Detecting Single-Base Mutations

Magnetically Amplified DNA Assays (MADA): Sensing of Viral DNA and Single-Base Mismatches by Using Nucleic Acid Modified Magnetic Particles**

*Fernando Patolsky, Yossi Weizmann, Eugenii Katz, and Itamar Willner**

Dedicated to Professor Dieter M. Kolb on the occasion of his 60th birthday

The detection of DNA has attracted significant recent research efforts directed to the analysis of genetic disorders, identification of pathogens, tissue matching, the detection of biological-warfare agents, and forensic applications.[1,2] The sensing of DNA involves two major challenges related to the sensitivity of the detection process and the specificity of DNA analysis, with the ultimate goal of detecting single-base mismatches. Amplified electronic DNA detection is accomplished by the coupling of biocatalytic (enzyme) conjugates to the nucleic acid/DNA recognition pair.[3–5] For example, the amplified analysis of the M13ϕ DNA was accomplished by the generation of biotin- or ferrocene-labeled replica followed by the biocatalyzed precipitation of an insoluble product on electrodes,[4] or the activation of a bioelectrocatalytic process,[5] respectively. Alternatively, the assembly of nanoarchitectures on surfaces with dendritic-type nucleic acid structures[6] or with layered functional particles[7] are extensively used to amplify DNA detection. Single-base-mismatch detection was reported by Heller and Caruana using thermally controlled hybridization.[8] The amplified single-base-mismatch detection was accomplished by the polymerase-induced incorporation of a biotin-labeled base into the primer sensing nucleic acid. Coupling of the avidin–enzyme[9a] or the avidin–Au nanoparticle[9b] conjugates enabled the secondary catalytic amplification of the sensing processes. Functional magnetic particles are often used in analytical assays for the separation of specific components from biological mixtures,[10] and for the elimination of non-specific adsorption.[11] Recently, functional magnetic particles were employed for the magnetoswitchable activation and deactivation of bioelectrocatalytic transformations[12] and of electrogenerated biochemiluminescence.[13] Also, magnetic particles were recently reported for the electrochemical analysis of DNA.[14] The use of rotating magnetic particles was recently reported as a means to enhance bioelectrocatalytic processes by convection-controlled (rather than diffusion-controlled) interactions of the substrate with the enzyme.[15] Wang and colleagues have recently employed nucleic acid functionalized magnetic particles for the amplified electrochemical detection of DNA by the catalytic deposition of silver on magnetic particles and Au-nanoparticle/DNA conjugates, followed by the electrochemical stripping of the resulting silver.[16] Herein we wish to report on the novel amplified detection of viral DNA and of single-base mismatches using nucleic acid functionalized magnetite particles and electrogenerated chemiluminescence as a transduction means. The magnetic particles have two complementary functions in the DNA detection schemes: a) They act as building units for the polymerase-induced incorporation of labels using thermally controlled replication cycles, b) the magnetic attraction of the labeled particles and their rotation on the electrode surface enables further amplified detection of DNA by electrogenerated chemiluminescence.

Magnetic particles (5-μm diameter) are modified with the thiolated primer **1** using the heterobifunctional cross-linker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester, as outlined in Figure 1. The average coverage of the magnetic particles was determined by using the OliGreen reagent (Molecular Probes, Inc.) and corresponded to approximately 50000 oligonucleotide units per particle. The number of nucleic acids that were associated with the particles and accessible to an external enzyme was estimated by subjecting the **1**-functionalized magnetic particles to DNase and by the subsequent determination of the content of the DNA that is cleaved off. We find that around 20000 oligo units per particle are cleaved off, which implies that only about 40% of the particle-linked nucleic acids are accessible to the enzyme.

