Amplified Telomerase Analysis by Using Rotating Magnetic Particles: The Rapid and Sensitive Detection of Cancer Cells

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*A highly sensitive telomerase detection method that involves amplified telomerase analysis and the use of rotating magnetic particles has been developed. Magnetic particles, functionalized with a primer (**1**) that is recognized by telomerase, are mixed with a nucleotide mixture that includes biotinylated-dUTP, and telomerase-induced elongation of the primers proceeds with simultaneous biotin incorporation. Avidin–Horseradish peroxidase conjugate, coupled to biotin labels, yields the biocatalytic functional particles. Mixing the resulting particles with naphthoquinone-modified magnetic particles enables the optoelectronic detection of telomerase. Attraction of the magnetic particles to an electrode, followed by rotation of the particles, causes the electrocatalytic reduction of O_2 to H_2O_2 and HRP-catalyzed oxidation of luminol (**3**); this results in chemilumunescence. The intensity of the emitted light depends on the telomerase content of the sample and the rotation speed of the particles. A minimum number of 10 cancer cells could be detected.*

Introduction

Telomerase is a ribonucleoprotein complex capable of synthesizing new telomers by the addition of telomeric repeats to the 3′-end of chromosomal DNA,[1] thereby providing cells with a mechanism to prevent gradual telomer erosion. Telomerase is thought to be responsible for the continuous and uncontrolled growth of cancer cells.[2–3] Nearly all cancer types have been screened, and a strong link between the presence of telomerase and malignancy has been established. Because of its involvement in carcinogenesis, telomerase is a versatile and useful biomarker in cancer diagnosis and therapy, and a promising prognostic tool for determining whether a particular tissue is likely to develop cancer.[4–6] Several analytical procedures for the quantification and characterization of telomerase activity have been reported.[7–11] The most frequently used assay is the TRAP method (telomeric repeat amplification protocol). The TRAP method suffers, however, from important disadvantages, especially for clinical uses, because it is time-consuming and depends on PCR, which is susceptible to inhibition by extracts of clinical samples. Furthermore, it is difficult to quantify telomerase activity because of the logarithmic amplification of telomerase product in the PCR step.[12,13] Also, due to the susceptibility of the TRAP assay to *Taq*-Polymerase inhibitors, false negative and false positive results are frequent.[14] Thus, the development of sensitive, accurate, and rapid methods to detect telomerase is of great importance for clinical purposes.

Recent activities in bioelectronics have addressed the amplified detection of DNA-sensing events. It has been demonstrated that replication of the analyzed DNA on electronic transducers, for example, electrodes or piezoelectric crystals, enables the incorporation of biotin[15,16] or redox labels[17] into nucleic acid replica. The coupling of biocatalysts to the biotin-labeled replica catalyzes the precipitation of insoluble products on electrodes or the conjugation of redox enzymes to the redox-active replica; this provides biocatalytic amplification paths for sensing DNA. Alternatively, electrogenerated chemiluminescence resulting from the formation of double-stranded DNA complexes on electrodes, has been employed as an optoelectronic signal for the detection of DNA.[18] In a series of recent reports, we have addressed the use of magnetic particles that are rotated by an external rotating magnet for the amplified detection of biorecognition events.[19,20] We found that, upon rotation of magnetic particles by the external magnet, the particles behave as rotating microelectrodes in which biocatalytic processes at the particle surface are controlled by convection rather than by the diffusion of substrates to the particle interface. As a result, biocatalytic transformations[19] and DNA-recognition processes[20] were enhanced and amplified. For example, the replication of analyte DNA on the magnetic particle by using polymerase-induced thermal cycles was used to incorporate appropriate labels into the probes associated with the particle, thereby amplifying the detection of the target DNA. Attraction of the magnetic particles to the electrode, followed by their rotation and electrogeneration of chemiluminescence led to unprecedented sensitivities in the detection of DNA. Here we report on the very rapid and ultrasensitive detection of telomerase in cancer cells by using rotating magnetic particles functionalized with telomerase-synthesized labeled telomers.

