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Magneto-Mechanical Detection of Nucleic Acids and Telomerase Activity in Cancer Cells

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Abstract: The ultra-sensitive magneto-mechanical detection of DNA, single-base-mismatches in nucleic acids, and the assay of telomerase activity are accomplished by monitoring the magnetically induced deflection of a cantilever functionalized with magnetic beads associated with the biosensing interface. The analyzed M13ϕ DNA hybridized with the nucleic acid-functionalized magnetic beads is replicated in the presence of dNTPs that include biotin-labeled dUTP. The resulting beads are attached to an avidin-coated cantilever, and the modified cantilever is deflected by an external magnetic field. Similarly, telomerization of nucleic acid-modified magnetic beads in the presence of dNTPs, biotin-labeled dUTP, and telomerase from cancer cell extracts and the subsequent association of the magnetic beads to the cantilever surface results in the lever deflection by an external magnetic field. M13ϕ DNA is sensed with a sensitivity limit of 7.1×10^{-20} M by the magneto-mechanical detection method.

The amplified detection of nucleic acids has been a subject of extensive research efforts.[1] Amplified electronic readout of DNA detection was accomplished by the coupling of enzymes to the DNA recognition processes occurring on electrodes. For example, the activation of redox enzymes,[2] the use of enzymes that precipitate an insoluble product on the electrode,[3] or the use of enzymes for the electrogeneration of chemiluminescence as a result of DNA hybridization[4] were used as biocatalytic amplifiers that enabled the electrochemical or photonic readout of the DNA detection processes. Nucleic acid-functionalized metal or semiconductor nanoparticles were used for the amplified electronic transduction of DNA analysis. Electroless catalytic deposition of metals on the nanoparticles enabled the amplified microgravimetric quartz crystal microbalance[5] or the electrical[6] detection of the DNA. Alternatively, dissolution of the metal[7] or semiconductor[8] nanoparticles allowed the voltammetric detection of the resulting ions. Amplified detection of DNA was reported by the replication of the analyzed nucleic acid, while incorporating labels such as biotin labels[9] or redox-active[10] units. Activation of secondary biocatalytic processes that precipitate products on electrodes or activate redox enzymes, respectively, enabled the amplified electrochemical detection of the respective DNA. Recently, nucleic acid-functionalized magnetic particles were employed as active matrixes for the replication and labeling of DNA.[11] Upon the rotation of the magnetic particles, the amplified detection of DNA was accomplished using electrogenerated chemiluminescence as transduction signal. In this system, the rotation of the magnetic particles led to the convection-controlled electrogenerated chemiluminescence.

Telomerase is a ribonucleoprotein complex that synthesizes and adds telomer repeats to the 3′-end of chromosomal DNA.[12] The telomer ends protect the chromosomes and their gradual erosion in cells and provide a signal for the termination of the cell life-cycle. In telomerase-containing cells the erosion of the telomer ends is prevented, leading to the formation of immortal cells.[13] It was found that almost all cancer cells include telomerase, and the linkage between malignancy and telomerase

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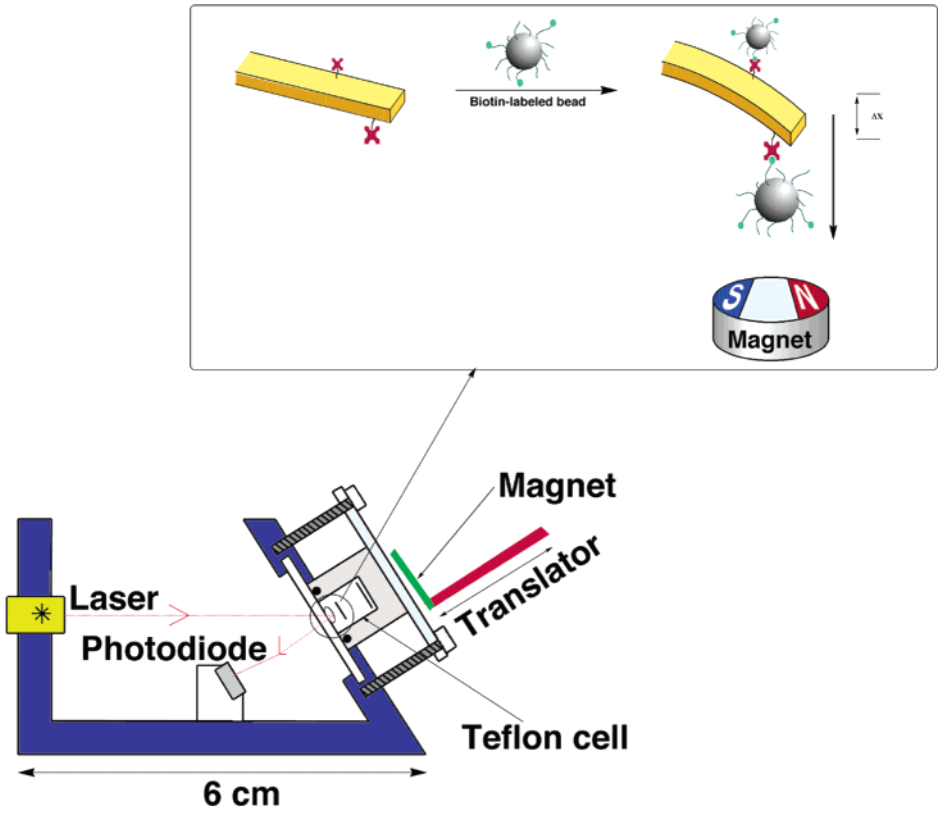

