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Label-Free Detection of Telomerase Activity Using Guanine Electrochemical Oxidation Signal

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Telomerase is an important biomarker for cancer cells and its activation in 85% of all cancer types confers a clinical diagnostic value. A label-free electrochemical assay based on guanine oxidation signal to measure telomerase activity is described. This developed technology combined with a disposable sensor, carbon graphite electrode (CGE), and differential pulse voltammetry (DPV) was performed by using PCR amplicons with/without telomeric repeats as the guanine oxidation signal observed at +1.0 V measured after the immobilization of PCR products. Guanine oxidation signal was chosen as a measure of telomerase activity because a substantial increase in the number of guanines was introduced by the action of telomerase which adds hexameric repeats $(TTAGGG)_n$ that contain 50% guanine. The developed assay was shown to specifically measure telomerase activity from cell extracts, and elongation rates increased linearly in a concentration dependent manner. Telomerase activity could be detected in cell extracts containing as low as 100 ng/μL of protein. All of the electrochemical measurements were also confirmed with the conventional TRAP-silver staining assay. Rapidity, simplicity, and the label-free nature of the developed assay make it suitable for practical use in quantitative determination of telomerase activity from clinical samples for diagnosis of cancer.

Telomeres are tandem repeats of DNA that are located at the termini of chromosomes to protect them from fusion and degradation.[1] In somatic cells, chromosomes lose 50−200 nucleotides from telomeric sequences[2] due to the inability of DNA polymerase to replicate the ends of linear DNA.[3] This progressive shortening of telomeres with each cell division leads to cellular senescence in somatic cells.[1] However, germ cells and immortal cells have mechanisms to preserve their telomeric length. The most widely used mechanism is the activation of a unique reverse transcriptase called telomerase. Telomerase is a ribonucleoprotein complex that adds hexameric TTAGGG repeats to the ends of chromosomes.[4] Telomerase activity has been evaluated for its diagnostic and prognostic value[5,6] since it is observed in most malignancies but not in most normal somatic tissues.[7]

A variety of techniques have been developed to analyze telomerase activity.[7−15] Initial assays used an oligonucleotide of telomeric sequence as a substrate for elongation by telomerase in cell extract and radioactive dNTP precursor(s).[8] However, these assays required high levels of tissue samples and radioactive precursors. Substantial increase in sensitivity was achieved by the introduction of polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP).[7] Several quantitative assays have been developed with the TRAP assay as a foundation.[9−11] However, they required use of harmful radioactive or expensive florescent substances. As a result they cannot be followed routinely in most laboratories. There are a number of reports overcoming those drawbacks by incorporating silver staining techniques with TRAP,[12−15] but they have the disadvantage of being quite time-consuming and laborious. Recently, alternative approaches have been developed to measure telomerase activity using optical,[16,17] surface plasmon resonance,[18] electrochemical, and quartz crystal microbalance[19] biosensors.