Results and Discussion

The method for the amplified detection of telomerase activity is depicted in Scheme 1. Amine-functionalized magnetic parti-

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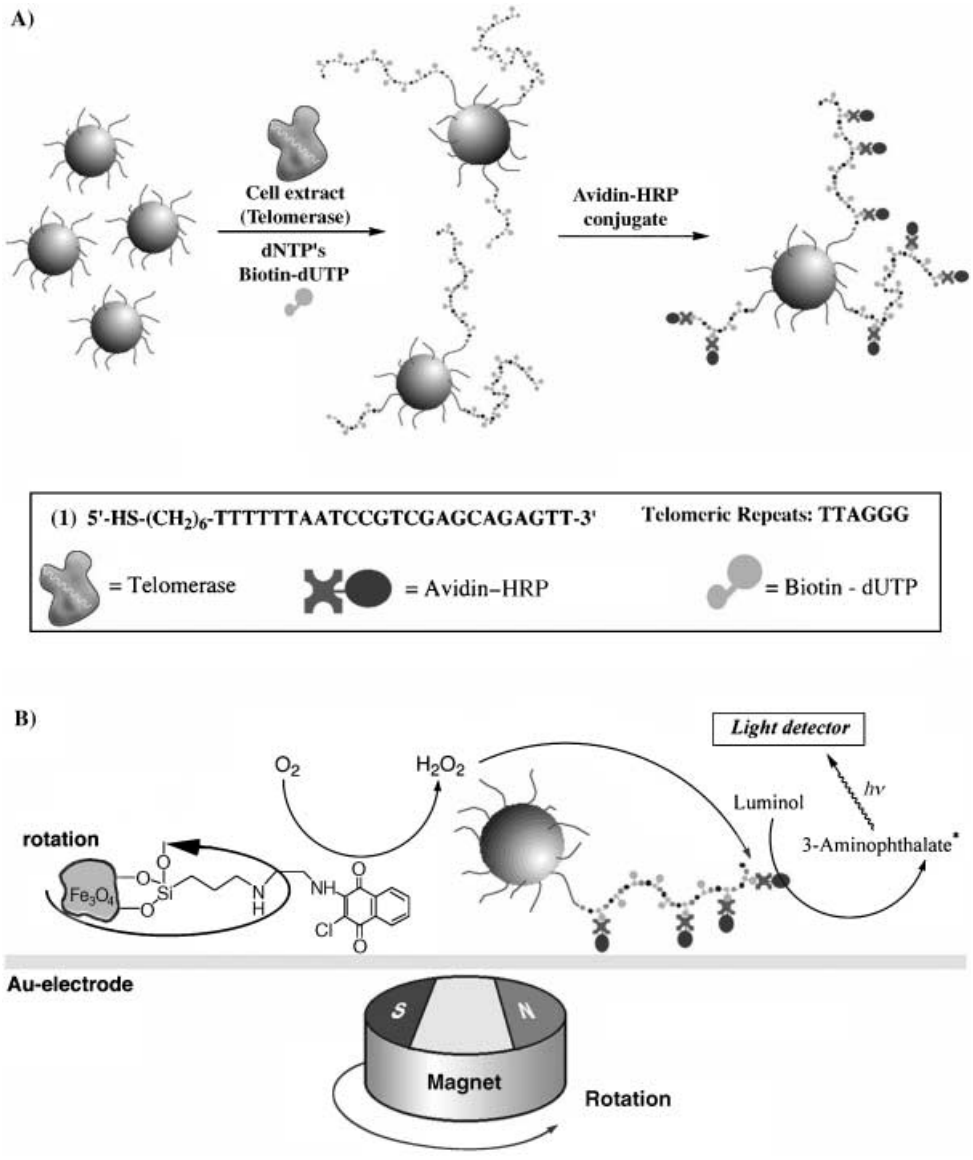
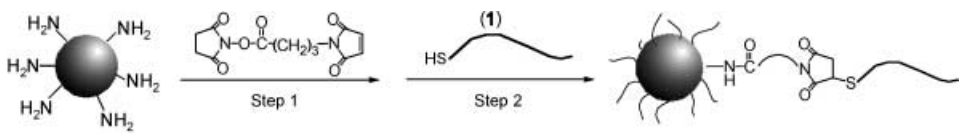
 DOI: 10.1002/cbic.200300820