Figure 2 depicts the method for the amplified detection of the viral M13ϕ DNA (**2**) using the functional magnetic particles. The **1**-modified magnetic particles are hybridized with the M13ϕ ssDNA (7229 bases) and are subjected to polymerization in the presence of a mixture of dGTP, dATP, dCTP, and biotinylated dUTP (b-dUTP). The polymerization introduces into the replica a high number of biotin labels. The replication is followed by thermal cycles that result in the dissociation of the analyzed M13ϕ DNA, its re-hybridization with other oligonucleotide primers associated with the magnetic particles, and the subsequent polymerization and formation of new replica containing a high number of biotin

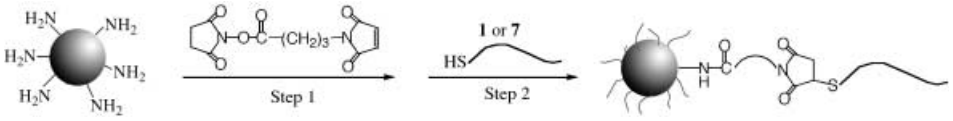

Figure 1. Alkylamine-coated magnetic particles are modified with cross-linker and then a thiolate primer.

[*] Prof. I. Willner, F. Patolsky, Y. Weizmann, Dr. E. Katz
Institute of Chemistry
The Farkas Center for Light-Induced Processes
The Hebrew University of Jerusalem
Jerusalem 91904 (Israel)
Fax: (+972) 2-6527-715
E-mail: willnea@vms.huji.ac.il

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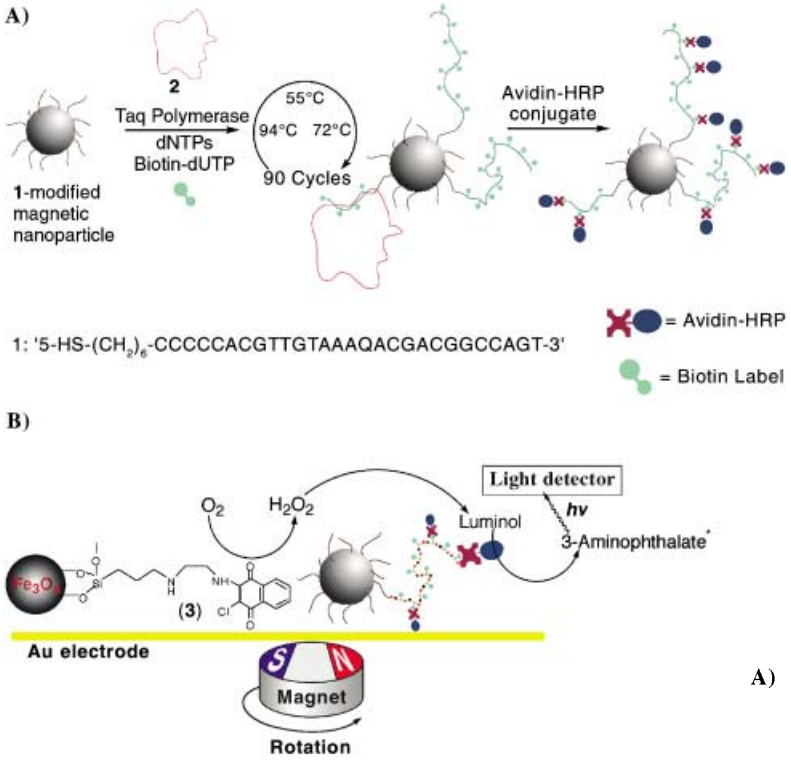

Figure 2. Amplified detection of viral DNA by multilabeled rotating magnetic particles: A) labeling of the nucleic acid replica on the particles with biotin units by using thermal cycles, B) generation of amplified chemiluminescence upon rotation of the functionalized magnetic particles on the electrode surfaces.