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(1) Greider, C. W. *Annu. Rev. Biochem.* **1996**, *65*, 337−365.
(2) Vaziri, H.; Schachter, F.; Uchida, I.; Wei, L.; Zhu, X.; Effros, R.; Cohen, D.; Harley, C. B. *Am. J. Hum. Genet.* **1993**, *52*, 661−662.
(3) Watson, J. D. *Nature New Biol.* **1972**, *239*, 197−201.
(4) Cech, T. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 34−43.
(5) Bonatz, G.; Klapper, W.; Barthe, A.; Heidorn, K.; Jonat, W.; Krupp, G.; Parwaresch, R. *Biochem. Biophys. Res. Commun.* **1998**, *253*, 214−221.
(6) Kubota, Y.; Nakada, T.; Sasagawa, I.; Yanai, H.; Itoh, K. *Cancer* **1998**, *82*, 176−179.
(7) Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. *Science* **1994**, *266*, 2011−2015.
(8) Counter, C. M.; Avilion, A. A.; LeFeuvre, C. E.; Stewart, N. G.; Greider, C. W.; Harley, C. B.; Bacchetti, S. *EMBO J.* **1992**, *11*, 1921−1929.
(9) Gelmini, S.; Caldini, A.; Becherini, L.; Capaccioli, S.; Pazzagli, M.; Orlando, C. *Clin. Chem.* **1998**, *44*, 2133−2138.
(10) Hou, M.; Xu, D.; Björkholm, M.; Gruber, A. *Clin. Chem.* **2001**, *47*, 519−524.
(11) Tatematsu, K.; Nakayama, J.; Danbara, M.; Shionoya, S.; Sato, H.; Omine, M.; Ishikawa, F. *Oncogene* **1996**, *13*, 2265−2274.
(12) Nemos, C.; Remy-Martin, J. P.; Adami, P.; Arbez-Gindre, F.; Schaal, J. P.; Jouvenot, M.; Delage-Mourroux. R. *Clin. Biochem.* **2003**, *36*, 621−628.
(13) Dalla Torre, C. A.; Maciel, R. M.; Pinheiro, N. A.; Andrade, J. A.; De Toledo, S. R.; Villa, L. L.; Cerutti, J. M. *Braz. J. Med. Biol. Res.* **2002**, *35*, 65−68.
(14) Wen, J. M.; Sun, L. B.; Zhang, M.; Zheng, M. H. *Mol. Pathol.* **1998**, *51*, 110−112.
(15) Eskiocak, U.; Iseri, O. D.; Kars, M. D.; Gunduz, U. *Chemotherapy,*submitted for publication.
(16) Schmidt, P. M.; Lehmann, C.; Matthes, E.; Bier, F. F. *Biosens. Bioelectron.* **2002**, *17*, 1081−1087.
(17) Schmidt, P. M.; Matthes, E.; Scheller, F. W.; Bienert, M.; Lehmann, C.; Ehrlich, A.; Bier, F. F. *Biol. Chem.* **2002**, *383*, 1659−1666.
(18) Maesawa, C.; Inaba, T.; Sato, H.; Lijima, S.; Ishida, K.; Terashima, M.; Sato, R.; Suzuki, M.; Yashima, A.; Ogasawara, S.; Oikawa, H.; Sato, N.; Saito, K.; Masuda, T. *Nucleic Acids Res.* **2003**, *31*, E4.

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After the discovery of nucleic acids electroactivity at the end of the 1950s by Palecek,[20] there has been much progress in the development of label-free electrochemical detection of DNA.[21−22]

Guanine as a purine base is the easiest oxidized base in DNA, and its irreversible peak was found at 1.00 V vs Ag/AgCl reference electrode. This peak height is dependent on the nucleic acid concentration and the guanine base composition in DNA material. The oxidation peak of guanine is reproducible and much higher than the pyrimidines.[23]

In recent years, carbon-based solid electrodes are in use to be electrochemical transducers and guanine or adenine oxidation signals can be measured at carbon surfaces.[24−28] Electrochemical DNA biosensors provide a rapid and inexpensive analysis method for the detection of molecular DNA.[29−32]

In this study, we have developed a fast and direct electrochemical assay based on guanine oxidation signal to measure telomerase activity. This sensitive assay is based on the label-free electrochemical detection of the telomeric repeats contained DNA by using differential pulse voltammetry (DPV) and a disposable carbon graphite electrode (CGE) as an electrochemical transducer. Half the amount of telomeric repeats are guanine bases; therefore, our cheap and simple detection protocol based on guanine response has an important advantage. There have not yet been any literature reports about the electrochemical detection of telomeric repeats from PCR-amplified products by using the guanine signal without any external labels. The features of the protocol are discussed here.

EXPERIMENTAL SECTION

Telomerase Assay. Activity of telomerase was determined with an improved version of TRAP[7] using TRAPeze telomerase detection kit (Chemicon, MA). Briefly, telomerase positive cell extract (included in the kit) was incubated in TRAP reaction mixture at 30 °C for 30 min for elongation of the substrate nucleotide (TS: 5′-AATCCGTCGAGCAG AGTT-3′) with TTAGGG repeats by telomerase. Then the reaction mixture was subjected to 33 cycles of PCR amplification (94 °C for 30 s, 59 °C for 30 s, and 72 °C for 60 s). Primers to generate 36 bp internal control (IC) of PCR amplification were also included in the reaction mixture. Each assay included a primer-dimer negative control (lysis buffer instead of protein extract) and a heat inactivated control (extracted protein incubated at 90 °C for 10 min).