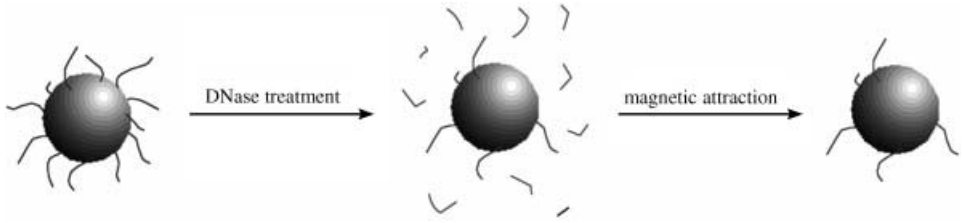
cles (5 μm diameter) were activated with the bifunctional reagent 3-maleimidopropionic acid-*N*-hydroxysuccinimide ester, as outlined in Scheme 2. The mercaptohexyl-modified nucleic acid **1** was then covalently linked to the magnetic particles. Primer **1** includes a six T-base linker unit followed by the characteristic sequence recognized by the telomerase. The average coverage of the magnetite particles with **1** was determined by using OliGreen® reagent and corresponds to approximately 50000 oligonucleotide units per particle. The number of nucleic acid molecules that were associated with the particles and accessible to an external enzyme was estimated by subjecting the **1**-functionalized magnetic particles to DNase, Scheme 3, followed by determination of the amount of residual DNA that is not cleaved from the particles (see Experimental Section). We found that approximately 20000 oligonucleotide units per particle were cleaved off; this implies that only about 40% of the particle-linked nucleic acids are accessible to the enzyme. We thus assume that the number of nucleic acids linked to the magnetic particles and accessible to DNase are also accessible to telomerase. The functional magnetic particles were treated with telomerase-containing cell extract in the presence of a dNTP mixture that included biotin-labeled dUTP. Telomerization resulted in the incorporation of biotin labels into the telomers that were linked to the magnetic particles. The subsequent binding of avidin–horseradish peroxidase (HRP) conjugate to biotin labels introduced the biocatalytic conjugate into the telomer chains. The magnetic particles were collected at the bottom of the analysis flask by means of an external magnet and washed to remove any residual cell extract or nonspecifically adsorbed avidin–HRP conjugate. The resulting particles were then mixed with naphthoquinone-functionalized magnetite particles, which were prepared by treating 2,3-dichloro-1,2-naphthoquinone (**2**) with the aminoethylaminesiloxane-modified magnetic particles. The mixture of particles was introduced into an electrochemical cell that included luminol (**3**). Upon application of a potential step that reduced the quinone to the hydroquinone, the electrocatalyzed reduction of O_2 to H_2O_2 proceeded. The resulting H_2O_2 mediated the HRP-catalyzed oxidation of luminol with the concomitant emission of light. Note



Scheme 1. *Amplified rapid detection of telomerase activity by multilabeled rotating magnetic particles. A) Multilabeling of magnetic particles with biotin by using telomerase enzyme activity. B) Generation of amplified chemiluminescence upon rotation of biotin-multifunctionalized magnetic particles on electrode surfaces.*



Scheme 2. *Activation of amine-functionalized magnetic particles with the bifunctional reagent 3-maleimidopropionic acid-*N*-hydroxysuccinimide ester, and the covalent binding of **1** to the particles.*



Scheme 3. *Estimation of the number of nucleic acid molecules associated with magnetic particles.*

that the electrogenerated luminescence is observed only if HRP conjugates bind to the telomerase units, and this occurs only when telomerase exists in the cell extract. Also, the intensity of the electrogenerated luminescence is controlled by the content of labels/avidin–HRP conjugates associated with the particles, which is determined by the amount of telomerase in the sample. Furthermore, the rotation of the magnetic particles by means of the external rotating magnet, amplifies the intensity of the emitted light. Upon rotation of the particles, the electrocatalyzed reduction of O_2 and the interaction of H_2O_2 with luminol are controlled by convection rather than by diffusion; this leads to enhanced (amplified) light emission. Note that this telomerase analysis method involves several amplification steps:

1) The telomerization process that introduces biotin/avidin–HRP labels.
2) The electrocatalyzed reduction of O_2 to H_2O_2 and the subsequent generation of light provide catalytic/biocatalytic processes that generate numerous photons as a result of the action of a single telomerase enzyme molecule.
3) The rotation of the magnetic particles amplifies the light emission.