label units. By controlling the number of thermal cycles, the replication on the surface of the particles yields very high densities of biotin-labeled nucleic acids. The thermal cycles were conducted for 75 s each, which translate to a replication efficiency of about 250 bases per cycle. The resulting biotin-labeled magnetic particles are then separated by means of the external magnet, treated with avidin–horseradish peroxidase (HRP), separated again by the external magnet, and washed with a phosphate buffer solution. A mixture of the avidin-functionalized magnetic particles (1 mg) and naphthoquinone **3**-modified magnetic particles[12b] (Figure 2B) is then introduced into the electrochemical cell. The magnetic particles are then attracted to the electrode by means of the external magnet. Upon the application of a potential to the electrode that reduces the naphthoquinone to the respective hydroquinone, the electrocatalyzed reduction of O_2 to H_2O_2 occurs. The HRP-catalyzed oxidation of luminol by the electrogenerated H_2O_2 results in chemiluminescence and the emission of light. Figure 3A, curve a, shows the emitted light intensity upon analyzing M13ϕ DNA as in Figure 2, and by the application of a potential step on the electrode from 0 V to −0.5 V and back (the E^o value for the **3**-modified particles at pH 7 is −0.4 V versus SCE). In a control experiment in which all the analysis steps were applied on a sample that lacked M13ϕ DNA, no light emission was observed, which indicates that no non-specific adsorption of the avidin–HRP conjugate on the electrode, or on the magnetic particles, takes place, Figure 3A, curve e. The effect of the rotation of the magnetic particles, caused by rotating the external magnet, is depicted in Figure 3A, curves b–d. As the rotation speed of the magnetic particles is increased, the emitted light intensity increases, and a linear relationship between the intensity of emitted light and $\omega^{1/2}$ is observed (Figure 3B). The increase in the emitted light intensity is attributed to the fact that the rotating magnetic particles behave as catalytic rotating microelements, where the electrocatalyzed reduction of O_2 to H_2O_2 and the subsequent generation of chemiluminescence in the presence of luminol are controlled by convection rather than by the diffusion of O_2 and luminol to the particles associated with the conductive support.

At a constant rotation speed of the particles, the intensity of emitted light is controlled by the surface coverage of the labeled nucleic

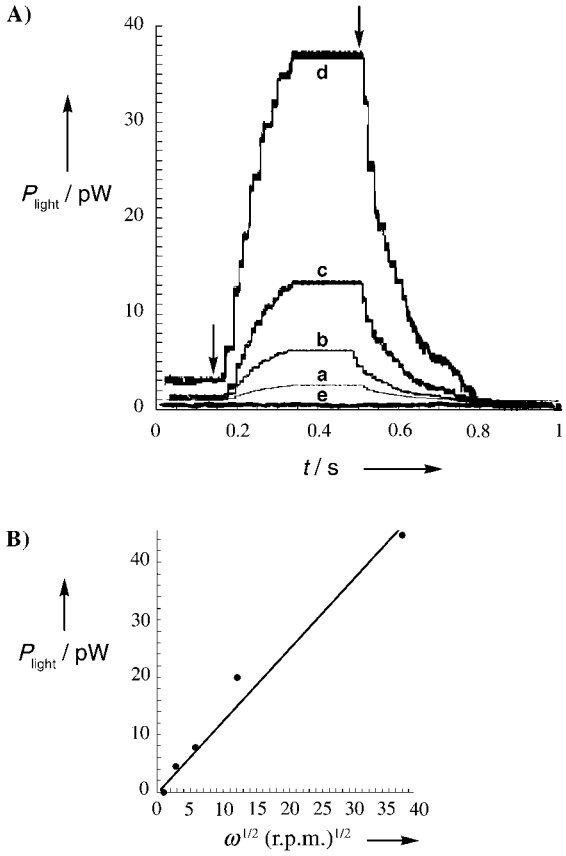

Figure 3. A) Chemiluminescence intensities upon the analysis of M13ϕ DNA (8×10^{-9} M) at different rotation speeds, a) 0 r.p.m., b) 60 r.p.m., c) 400 r.p.m., d) 2000 r.p.m., and curve e) chemiluminescence signal upon in the absence of M13ϕ DNA at 2000 r.p.m. B) Chemiluminescence intensities as a function of $\omega^{1/2}$ (ω = rotation speed). In all the experiments the chemiluminescence is generated by the application of a potential step from $E = 0.0$ V to $E = -0.5$ V and back versus SCE. Arrows indicate the times at which the potential was switched to −0.5 V and to 0.0 V, respectively. Data recorded in 0.01 M phosphate buffer pH 7.4 that includes luminol (1×10^{-6} M) under air.

acid associated with the magnetic particles, and this relates to the concentration of M13ϕ DNA in the analyzed sample during the replication cycles. Figure 4 shows the emitted light intensity upon the analysis of different concentrations of M13ϕ DNA at a rotation speed of $\omega = 2000$ r.p.m. At $\omega =$ 2000 r.p.m. (Figure 4) we are able to sense the M13ϕ DNA with a detection limit that corresponds to 8.3×10^{-18} M, that translates to 50 copies per 10 μL.