Silver-Stain Detection. PCR products (25 μL) were electrophoresed in 0.5X tris−borate−EDTA buffer on 10% nondenaturating polyacrylamide gel at 300 V for 2 h. Characteristic ladder of TRAP products with 6 bp increments starting from 50 bp and the 36 bp internal control was revealed by silver staining.[15] Densitometric analysis of the gel was performed by ImageJ software (National Institutes of Health, Maryland)[33] and relative telomerase activity (RTA) was calculated according to following formula:

$$\mathrm{RTA} = \frac{(X_{\mathrm{TP}}/X_{\mathrm{IC}})}{(\mathrm{Tel}_{\mathrm{TP}}/\mathrm{Tel}_{\mathrm{IC}})}$$

where densitometric band intensities were designated by X and Tel for the samples and telomerase positive control, respectively. Values for TP (telomerase products) were obtained from the area containing all the telomerase elongated bands whereas values for IC was obtained only from the densitometric signal of the 36 bp band.

The similar formula can be obtained for electrochemical systems. i_{TP} is the voltammetric guanine signal for TP, and i_{IC} is the voltammetric guanine signal for internal control/negative control. For instance, in Figure 4B, five telomeric active samples RTA vs protein concentration of extracts can be seen. In this figure, sample 1 can be used as a control and at the same time as a sample, in this case, RTA is equal to 1. The other RTA values of samples can be calculated accordingly. $\mathrm{Tel}_{\mathrm{TP}}$ and $\mathrm{Tel}_{\mathrm{IC}}$ are the telomerase activities (concentration of the proteins) for sample and control, respectively.

The electrochemical RTA value $\mathrm{RTA}_{\mathrm{EC}}$ is equal to

$$\mathrm{RTA}_{\mathrm{EC}} = \frac{i_{\mathrm{TP}}/i_{\mathrm{IC}}}{\mathrm{Tel}_{\mathrm{TP}}/\mathrm{Tel}_{\mathrm{IC}}}$$

Electrochemical Detection. *Electrochemical Assay.* An AUTOLAB-PGSTAT 30 electrochemical analysis system, GPES 4.8 software package (Eco Chemie, The Netherlands), and DPV analysis method were used for the investigation of the oxidation signals of guanine. The raw data were also treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by the moving average baseline correction with a "peak width" of 0.01.[34,35]

The three-electrode system consisted of the carbon graphite working electrode, an Ag/AgCl reference electrode (model RE-1, BAS, W. Lafayette, USA), and a platinum wire as the auxiliary electrode.

The interconvertible carbon graphite electrode that was described in the study of Ozsoz et al.[36] was used in voltammetric measurements for the electrochemical detection of telomeric repeats contained PCR products. A Noki pencil model 2000 (Japan) was used as a holder for the graphite lead (Tombo, Japan).

(19) Pavlov, V.; Willner, I.; Dishon, A.; Kotler, M. *Biosens. Bioelectron.* **2004**, *20*, 1011−1021.
(20) Paleček, E. *Talanta* **2002**, *56*, 809−819.
(21) Paleček, E.; Fojta, M. Electrochemical DNA Sensors. In *Bioelectronics: From Theory to Applications*; Willner, I., Katz, E.,Eds.; Wiley-VCH Verlag GmbH and Co.: Weinheim, Germany, 2005, pp 127−192.
(22) Stempkowska, I.; Ligaj, M.; Jasnowska, J.; Langer, J.; Filipiak, M.; *Bioelectrochemistry* **2007**, *70*, 488−494.
(23) Steenken, S.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1997**, *119*, 617−618.
(24) Oliveira-Brett, A. M.; Diculescu, V.; Piedade, J. A. P. *Bioelectrochemistry* **2002**, *55*, 61−62.
(25) Wang, H. S.; Ju, H. X.; Chen, H. Y. *Anal. Chim. Acta* **2002**, *461*, 243−250.
(26) Zen, J. M.; Chang, M. R.; Ilangovan, G. *Analyst* **1999**, *124*, 679−684.
(27) Ferapontova, E. E.; Dominguez, E. *Electroanalysis* **2003**, *15*, 629−634.
(28) Drummond, T. G.; Hill, M. G.; Barton, J. K. *Nat. Biotechnol.* **2003**, *21*, 1192−1199
(29) Gooding, J. J. *Electroanalysis* **2002**, *14*, 1149−1156.
(30) Labuda, J.; Buckova, M.; Heilerova, L.; Silhar, S.; Stepanek, I. *Anal. Bioanal. Chem.* **2003**, *376*, 168−173.
(31) Lucarelli, F.; Marrazza, G.; Turner, A. P. F.; Mascini, M. *Biosens. Bioelectron.* **2004**, *19*, 515−530.
(32) Wang, J. *Anal. Chim. Acta* **2002**, *469*, 63−71.
(33) Abramoff, M. D.; Magelhaes, P. J.; Ram, S. J. *Biophotonics Int.* **2004**, *11*, 36−42.
(34) Ozkan, D.; Erdem, A.; Kara, P.; Kerman, K.; Meric, B.; Hassmann, J.; Ozsoz, M. *Anal. Chem.* **2002**, *74*, 5931−5936.
(35) Palecek, E.; Kizek, R.; Havran, L.; Billova S.; Fojta, M. *Anal. Chim. Acta* **2002**, *496*, 73−83.
(36) Erdem, A.; Kara, P.; Kerman, K.; Ozkan, D.; Ozsoz, M. *Electroanalysis* **2003**, *15*, 613−619.