Figure 1. Schematic configuration of the instrumentation setup and concept for the magneto-mechanical analysis of biorecognition processes on functionalized cantilevers.

activity was established.[14] Several analytical procedures for the quantification and analysis of telomerase activity have been developed.[15,16]

A major challenge in future biosensing will involve the ability to handle low-volume samples and the development of micro (nano)-sensing devices. The force interactions between complementary components of biological complexes such as antigen−antibody or double-stranded DNA were used to probe recognition events at the molecular level.[17,18] Also, flexible biomaterial-functionalized nano-elements such as carbon nanotubes were reported[19] to sense the individual interactions of biological complexes such as biotin−avidin or antigen−antibody complexes.

The possibility of chemically modifying cantilevers and optically monitoring their nanometric deflections turn these micro-elements into ideal mechanical sensors. [20] Indeed, stress interactions occurring on the surface of the cantilevers were reported to induce their mechanical deflection. For example, a thin polyaniline film associated with a lever was reported to act as a reversible electrochemically driven mechanical device.[21] Oxidation of the polymer film leads to the electrostatic repulsion of the polymer chains, resulting in stress on the cantilever and its deflection. The reduction of the oxidized polymer removes the surface stress, and the lever is retracted to its original position. Biorecognition events, such as antibody−antigen[17] or nucleic acid−DNA[18] binding processes were similarly reported to stimulate the mechanical deflection of cantilevers. For example, the electrostatic repulsions between surface-hybridized DNA were reported to induce a surface-stress, resulting in the mechanical deflection of the lever.[17] The force exerted on a lever modified with magnetic particles and subjected to an external magnetic field could drive the deflection of the cantilever. Indeed, upon the mounting of magnetic particles on a cantilever, a highly sensitive magnetic-field sensor was developed.[17] The deflection of the cantilever could monitor magnetic field changes as low as 10 nT. The extensive use of magnetic particles as supports for biosensing processes[22] suggests that the amplified magneto-mechanical detection of biorecognition processes should be feasible. Here we report on the magneto-mechanical detection of DNA, the analysis of single-base mismatches in DNA, and the detection of the telomerase activity in cancer cells using cantilevers modified with functionalized magnetic particles.

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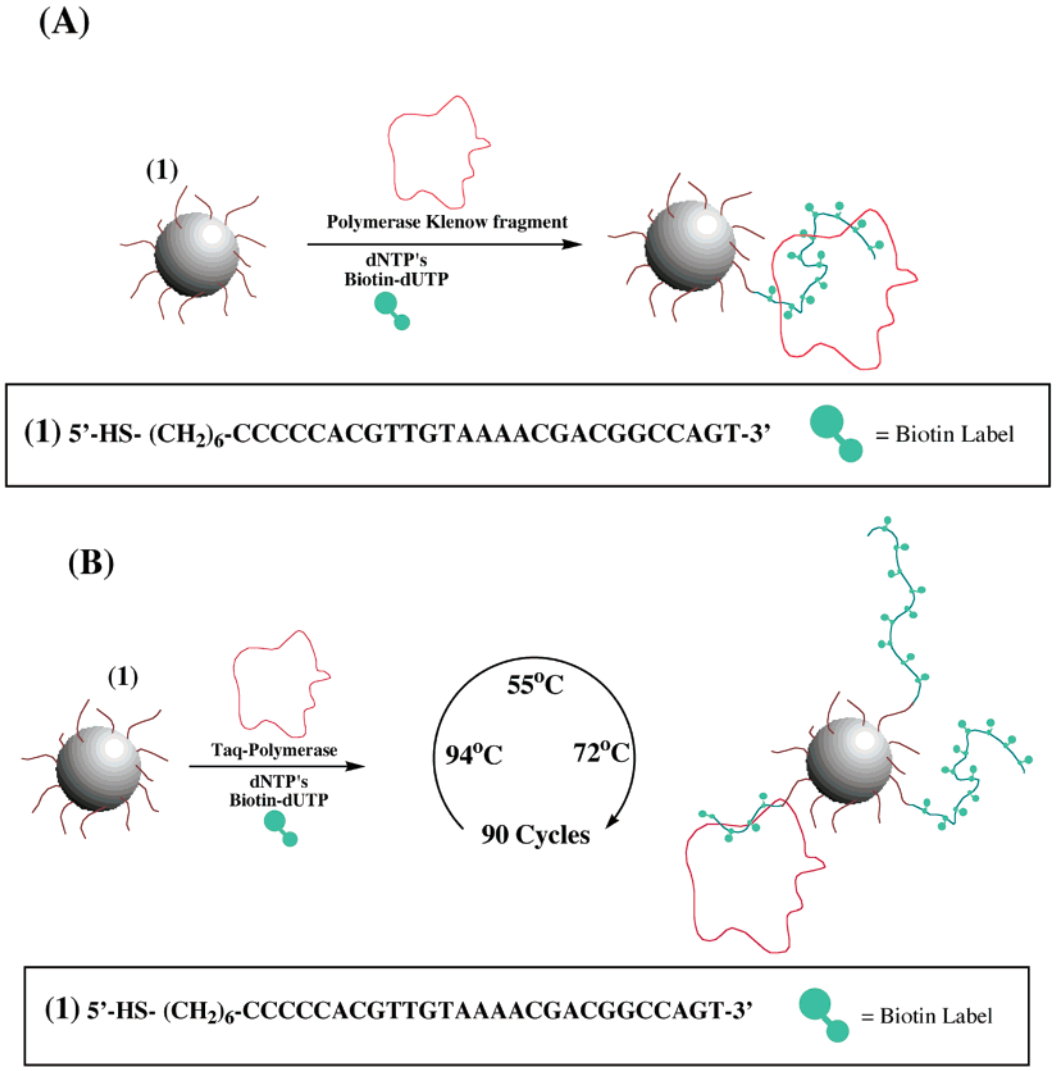