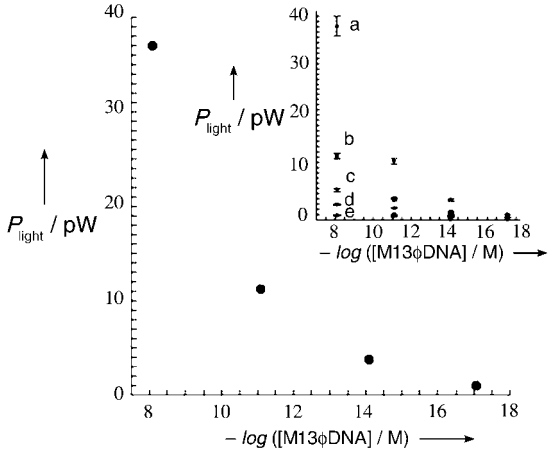

Figure 4. Calibration curve corresponding to the chemiluminescence intensities upon analyzing different concentrations of M13ϕ DNA at 2000 r.p.m. Inset: Calibration curve corresponding to the chemiluminescence intensities upon analyzing different concentrations of M13ϕ at a) 2000 r.p.m., b) 400 r.p.m., c) 60 r.p.m., d) 20 r.p.m., e) 0 r.p.m.

A related amplification path that uses functional magnetic particles was employed for the detection of single-base mismatches in DNA. This method is exemplified by the analysis of the mutant sequence **5**, where the G-base exchanges the A-base in the normal sequence gene **6** (Figure 5). The magnetic particles are functionalized with the nucleic acid **7** that is complementary to the mutant sequence **5** and the normal gene sequence **6**, up to one base prior to the mutation site. Interaction of the **7**-modified magnetic particles with the samples that include either **5** or **6** results in their hybridization with the particles. Treatment of the hybridized assemblies associated with the magnetic particles with polymerase and biotinylated-dCTP, followed by thermal dissociation/annealing/labeling cycles results in the multilabeling with biotin units of the magnetic particles incorporating **5**, whereas no biotin labels are introduced to the magnetic particles incorporating **6**. The subsequent interaction of the particles with the avidin–HRP conjugate, followed by the separation of the particles by means of the external magnet yield the biocatalytically labeled particles. Mixing of the resulting particles with the naphthoquinone **3**-modified particles in the electrochemical cell, followed by their attraction to the electrode by means of the external magnet, upon application of a potentential corresponding to −0.5 V, leads to the electrocatalyzed reduction of O_2 to H_2O_2. The electrogenerated H_2O_2 leads, in the presence of luminol, to the emission of light upon the analysis of **5**, while no light is detected upon the analysis of **6**. Rotation of the magnetic particles by means of the external magnet is then expected to amplify the emitted light since the electrogenerated chemiluminescence is controlled by convection of the respective substrates to the particles.

Figure 6A shows the emitted light intensity at rotation speeds of up to 2000 r.p.m. upon analyzing **5** and **6** as in Figure 5. A potential step from 0 V to −0.5 V and back is applied on the electrode to activate the electrocatalyzed reduction of O_2, and to drive the secondary chemiluminescent process. No light emission is observed upon the analysis of **6**, which indicates that no biotin labels were incorporated into the nucleic acid modified magnetic particles. Clearly, light emission is observed only upon the analysis of the mutant sequence. The intensity of the emitted light increases as the rotation speed of the particles is elevated, ($P_{light} \propto \omega^{1/2}$), again implying that the processes at the electrode support are controlled by convection. Figure 6B shows the light emitted upon analyzing different concentrations of **5**, at different rotation speeds. The mutant sequence **5** is analyzed with a detection limit of 1×10^{-17} M. Note that the concentration of **6** in the analyzed sample is 10^3-fold higher that of **5**, and still no light emission is observed upon analyzing **6**. This result implies that no non-specific association of the HRP conjugate to the magnetic particles occurs.