This last process is controlled by the rotation speed of the particles (vide infra). It should be noted that besides the function of the magnetic particles in amplifying the signal through their mechanical rotation, they fulfill an important complementary function in separating specific telomerase activity from other nonspecific ingredients in crude cell extracts. Therefore, the separation and purification of telomer-synthesized modified particles from cell extracts by the external magnet enables the subsequent unperturbed optoelectronic analysis of the functionalized particles.

Figure 1 A depicts the analysis of 293-kidney cancer-cell-line extracts according to Scheme 1. It shows the light emitted from the system upon analysis of an extract from 100 000 cells, while applying a potential step of 0 to −0.5 V and rotating the particles at different speeds. The intensity of the light emitted from the system is enhanced as the rotation speed increases. Provided that the functional magnetic particles behave as rotating microelectrodes in the analytical system, and that the electrocatalyzed generation of light is controlled by convection of substrates to the rotating electrode, a linear dependency between the emitted light and $\omega^{1/2}$ should exist (ω = rotation speed).[21] Figure 1 B shows that a linear relation between the electrogenerated light and $\omega^{1/2}$ indeed exists. In control experiments in which naphthoquinone-functionalized magnetic particles or avidin–HRP conjugate are excluded from the system, no light emission was detected, irrespective of the rotation speed of the particles. These experiments confirm that the electrogenerated chemiluminescence originates from the primary electrocatalyzed reduction of O_2 to H_2O_2 and subsequent HRP-mediated oxidation of luminol by H_2O_2. The electrogenerated chemiluminescence at constant rotation speed is dependent on the number of cancer cells in the extract. Figure 2 shows the light emitted from the electrochemical cell upon the analysis of telomerase originating from different concentrations of 293-kidney cancer cells at a constant rotation speed of

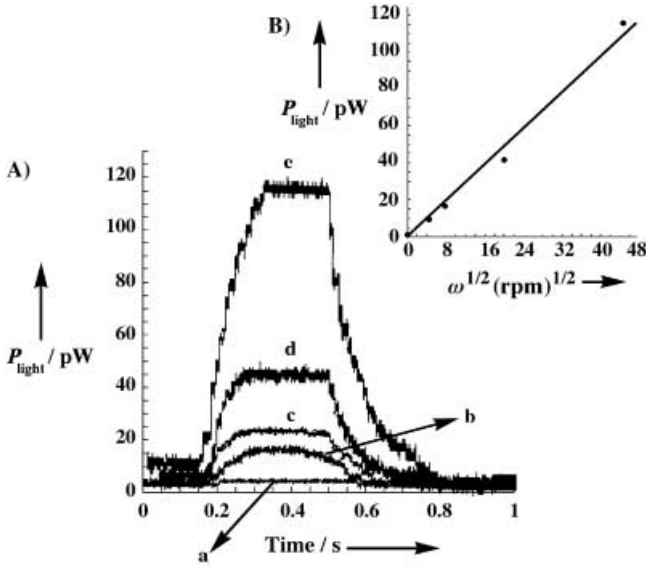


Figure 1. *A) Chemiluminescence intensities observed with 293-kidney cell line extract (100 000 cells) at different rotation speeds. a) 0 rpm, b) 20 rpm, c) 60 rpm, d) 400 rpm, e) 2000 rpm. B) Chemiluminescence intensities as a function of $\omega^{1/2}$ (ω = rotation speed). In all experiments chemiluminescence is generated by the application of a potential step from $E_1 = 0.0$ V to $E_2 = -0.5$ V and back vs. SCE. Data recorded in 0.01 M phosphate buffer, pH 7.4, containing luminol (1×10^{-6} M) under air.*