Figure 2. (A) Replication of M13ϕ DNA hybridized with **(1)**-functionalized magnetic beads with the incorporation of biotin labels into the replica by Klenow fragment. (B) Replication of M13ϕ DNA hybridized with **(1)**-functionalized magnetic beads with the incorporation of biotin labels into the replica using Taq-polymerase and appropriate thermal cycles.

Results and Discussion

The experimental setup and concept for the magneto-mechanical detection of the biorecognition events is schematically shown in Figure 1. Magnetic beads are functionalized with a sensing interface, and the analysis of the biorecognition event on the magnetic beads involves the labeling of the resulting beads with biotin labels. The association of the labeled magnetic beads onto an avidin-functionalized AFM cantilever and the subsequent deflection of the modified cantilever by means of the external magnetic field provides the route for the amplified magneto-mechanical detection of the primary recognition event. The deflection of the lever is monitored by following the reflection of a laser beam on a conventional AFM photodiode detector. The concept for the magneto-mechanical detection of the M13ϕ phage DNA is shown in Figure 2A and 2B. The probe nucleic acid, (**1**), is linked to the magnetic particles. The magnetic particles are hybridized with the analyte M13ϕ DNA and subsequently polymerized in the presence of the mixture of nucleotides, dNTPs, that includes biotinylated-dUTP. Replication of the hybridized DNA leads to the biotin-labeled replica associated with the particles. An Au-coated cantilever functionalized with an avidin monolayer is used as a versatile binding interface for the biotin-labeled nucleic acid-functionalized magnetic particles. The association of the biotinylated replica DNA-functionalized particles to the surface, followed by the mechanical deflection of the lever using the external magnet, provides the amplified sensing mechanism for the primary hybridization. Figure 3A shows the reversible magneto-mechanical deflection and retraction of the cantilever upon positioning the external magnet below the lever and upon its removal from the lever, respectively. Upon the removal of the external magnet, the lever is retracted to its original position. In the experiment shown in Figure 3A the concentration of M13ϕ DNA that is analyzed is 4.3×10^{-12} M, and its detection proceeds with a signal-to-noise ratio that is ca. 3. At lower concentrations, it is difficult to observe the deflection of the cantilever, whereas the magnitude of deflection increases as the concentration of the analyte DNA is higher. Control experiments indicate that the interaction of the (**1**)-modified magnetic particles with a mixture of partially cleaved λ-DNA, followed by an attempt to replicate a possible double-stranded assembly that includes biotin labels, does not yield to any observable mechanical deflection of the lever upon interaction with the avidin-modified lever and its attraction by the external magnet. Also, magnetic particles that

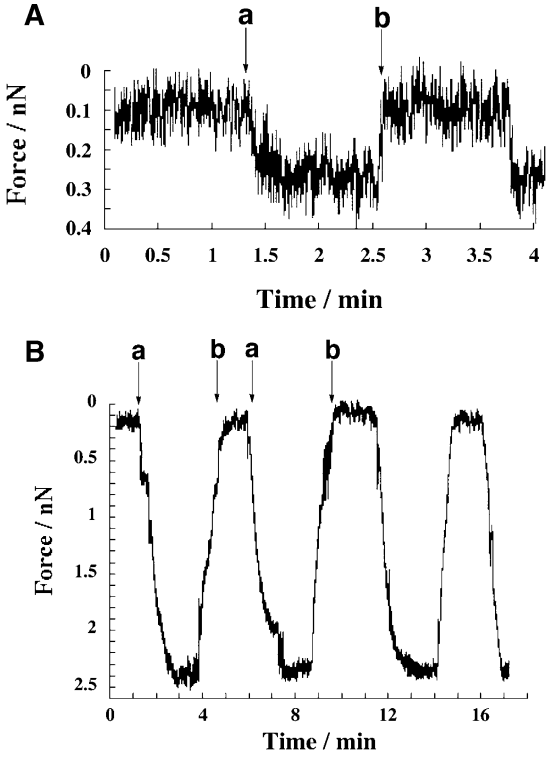

Figure 3. Magneto-mechanical deflection or retraction of the cantilever analyzing M13ϕ DNA according to: (A) using Klenow fragment as the replication catalyst according to Figure 2A, M13ϕ DNA, 4.3×10^{-12} M, (B) using Taq-polymerase and the thermal cycles for replication and labeling according to Figure 2B, M13ϕ DNA, 7.1×10^{-12} M. At points (a) the cantilever is subjected to the external magnet. At points (b) the external magnet is removed.