In conclusion, the present study has described the "magnetically amplified DNA analysis" (MADA) process. Several consecutive steps in the process lead to the overall amplification: 1) The thermal cyclic replication of the analyte on the magnetic particles leads to the incorporation of a high number of label units into the nucleic acids linked to the particles, 2) the electrocatalytic generation of O_2 at the electrode, and the coupled biocatalyzed light emission yield numerous product molecules or photons as a result of a single recognition event, 3) the rotation of the magnetic particles leads to the amplified light emission since the transport of the substrates for the electrocatalytic and biocatalytic processes at the particles are convection-controlled. Using these methods unprecedented sensitivities were achieved. The observed detection limit for the M13ϕ DNA is 8.3×10^{-18} M, (50 copies per 10 μL), and for the mutant sequence **5**, the detection limit corresponds to 1×10^{-17} M. These values were achieved with no attempt to optimize the analysis process (for example, optimization of

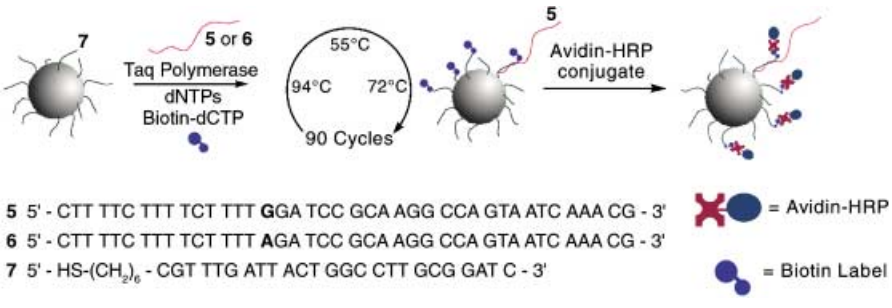

Figure 5. Amplified detection of a single-base mismatch in DNA by using rotating magnetic particles. See text for details.

