyzed reduction of O_2 to H_2O_2. The electrogenerated H_2O_2 leads, in the presence of luminol **5** and the enzyme label HRP, to the generation of the chemiluminescence signal. Note that the avidin-HRP binds to the magnetic particles only if the target DNA hybridizes with the magnetic particles, provided that nonspecific adsorption does not take place. Thus, chemiluminescence occurs only if the target DNA **2** is in the analyzed sample. Furthermore, the light intensity relates directly to the number of recognition pairs of **1** and **2** associated with the electrode, and thus it provides a quantitative measure of the concentration of **2** in the sample. The subsequent rotation of the particles on the surface by means of the rotating external magnet results in the enhanced electrogenerated chemiluminescence, because the magnetic particles behave as rotating microelectrodes, where the interaction of O_2 and luminol with the catalysts on the electrode is controlled by convection rather than by diffusion. Thus, the rotation of the magnetic particles is anticipated to yield the amplified detection of DNA.

A sample of a **1**-functionalized magnetic particle was interacted with **2**, 1.4×10^{-8} M, in the presence of the biotinylated nucleic acid **3**, 2×10^{-7} M. The resulting double-stranded **1**/**2**/**3** three-component system was collected by the external magnet, washed with 0.2 M phosphate buffer (pH 7.4), and then reacted with the avidin-HRP conjugate and again collected by the external magnet. The resulting particles were added to the electrochemical cell together with the naphthoquinone **4**-modified magnetic particles, 2 mg mL^{-1}. Figure 1A, curve a, shows the emitted light intensity upon the collection of the magnetic particles on the electrode by means of the external magnet, and the application of a potential step on the electrode from 0 to -0.5 V and back. Figure 1A, curves b−d, shows the emitted light intensities upon the rotation of the particles by means of the external magnet, using different rotation speeds. Increase of the rotation speed enhances the intensity of the emitted light, and the resulting light intensity relates linearly to $\omega^{1/2}$ (ω = rotation speed), as expected for electrocatalytic rotating microelectrodes, Figure 1a, inset. In a control experiment that lacks **2** in the hybridization step, no light emission is detected, indicating that no nonspecific adsorption of **3** or the avidin-HRP conjugate takes place. The light intensity emitted from the system relates to the surface coverage of the avidin-HRP conjugate, and this is controlled by the amount of **2**/**3** associated with the particles and thus determined by the concentration of **2**. Figure 1B shows the derived calibration curves corresponding to the emitted light intensities upon analyzing different concentrations of **2**, recorded at different rotation speeds. Figure 1A, curve e, shows the light intensity observed upon the analysis of the mutant **2a**, that includes a seven-base mutation sequence with respect to **2**, 1×10^{-7} M, at a rotation speed of 2000 rpm according to Scheme 1. No emitted light due to nonspecific adsorption of the avidin-HRP conjugate on the surface is observed. This light intensity is considered as the background signal, and thus **2** can be sensed with a detection limit

10.1021/ja028850x CCC: $25.00 © 2003 American Chemical Society

Scheme 1. (**A**) Preparation of DNA-Functionalized Magnetic Particles Labeled with the Avidin-HRP Conjugate and (**B**) Amplified Detection of DNA by the Rotation of the Labeled DNA-Functionalized Magnetic Particles and Quinone-Modified Magnetic Particles by an External Rotating Magnet and the Electrocatalyzed Generation of Chemiluminescence

(1) 5'-TCTATCCTACGCT-$(CH_2)_6$-SH-3'
(2) 5'-AGCGTAGGATAGATATACGGTTCGCGC-3'
(2a) 5'-AGCGCTCCAGTGATATACGGTTCGCGC-3'
(3) 5'-biotin-GCGCGAACCGTATA-3'

Figure 1. (A) Chemiluminescence intensities upon the analysis of **2**, 1.4 $\times$ 10^{-8} M, according to Scheme 1, at different rotation speeds: (a) 0 rpm; (b) 60 rpm; (c) 400 rpm; (d) 2000 rpm; (e) analysis of mutant **2a**, 1 $\times$ 10^{-7} M, at 2000 rpm. Inset: Peak light intensity as a function of $\omega^{1/2}$ (ω = rotation speed). (B) Light intensities observed upon the analysis of different concentrations of **2** at variable rotation speeds: (a) 0 rpm; (b) 60 rpm; (c) 2000 rpm. Inset: Enlargement of the results in the lower concentration range. For all experiments, the chemiluminescence signals are produced by applying a potential step on the electrode from 0 to -0.5 V and back (vs SCE).

of 1 $\times$ 10^{-14} M at $\omega = 2000$ rpm (S/N > 3). The sensitivity reached by the present analytical protocol is ca. 10-fold higher than that obtained by a related biocatalytic amplification route that generates redox-active nucleic acid replica that activate a bioelectrocatalytic process.[4d]

Similar amplification of antigen−antibody recognition events was accomplished by the rotation of functionalized magnetic particles.