include a nucleic acid probe that is noncomplementary to the M13ϕ DNA do not yield, after attempts to hybridize with M13ϕ DNA and to generate the biotin-labeled replica, to any deflection of the avidin-modified lever, upon subjecting the system to the external magnetic field. Although these experiments confirm the concept of amplified and specific magneto-mechanical detection of DNA, the results are unsatisfactory in respect to the sensitivity of the method. To overcome this limitation, the (**1**)-functionalized magnetic particles were subjected to a pre-amplification labeling procedure as outlined in Figure 2B. In this process, the (**1**)-functionalized magnetic particles are interacted with the M13ϕ DNA and subjected to 90 cycles of replication with the incorporation of biotin labels. By these procedures, the particles are repeatedly hybridized with M13ϕ DNA, replicated in the presence of the dNTPs nucleotide mixture that includes biotin-dUTP in the presence of *Taq polymerase*, melted to dissociate the double-stranded assembly, and the functionalized magnetic particles are further hybridized, polymerized while labeling, etc. This procedure introduces a linear amplification of the labeled chains associated with the magnetic particles. Figure 3B shows the reversible magneto-mechanical deflection and retraction of the avidin-modified cantilever that was interacted with the preamplified labeled particles. Positioning of the external magnet below the lever attracts the lever that is modified with the particles and results in its mechanical deflection, whereas the removal of the external magnet retracts the lever to its original position. This latter experiment uses a 7.1×10^{-12} M concentration of the analyzed M13ϕ DNA. We observe a ca. 10-fold amplification in the

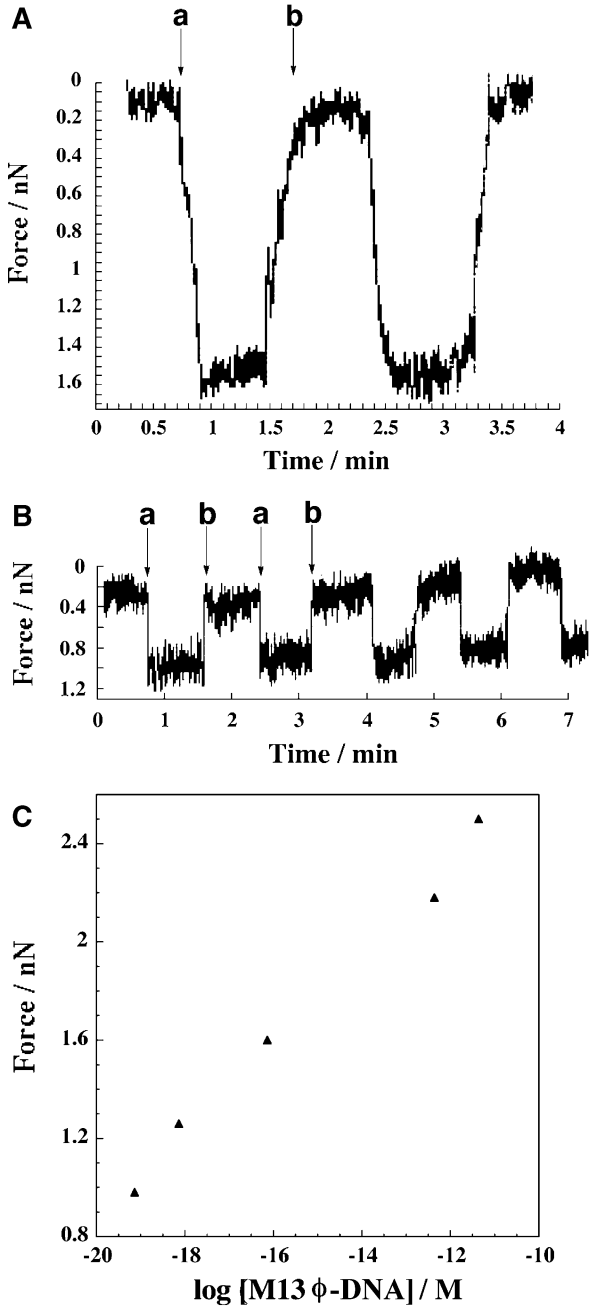

Figure 4. Magneto-mechanical analysis of different concentrations of M13ϕ DNA according to Figure 2B: (A) Analysis of M13ϕ DNA, 7.1×10^{-17} M. (B) Analysis of M13ϕ DNA, 7.1×10^{-20} M. (C) Dependence of the deflection signal on the M13ϕ DNA concentration.

deflection magnitude of the lever as a result of the preamplified labeling of the particles by the thermal cycles. The preamplified labeling of the magnetic particles enables us to analyze substantially lower quantities of the M13ϕ DNA. The advantages of the present method in comparison to coventional PCR-based techniques are, however, obvious. While PCR introduces errors into the replicated product and the error-containing products are amplified, the present method lacks this limitation, since any generated error on the magnetic beads is not further amplified. Figure 4 shows the magneto-mechanical analysis of M13ϕ DNA concentrations that correspond to 7.1×10^{-17} and 7.1×10^{-20} M, Figure 4, A and B, respectively. As the original concentration of the analyzed DNA is lower, the magnitude