2000 rpm, in accordance with the protocol depicted in Scheme 1 A and B. In these systems, a potential step from 0.0 V to −0.5 V is applied to the functional particles. Figure 2, inset, shows the light emitted from extracts that include 100 and 10 293-kidney cancer cells. For comparison, no generation of light was observed when the procedure was carried out in the absence of cells or in the presence of 293-kidney cell extracts heated to 90 °C for 20 minutes in order to inactivate the telomerase prior to analysis (Figure 2, curves b and c respectively). This implies that no nonspecific binding of the avidin–HRP conjugate to the magnetic particles or the electrode takes place.

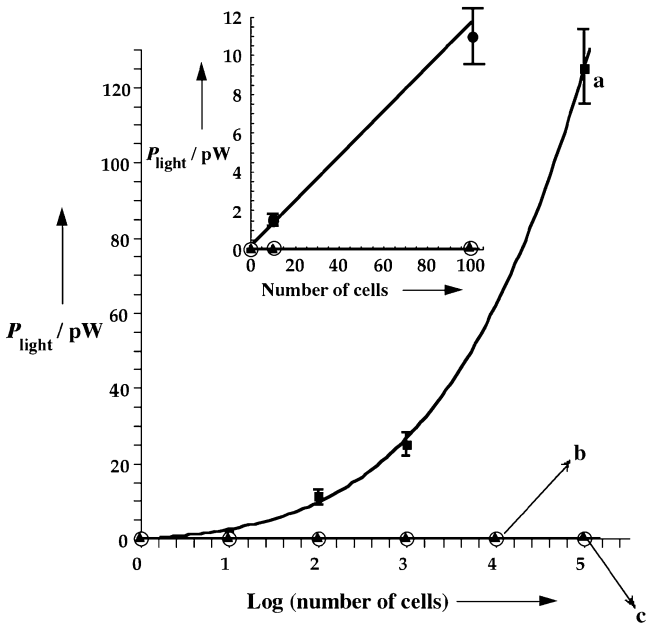


Figure 2. *Calibration curve corresponding to the chemiluminescence intensities of: a) 293-kidney cell line extracts with different cell numbers at a constant rotation speed of 2000 rpm (■); b) cell-free control samples (○); c) heat-inactivated extract (▲). Inset: Enlargement of the calibration curve showing chemiluminescence signal intensities obtained from extracts containing 100 and 10 cells. The conditions for chemiluminescence recording are detailed in Scheme 1.*

Similar results were observed upon the analysis of telomerase when using cultured HeLa cell-line extracts. Figure 3 A shows electrogenerated chemiluminescence with an extract from 100 000 HeLa cells, according to Scheme 1, and by using different rotation speeds of the magnetic particles. The intensity of emitted light is enhanced when the rotation speed of the particles is increased, and a linear relationship between the intensity of generated light and $\omega^{1/2}$ is observed. The intensities of electrogenerated chemiluminescence obtained from the analysis of different concentrations of HeLa cell extracts are shown in Figure 3 B. In these experiments, magnetic particles were rotated at a constant rotation speed of 2000 rpm. The light emitted from 10 HeLa cells is easily detectable. The amplified light emission observed upon rotation of the magnetic particles is attributed to enhanced transport of the reactants to the electrode surface, where chemiluminescence takes place. One might argue that a similar effect could be accomplished by vortexing or sonication of the samples. However, it should be noted that simple mixing does not lead to the beneficial results described in the present method. In order to produce H_2O_2 the quinone-functionalized magnetic particles must be in close contact with the electrode surface, and this is accomplished only by their fixation with the external magnet.

A major issue that needs to be addressed relates to the differentiation of normal, nonmalignant cells from cancer cells by the analysis of telomerase activity in respective extracts. Figure 4 shows electrogenerated chemiluminescence obtained from the analysis of telomerase activity of HeLa cells, curve a, 293-kidney cells, curve b, NHF (Normal Human fiberblast) cells, curve c, and heat-treated HeLa cell extracts, point d. The electrogenerated chemiluminescence observed with a 100-fold higher content of normal cells is about 200-fold lower than the light emitted from 1000 293-kidney cells. The minute light emitted from the NHF cell extract may be attributed to nonspecific adsorption of residual quantities of the avidin–HRP conjugate to the magnetic particles. The light generated in the system that includes the NHF cells may be considered as the background light level of the analysis protocol.