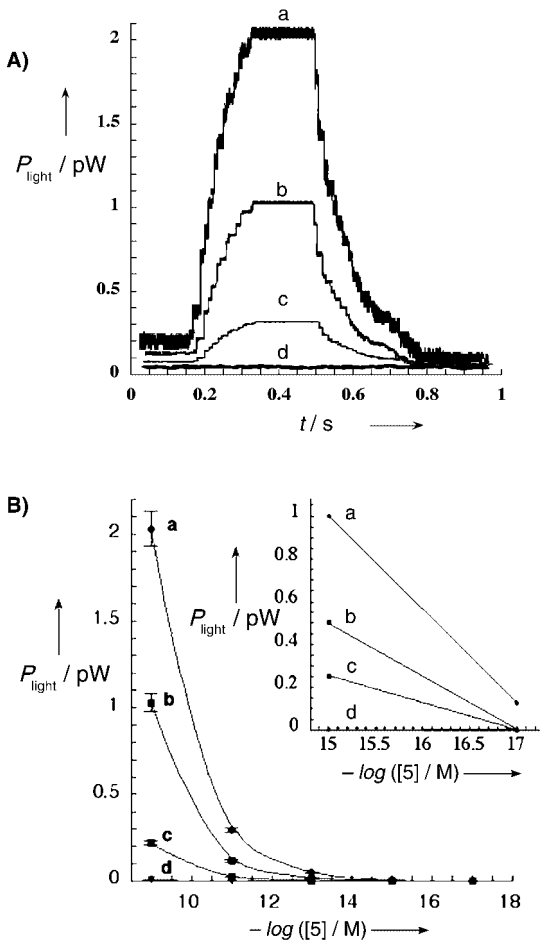

Figure 6. A) Chemiluminescence intensities upon the analysis of the mutant sequence **5** (1.4×10^{-9} M) at: a) 2000 r.p.m., b) 400 r.p.m., c) 60 r.p.m. The chemiluminescence intensity upon the analysis of the normal sequence **6** (1.4×10^{-6} M) at 2000 r.p.m. according to the method in Figure 5 is shown in curve (d). The conditions for the recording of the chemiluminescence are detailed in Figure 3. B) Calibration curves corresponding to the analysis of different concentrations of the mutant **5** at different rotation speeds: a) 2000 r.p.m., b) 400 r.p.m., c) 60 r.p.m. , d) 0 r.p.m. Inset: Enlargement of calibration curves showing the chemiluminescence intensities at low concentrations of **5**.

the thermal incorporation of the biotin labels by the polymerization cycles). We believe, that by optimization of the process and further electronic amplification of the detected light signal, the method may be suitable for analyzing a few DNA molecules.

Experimental Section

Amine-functionalized borosilicate-based magnetic particles (5 μm, MPG Long Chain Alkylamine, CPG Inc.) and Biotin-21-dUTP (Clontech) were obtained from the firms indicated. The heterobifunctional cross-linker 3-maleimidopropionic acid N-hydroxysuccinimide ester, oligonucleotides **1**, **5**, **6**, and **7**, Avidin–HRP conjugate, dNTPs, Biotin-11-dCTP, Taq Polymerase, 10×PCR buffer, and all other compounds were purchased from Sigma and used as received.

Preparation of DNA-functionalized magnetic particles: the amino-functionalized magnetic particles (30 mg) were activated by reaction with the heterobifunctional cross-linker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (10 mg, Sigma) in DMSO (1 mL). After 4 h of incubation at room temperature, the particles were collected by using an external magnet and thoroughly washed with DMSO and water. The maleimido-activated particles were then reacted with 20–30 O.D. of the thiolated oligonucleotide in phosphate buffer 0.1M, pH 7.4 for a period of 8 h. (The thiolated nucleotide was freshly reduced with threo-4,4-dimercapto-2,3-butanediol (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 0.1M, pH 7.4. To keep the DNA-modified particles for periods longer than one week, 1% w/v sodium azide was added, and the particles were kept at 4°C. The oligonucleotide content on the magnetic particles, before and after enzymatic DNase treatment (10 units DNase, 30 min at 37°C) was measured by the use of the OliGreen reagent (ssDNA Quantitation Assay Kit Molecular Probes, Inc.).

Conditions for Thermal Cycles: a) For single-point-mutation detection: denaturation 30 s, 94°C; annealing 30 s, 55°C; polymerization 5 s, 72°C; b) For Viral-DNA detection: denaturation 30 s, 94°C; annealing 30 s, 55°C; polymerization 15 s, 72°C.

An Au-coated (50 nm gold layer) glass plate (Analytical-μSystem, Germany) was used as a working electrode (0.3 cm^2 area exposed to the solution). An auxiliary Pt electrode and a quasi-reference Ag electrode were made from wires of 0.5-mm diameter and added to the cell. The quasi-reference electrode was calibrated versus saturated calomel electrode (SCE) and the potentials are given versus SCE. An open electrochemical cell (230 μL) that includes the Au electrode in a horizontal position and a light detector linked to a fiber optic enabled easy light-emission measurements upon application of the appropriate potential to the modified working electrode. The electrochemical measurements were performed using a potentiostat (EG&G, model 283) connected to a computer (EG&G Software 270/250). All the measurements were performed in 0.01M phosphate buffer solution, pH 7.0, at room temperature. The electrochemically induced chemiluminescence was measured with a light detector (Laserstat, Ophir) linked to an oscilloscope (Tektronix TDS 220). The light detector was connected to the electrochemical cell by an optical fiber. The background electrolyte solution was equilibrated with air and included luminol, 1×10^{-6} M. A permanent magnet (NdFeB/zn-coated magnet 0.2 kOe) mounted on a rotating disx electrode system (EG&G, model 638) was used to rotate the particles.

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