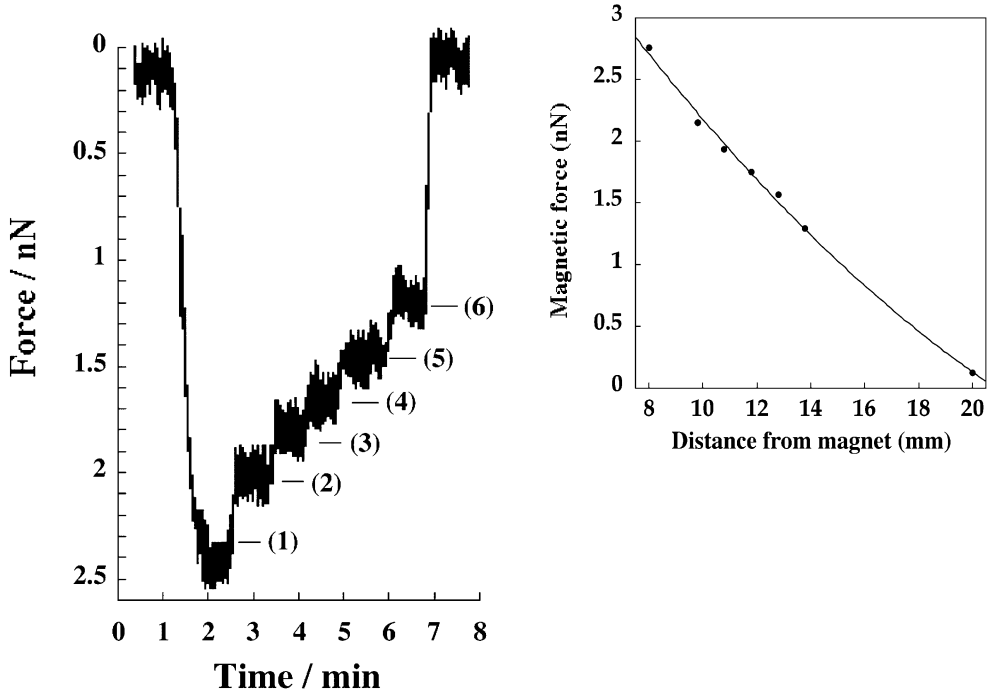

Figure 5. Stepwise retraction of the cantilever analyzing M13ϕ DNA, 7.1×10^{-12} M, according to Figure 2B, upon gradual removal of the external magnet from the analyzing cell. (Inset) Magnetic forces exerted on the cantilever as a function of the distance between the external magnet and the cantilever.

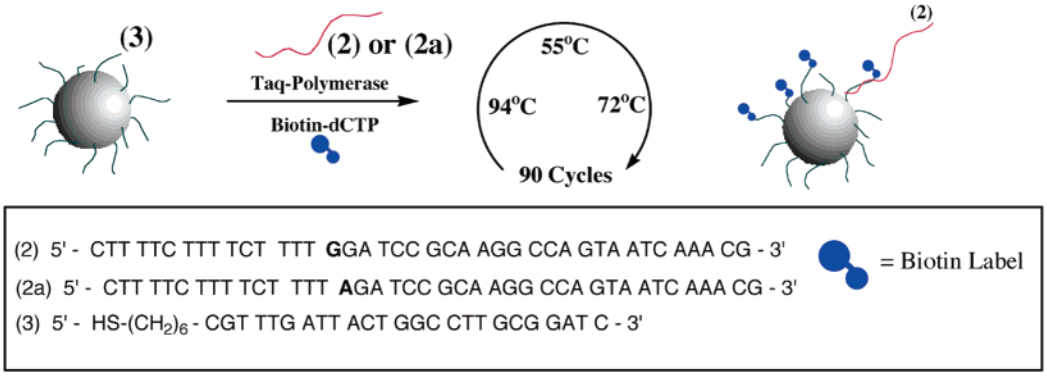

Figure 6. Detection of a single-base mismatch in a nucleic acid by the Taq polymerase-induced incorporation of a biotin label into the **(3)**-functionalized magnetic beads.

corresponding to the mechanical deflection of the lever decreases, due to the lower coverage of the cantilever with magnetic particles. Note, however, that the preamplified labeling of the magnetic particles upon the analysis of M13ϕ DNA at a concentration that corresponds to 7.1×10^{-20} M yields a mechanical deflection of the cantilever that is ca. 2-fold higher than the deflection magnitude observed for the analysis of M13ϕ DNA at a concentration of 4.3×10^{-12} M, with no preamplification process (Figure 4B vs Figure 3A). It should be noted that attempts to analyze the DNA at concentrations that are lower than 7.1×10^{-20} M using the preamplification labeling procedure led to the measurable deflection of the levers, but to poor reproducibilities (ca. 40%), and thus we prefer to exclude these results from our discussion. A possible origin for the poor reproducibility observed when analyzing very low concentrations of the analyte DNA may be the random distribution of the magnetic beads on the lever. The deflection of the cantilever is controlled by the number of magnetic particles associated with the lever, and the respective spatial position of the magnetic particles on the lever (particles at the end of the lever contribute more to the lever deflection). Upon the association of a large number of particles with the lever, (at high concentrations of labeled DNA), the relative particle positions are statistically averaged. On the other hand, for very dilute solutions of the analyzed M13ϕ DNA, the very few particles deposited on the lever differ substantially from one experiment to another, leading to the limited reproducibility (for example upon analyzing the M13ϕ DNA, 7×10^{-12} M, we counted by optical microscopy 14 magnetic particles, 5 μm diameter, on the lever; yet smaller particles are certainly co-deposited). Within the concentration range of 10^{-12}–10^{-20} M of the M13ϕ DNA, we find, however, an almost linear relation between the force exerted on the cantilever (derived from the appropriate magnitudes of deflection) and the concentration of the analyzed DNA, Figure 4C.