In order to probe the applicability of the amplified-telomerase assay that applies rotating magnetic particles to analyzing cancer cells, tissues from patients with lung cancer were assayed. The electrogenerated chemiluminescence intensities obtained from adenocarcinoma and squamous epithelial carcinoma cells are shown in Figure 5 and compared to light signals obtained from healthy tissue or normal cell extracts. The chemiluminescence signals (telomerase activity) obtained from the carcinoma tissues were significantly higher than the minute chemiluminescence signal obtained from healthy cell extracts.

It should be noted that, for all systems discussed in this study, the direct addition of H_2O_2 to the system would generate chemiluminescence, and the use of quinone-functionalized magnetic particles could be, in principle, eliminated. We emphasize,

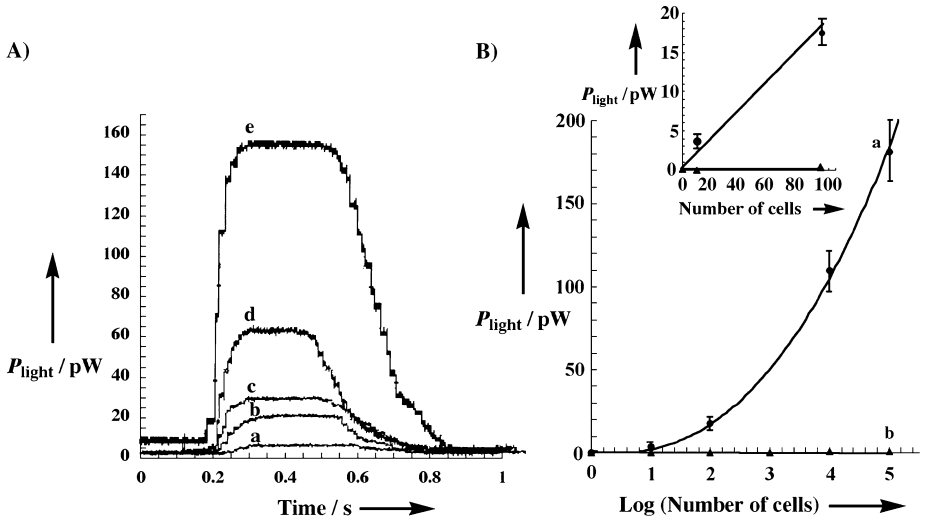


Figure 3. *A) Chemiluminescence intensities obtained with HeLa cell line extracts (100 000 cells), at different rotation speeds: a) 0 rpm, b) 20 rpm, c) 60 rpm, d) 400 rpm, e) 2000 rpm. Conditions for chemiluminescence recordings are detailed in Scheme 1. B) Calibration curve corresponding to chemiluminescence intensities of extracts containing: a) varying numbers of HeLa cells at a constant rotation speed of 2000 rpm (●); b) cell-free control sample (▲). Inset: Enlargement of calibration curve showing chemiluminescence signal intensities obtained from extracts containing 100 and 10 cells.*

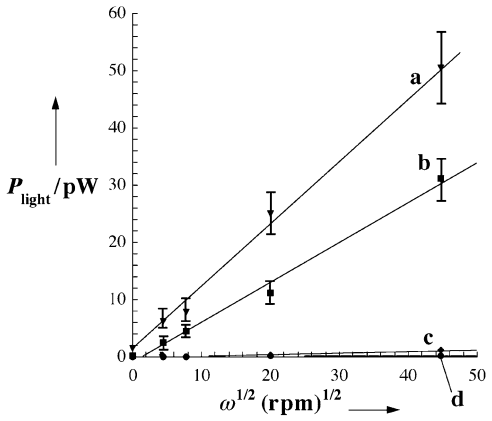


Figure 4. *Electrogenerated chemiluminescence intensities obtained from extracts containing different types of cells at variable rotation speeds: a) 1000 HeLa cells (▼); b) 1000 293-kidney cells (■); c) 100000 NHF cells (◆); d) 100000 HeLa cells heat-treated at 95°C for 20 min (●).*