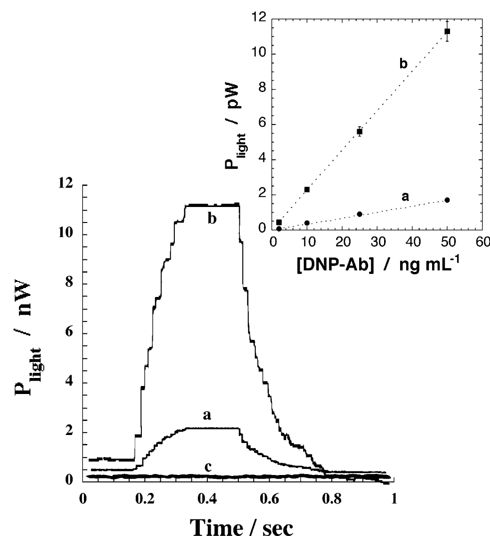

Figure 2. Chemiluminescence intensities upon the analysis of the DNP-Ab, 50 ng mL^{-1}, at different rotation speeds: (a) 0 rpm; (b) 400 rpm; (c) control experiment without DNP-Ab, 400 rpm. Inset: Peak chemiluminescence intensities observed in the presence of different concentrations of the DNP-Ab: (a) at 0 rpm; (b) at 400 rpm. For all experiments, the chemiluminescence signals are produced by applying a potential step on the electrode from 0 to -0.5 V and back (vs SCE).

The dinitrophenyl (DNP)-antigen, **6**, was covalently coupled to amino-functionalized magnetic particles. The resulting antigen-modified particles, 2 mg, were reacted with the dinitrophenyl antibody, DNP-Ab, at different concentrations, and the resulting particles functionalized with the antigen/DNP-Ab were reacted with the HRP-anti-DNP-Ab conjugate, 0.1 mg mL^{-1}. (For the detailed scheme describing the assembly of the respective functionalized magnetic particles, see Supporting Information.) The resulting magnetic particles were collected by means of the external magnet mixed with the naphthoquinone **4**-functionalized magnetic particles, 2 mg, and introduced into the electrochemical cell that included luminol **5**, 5 μM. Figure 2, curve a, shows the resulting light intensity upon analyzing the DNP-Ab, 50 ng mL^{-1}, by the attraction of the particles to the electrode by means of the external magnet and the application of a potential step from 0 to -0.5 V and back.

Figure 2, curve b, depicts the light intensity detected upon the rotation of the particles at a rotation speed of 400 rpm by means of the external rotating magnet. Clearly, the rotation of the particles amplifies the emitted light intensity because the electrocatalytic reduction of O_2 by the quinone, as well as the HRP-mediated biocatalyzed chemiluminescent process originating from the reaction of H_2O_2 and luminol, is controlled by convection. A control experiment where the DNP-Ab is omitted from the analyzed sample, but all other analysis steps were performed, led to a minute light emission (not seen at the given scale), Figure 2, curve c. This result indicates that no significant nonspecific adsorption of the biotinylated anti-DNP-Ab or avidin-HRP components on the electrode or on the magnetic particles occurs. The trace signal of emitted light may be considered as the noise level of the system.

At a constant rotation speed (or with no rotation), the emitted light intensity is controlled by the coverage of the electrode with the antigen/DNP-Ab/HRP-anti-DNP-Ab complex, and this relates to the bulk concentration of the analyzed DNP-Ab. Figure 2, inset, shows the calibration curves corresponding to the emitted light intensities upon analyzing different concentrations of the DNP-Ab by the magnetic particles at different rotation speeds. It can be seen that at a rotation speed of $\omega = 400$ rpm, the DNP-Ab is analyzed with a detection limit of 5 ng mL^{-1}. Using higher rotation speeds, we found that the sensitivity of the antibody detection can be further improved, with a detection limit of 50−100 pg mL^{-1} at 2000 rpm.

In conclusion, the present study has demonstrated that the rotation of the nucleic acid- or the antigen-functionalized magnetic particles, together with the naphthoquinone **4**-modified magnetic particles, yields, in the presence of HRP-bioconjugates and luminol **5**, enhanced electrogenerated chemiluminescence. The enhanced light emission enables the highly sensitive amplified detection of DNA or the DNP-Ab. The rotation of the functionalized magnetic particles and their electrochemical activation is analogous to the function of a rotating disk electrode (RDE).[17] One should, however, realize the advantages of the rotating magnetic particles over a conventional RDE that include the possibility of collecting and purifying the analyzed sample from a complex biological mixture, the possibility of regenerating quickly the sensing interface, and most importantly the high surface area of the rotating magnetic particles.

Acknowledgment. This study is supported by the German-Israeli Binational Program (DIP).

Supporting Information Available: Scheme showing the preparation of the dinitrophenyl antigen-functionalized magnetic particles and the amplified detection of the DNP-Ab by the rotation of the magnetic particles with an external rotating magnet (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Amino-functionalized magnetic particles (30 mg) (MPG Long Chain Alkylamine, CPG Inc.) were activated by reaction with the heterobifunctional cross-linker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (Sigma) in 1 mL of DMSO. After 4 h of incubation at room temperature, the particles were thoroughly washed with DMSO and water. The maleimido-activated particles were then reacted with 20−30 OD of the thiolated oligonucleotide in phosphate buffer 0.1 M, pH 7.4 for a period of 8 h. (The thiolated nucleotide was freshly reduced with DTT and separated on a Sephadex G-25 column prior to the reaction with the functionalized particles.) Finally, the magnetic particles were washed with water and phosphate buffer 0.1 M, pH 7.4. To keep the DNA-modified particles for periods longer than 1 week, 1% w/v sodium azide was added, and the particles were kept at 4 °C.

(16) The oligonucleotide content on the magnetic particles (0.1 mg•particles) was measured by the use of the Oligreen reagent (ssDNA Quantitation Assay Kit Molecular Probes, Inc.).

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JA028850X