An experimental support that the magneto-mechanical deflection of the cantilever is controlled by the external magnetic field intensity that exerts a force on the lever, is depicted in Figure 5. In this experiment, the M13ϕ DNA, 7.1×10^{-12} M, is analyzed according to Figure 2B, and the lever is deflected by the external magnet. The external magnet is then gradually

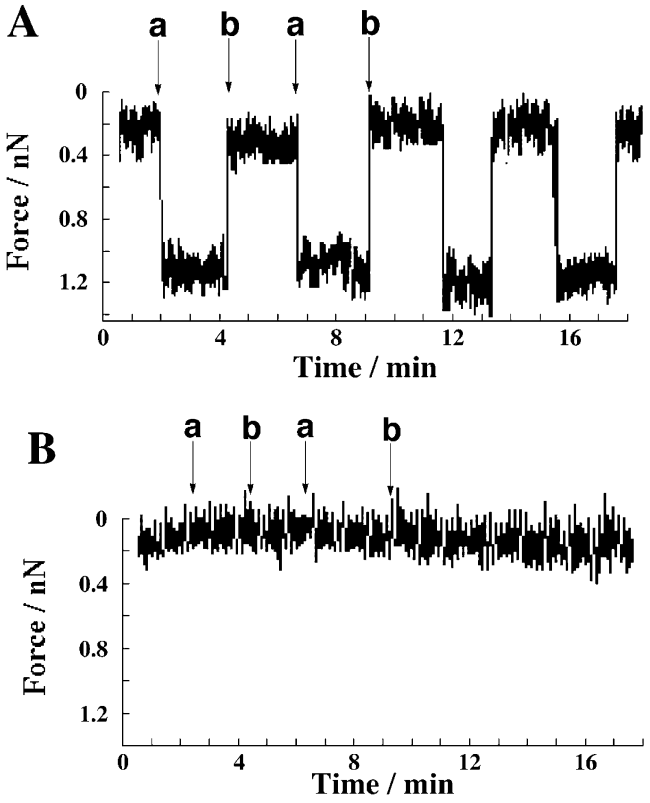

Figure 7. (A) Magneto-mechanical analysis of the single-base mismatch in the mutant **(2)**, 2.5×10^{-13} M, according to Figure 6. At points (a) the cantilever is subjected to the external magnetic field. At points (b) the external magnet is removed. (B) Magneto-mechanical detection of the normal gene (**2a)** by the **(3)**-functionalized magnetic beads. At points (a) the cantilever is subjected to the external magnet. At points (b) the external magnet is removed.

removed from the lever, by the appropriate positioning of the external magnet on the translator, leading to the stepwise decrease of the intensity of the external magnetic field. That is, the distance between the cantilever and the external magnet on the translator is gradually increased. In steps 2−5 in Figure 5, the magnet is equally separated, by 1 mm from the lever, while in step 1 the lever is separated from the magnet by 1.8 mm. At step 6 the external magnet reached the distance at which the magnet is fully eliminated from the system. The lever is stepwise retracted to its original rest position upon weakening of the external magnetic field intensity. The inset in Figure 5 shows the magnetic force exerted onto the cantilevers at the different distances separating the external magnet and the cantilever. Clearly, as the distance separating the magnet from the lever increases, the magnetic force on the lever decreases.

The magneto-mechanical amplification of biosensing events was also applied for the detection of single-base mismatches in DNA, Figure 6. Here, we exemplify the magneto-mechanical detection of the mutant (**2**) that includes the exchange of the single A-base in the normal gene (**2a**) with the G-base in the mutant. To analyze the mutant, the magnetic particles are functionalized with the primer (**3**) that is complementary to (**2**) or (**2a**) up to one base before the mutation site. The particles are then hybridized with the samples that include either the mutant (**2**) or the normal gene (**2a**). Subsequently, the particles are reacted with biotinylated-dCTP in the presence of Taq-polymerase. The labeled base is incorporated only into the double strand that includes the mutant (**2**). Accordingly, only the labeled magnetic particles are associated with the avidin-modified cantilever, and only the lever that senses the mutant is deflected by the external magnet. Figure 7A shows the magneto-mechanical deflection of the lever upon the analysis of the mutant (**2**), 2.5×10^{-13} M. Figure 7B shows the responses of the lever upon the analysis of the normal gene (**2a**) in the presence of the external magnet. Clearly, no deflection of the lever is observed, indicating that no nonspecific adsorption of magnetic particles onto the avidin-functionalized cantilever takes place.