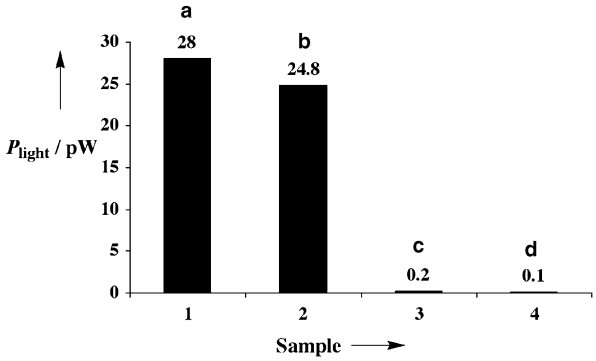


Figure 5. *Electrogenerated chemiluminescence intensities obtained by analyzing extracts from: a) lung adenocarcinomas, b) lung squamous epithelial carcinomas, c) healthy tissues, d) normal NHF-cell extract.*

however, that the use of the quinone-functionalized magnetic particles and in situ generation of H_2O_2 has two important advantages:

1) The system represents the electroswitchable, "ON" and "OFF", generation and extinguishing of chemiluminescence. Thus, in the "OFF" state, the system provides the background light intensity that is an important parameter in analyzing low concentrations of cancer cells. The "ON" state, provides only the light originating from the biological assay.
2) Luminol is sparingly soluble in aqueous buffer solutions. Thus, after the addition of H_2O_2, the chemiluminescence is rapidly reduced due to the consumption of luminol. In the present configuration, this disadvantage is resolved by the instantaneous generation of H_2O_2 near the particle surface and repeated generation of light signals by switching the electrical potential on the electrode.

Conclusion

In conclusion, the present study has introduced a novel method for detecting telomerase activity in cancer cell extracts. The method is based on a multiamplification analytical path that includes telomerase-induced synthesis of biotin-labeled telomers on functionalized magnetic particles, the electrobiocatalyzed generation of chemiluminescence, and the amplified emission of light by the rotation of magnetic particles. We have demonstrated unprecedented sensitivity in the detection of telomerase; without optimizing the procedure about 10 cancerous cells could be detected. All the analytical procedures could be completed within one hour. It should be noted that previous studies have claimed detection of telomerase activity from a few cells.[22] These studies, however, use PCR as a preamplification step. We suggest the examination of telomerase with amplified telomerase analysis by using rotating magnetic particles as a rapid method of identifying cancer cells and for monitoring anticancer therapeutic treatments. Since the method does not require a PCR amplification step, as the TRAP-derived procedures do, the determined telomerase activity is directly related to the actual telomerase content in the sample. Furthermore, since the analytical procedure is not affected by the presence of *Taq*-polymerase inhibitors in clinical samples, false-negative results are eliminated, thus improving the reliability and applicability of the analytical method as a new diagnostic tool. Moreover, this novel procedure combines the advantages of homogeneous and heterogeneous detection devices, thus converting the method into an ultrasensitive, rapid and reliable diagnostic tool for the detection of cancer cells.

Experimental Section

Amine-functionalized borosilicate-based magnetic particles (5 μm, MPG® Long Chain Alkylamine, CPG Inc.), Biotin-21-dUTP (Clontech), heterobifunctional crosslinker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester, oligonucleotide **1**, avidin–HRP conjugate, dNTPs, (all from Sigma), were used with no further purification. Magnetic particles (Fe_3O_4, saturated magnetization ca. 65 emu g^{-1}, average diameter 1 μm) were functionalized with aminonaphthoquinone according to the published procedure.[20]