The third magneto-mechanical assay was developed for the analysis of telomerase activity in HeLa cancer cells. Telomerase is a ribonucleoprotein that adds the telomer repeats to the ends of the chromosomes. The magneto-mechanical detection of telomerase is schematically depicted in Figure 8. The magnetic particles are modified with the primer (**4**) that is recognized by telomerase. Interaction of the magnetic particles with the HeLa cell extract in the presence of a mixture of nucleotides that

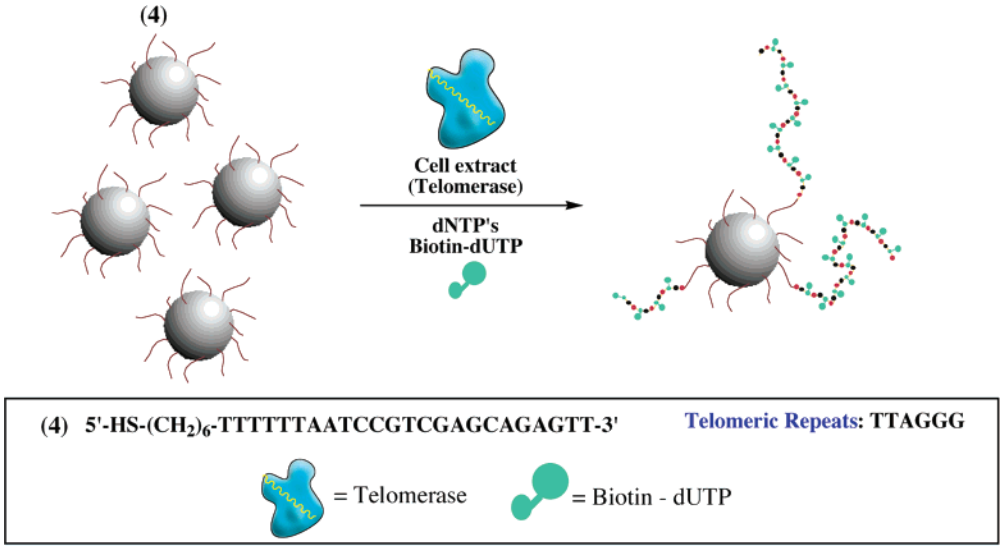

Figure 8. Detection of telomerase activity by the telomerization of the **(4)**-functionalized magnetic beads and the incorporation of biotin labels into the telomeric units.

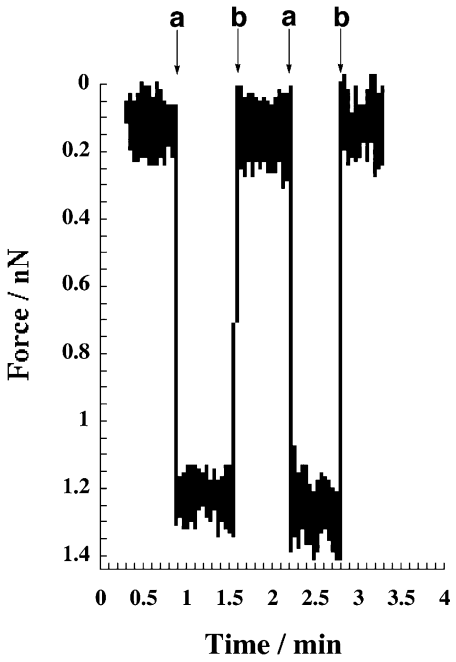

Figure 9. Magneto-mechanical detection of telomerase activity originating from 10000 HeLa cells according to Figure 8. At points (a) the cantilever is subjected to the external magnet. At points (b) the external magnet is removed.

includes biotinylated dUTP leads to telomerization while labeling of the beads with biotin. The association of the biotin-labeled telomer-functionalized magnetic particles to the avidin-modified cantilever leads, in the presence of the external magnet, to the deflection of the lever. Figure 9 shows the magneto-mechanical detection of the telomerase extracted from 10000 cells. Control experiments reveal that the cantilever is not deflected upon analyzing non-telomerase-containing cells (NHF cells). Also, attachment of an oligonucleotide that is not recognized by telomerase, e.g. **1**, to the magnetic particles did not yield, upon the interaction with the HeLa cell extract, any active particles that bind to the cantilever and affect its deflection. The method was applied to analyze telomerase cell activity that originates from variable number of HeLa cells. We were able to detect the cancer cells with a sensitivity limit of 100 cells (S/N > 4). This impressive sensitivity is certainly higher than conventional optical methods[23] and is comparable to that in the TRAP assay that includes PCR amplification.[15,16]

In conclusion, the present paper has introduced a new concept for the amplified detection of DNA and of telomerase activity. The method involves the association of magnetic particles that carry the biorecognition complex to the functionalized cantilever and the magneto-mechanical deflection of a cantilever in the presence of an external magnet. A major advantage of this method is the fact that the sensing element has micrometer-size dimensions. This will enable the handling and analysis of very small sample volumes, thus leading to enhanced sensitivities. Furthermore, the collection of the biotin-labeled beads by an external magnet provides a means to purify and concentrate the active sensor units from complex biological samples. Also, the production of arrays of cantilevers is already a viable practice. Thus, by the immobilization of different primers on the cantilevers, the high throughput magneto-mechanical analysis of genes may be accomplished. The magneto-mechanical detection of biosensing events may be broadened to many other biorecognition pairs.