Preparation of DNA-functionalized magnetic particles: 30 mg of the amino-functionalized magnetic particles (MPG® Long Chain Alkylamine, CPG Inc.) were activated by reaction with the heterobifunctional crosslinker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (10 mg, Sigma) in DMSO (1 mL). After incubation at room temperature for 4 h, the particles were collected with an external magnet and thoroughly washed with DMSO and water. The maleimido-activated particles were then mixed with 20–30 O.D. of thiolated oligonucleotide in phosphate buffer (0.1 M, pH 7.4) for 8 h. (The thiolated nucleotide was freshly reduced with DTT and separated on a Sephadex G-25 column prior to the reaction with functionalized particles). Finally, the magnetic particles were washed with water and phosphate buffer (0.1 M, pH 7.4). The DNA-modified particles can be stored in phosphate buffer containing sodium azide (1% *w/v*) at 4°C, for longer than one week. The oligonucleotide content on the magnetic particles, before and after enzymatic DNase treatment (10 units DNase, 30 min at 37°C) was measured with OliGreen® reagent (ssDNA Quantitation Assay Kit, Molecular Probes, Inc.).

The OliGreen stock solution was diluted 200-fold with TE buffer solution (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). Stock solutions con-

taining thiolated primer **1** in TE buffer at 200, 100, 50, 20, and 10 ng mL^{-1} were prepared. A mixture consisting of 1 mL of each of the primer solutions and OliGreen (1 mL) stock solution were prepared, and the samples incubated in the dark for 5 min. The fluorescence of each sample was measured at $\lambda_{em} = 520$ ($\lambda_{excitation} =$ 480 nm). Background fluorescence originating from the TE buffer was similarly detected and subtracted from the fluorescence intensity values of the DNA-containing samples. The fluorescence values were used to obtain a calibration curve. The **1**-modified magnetic beads (1.5 mg), before and after treatment with DNase, were introduced into TE buffer (1 mL) and to each of the analyzed samples OliGreen analyzing solution was added (1 mL). After incubation for 5 min in the dark, fluorescence from two different magnetic bead samples was measured. By subtracting the background fluorescence obtained from magnetic beads that lack the nucleic acid primer, the net fluorescence corresponding to the DNA associated with the particles before and after treatment with DNase was determined. By using these fluorescence values and applying the derived calibration curve, the loading of the magnetic particles with DNA before and after treatment with DNase was derived.

Telomerazation on magnetic particles: The telomerization reaction was performed by using **1**-modified magnetic beads (1.5 mg), in the presence of dGTP, dATP, Biotin-21-dUTP (0.2 mM each), and telomerase solution (20 mM Tris-HCl buffer, pH 8.3, 1.5 mM $MgCl_2$, 0.63 mM KCl, 0.05% Tween 20, 1 mM EGTA) at 30 °C for 1 h. (Telomerase solution consisted of telomerase originating from the specified number of cancer cell lines).

Cells and tissues: HeLa and 293-kidney cancer cell lines, and NHF cells, were stored as pellets at −80 °C until extraction. Lung tissue samples were obtained from patients with lung carcinoma. The diagnosis of each tissue sample was histologically determined by a pathologist. All surgical specimens were kept at −80 °C until extraction. Cell pellets and tissue samples were lysed by using the CHAPS Lysis buffer (Intergren Co.).

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). The Au working electrode was modified with a cystamine monolayer. An auxiliary Pt electrode and a quasireference Ag electrode were made from 0.5 mm diameter wires and added to the cell. The quasireference electrode was calibrated against saturated calomel electrode (SCE); 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 optics, enabled the detection of emitted light intensities upon the application of the appropriate potential to the modified working electrode. Electrochemical measurements were performed by using a potentiostat (EG&G, model 283) connected to a computer (EG&G Software 270/250 for). All measurements were performed in phosphate buffer solution (0.01 M, pH 7.0) at room temperature. 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-disk electrode system (EG&G, model 638) was used to rotate the particles.

The human samples used in this study were provided by the University Medical Center, with the patients' understanding that the samples may be used for research under anonymous conditions.

Acknowledgements

This research is supported by the Max Planck Award for International Cooperation.

Keywords: biosensors · cancer · DNA · enzymes · telomerase

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Received: November 14, 2003