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Experimental Section

Materials and Methods. Amine-functionalized borosilicate-based magnetic particles (5 μm, MPG Long Chain Alkylamine, CPG Inc.), biotin-21-dUTP (Clontech), the heterobifunctional cross-linker, 3-maleimideopropionic acid *N*-hydroxysuccinimide ester, all oligonucleotides, avidin, dNTPs, biotin-11-dCTP (all from Sigma), Taq-polymerase, 10×PCR buffer, and Klenow fragment (Sigma) were used with no further purification. AFM silicon cantilevers (CSC 12, cantileverE) were purchased from MicroMash, Estonia).

Preparation of DNA-Functionalized Magnetic Particles. Thirty milligrams of the amino- functionalized magnetic particles were activated by reaction with the heterobifunctional cross-linker 3-maleimidepropionic acid *N*-hydroxysuccinimide ester (5 mg, Sigma) in 1 mL of DMSO. After 4 h of incubation at room temperature, the particles were collected with an external magnet and 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. The DNA-modified particles were kept for periods longer than six months, in phosphate buffer that included 1% w/v sodium azide, 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.).

Cells and Tissues. HeLa cells and NHF cells were stored as pellets at −80 °C until extraction. Cell pellets were lysed by the CHAPS lysis buffer method.

Conditions for thermal cycles (Taq-polymerase 0.1 unit/μL, dNTPs 0.2 mM each): (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.

Conditions for Klenow fragment elongation: Klenow fragment 0.1 unit/μL, dNTPs 0.2mM.

Cantilever Modification. Gold-coated silicon cantilevers were incubated in 3,3′-dithiodipropionic acid bis(*N*-hydroxysuccinimide ester), 10 mM in dry DMSO, for a period of 2 h to obtain an active-ester monolayer. The resulting cantilevers were washed thoroughly with dry DMSO (three times) and finally washed shortly (5 s) with phosphate buffer, pH 7.4. The NHS-activated cantilevers were then immersed in an avidin solution (2−3 mg in phosphate buffer, 0.1 M, pH 7.4) and allowed to react overnight. The resulting cantilevers were washed with phosphate buffer, pH 7.4, prior to their use.

Telomerization reactions were performed in a solution of 1×CHAPS buffer containing a specific number of cells (cancer or normal cells), the probe (**4**)-modified magnetic particles (~1.5 mg), and a mixture of dATP, dCTP, dGTP, and biotin-21-dUTP (0.2 mM each), for a period of 30 min, at 30 °C. After the incubation period the magnetic particles were washed throughly with phosphate buffer.

Magneto-Mechanical Instrumentation System. The experimental setup for the magneto-mechanical detection of DNA and telomerase activity is depicted in Figure 1. A home-built instrument consists of an AFM Au-coated cantilever that is mounted on a piece of silicon, glued to the surface of a glass slide. A laser beam (SM 670-5 mW laser diode, NVG Inc.) is deflected from the cantilever on a four-

quadrant photodiode (UV-140-4 EG&G Photon Devices) to monitor the cantilever position. The detector is linked to an acquisition board (PCI-6034E, National Instruments) for signal processing and display. A neodynium iron boron magnet (50 mm × 50 mm × 10 mm) is mounted on a moveable stage in parallel to the cantilever surface. The magnetic field strength as a function of the distance was determined using a digital gaussmeter (DGM-102, Ses Techno, India).

The photodiode position sensitivity ($S = \Delta V/\Delta X$) was calibrated, and the voltage difference between the top and bottom parts of the detector was used to determine the cantilever deflection.

The avidin-modified cantilevers were interacted with the biotin-labeled magnetic particles obtained upon the replication of the DNA, as a result of analyzing single-base mismatches, or with the particles functionalized with the biotin-labeled telomers. The resulting cantilevers were mounted on the measuring cell and analyzed in the absence or presence of the external magnetic field. Since the deposition of the magnetic particles occurs randomly on the lever, we assume a uniformly distributed magnetic force acting on the cantilever. Under these conditions, the deflection of the end of the lever, ΔZ, and the deflection angle, $\Delta\theta$, are given by eqs 1 and 2, where L is the cantilever length, F_L is the force per unit length, E is the Young modulus of Si and I is the moment of inertia of the rectangular cantilever.[24,25]

$$\Delta Z = \frac{F_L L^4}{8EI} \tag{1}$$

$$\Delta\phi = \frac{4\Delta Z}{3L} \tag{2}$$

The relation between the beam displacement on the photodetector, ΔX, and the beam angle deflection, 2θ, is given by eq 3, where D is the distance between the cantilever and the photodiode (6 cm). Combining eq 3 with eq 2 enables us to derive an expression for the cantilever deflection, ΔZ, using eq 4, where $A = 3L/8D = 2.2 \times 10^{-3}$.

$$\Delta X = 2\Delta\theta \text{ x } D \tag{3}$$

$$\Delta Z = \frac{3L}{8D}\frac{\Delta V}{S} = A\frac{\Delta V}{S} \tag{4}$$

The relation between the magnetic force, F, acting on the lever modified with the magnetic particles and the cantilever deflection, ΔZ, is given by eq 5, where k is the spring constant of the cantilever (typically 0.03 N/m).

$$F = k \text{ x } \Delta Z \tag{5}$$

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