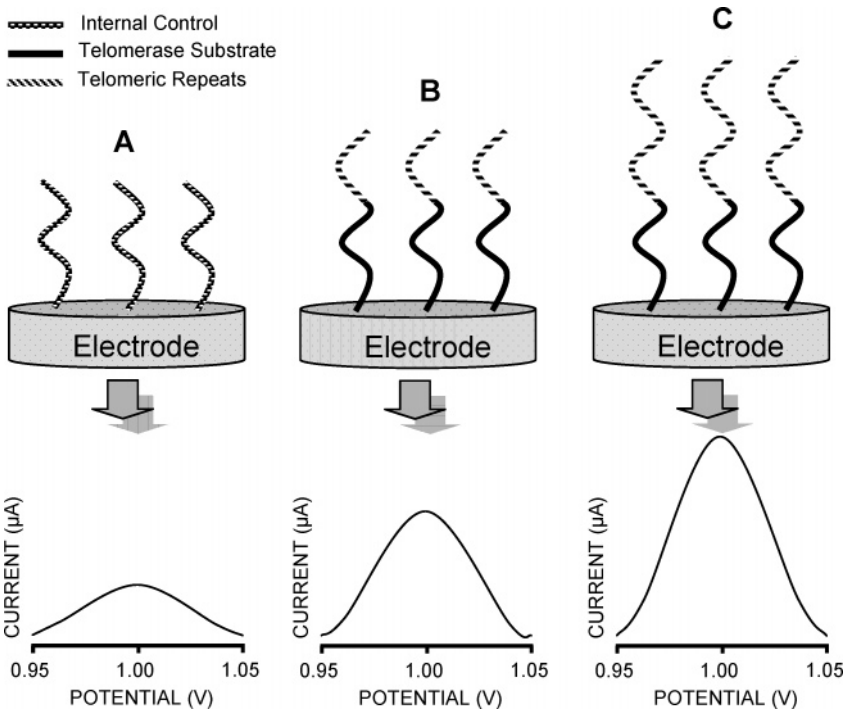

Figure 1. Schematic illustration of guanine oxidation signals of PCR products from (A) primer-dimer and heat inactivated negative controls, telomerase positive cell extracts with (B) moderate, and (C) strong telomerase activity.

Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was held vertically with 14 mm of the lead extruded outside (8 mm of which was immersed in the sample or buffer solution).

Characteristics of the PCR Samples. Only 36 bp IC products were present in the primer-dimer and heat inactivated negative controls. The base sequence of IC was 5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′. The base sequence of telomerase elongated products were 5′-AATCCGTCGAGCAGAGTT-$(AGGGTT)_n$-3′ where n ranges between 3 and 14 depending on telomerase activity. The characteristics of each sample used for the electrochemical assay was also confirmed by silver staining.

More dilute solutions of PCR amlicons were prepared using 20mmol/L tris−HCl buffer solution containing 20 mmol/L NaCl (pH 7.00, TBS).

Procedure for Voltammetric Assay. The detection of PCR amplicons was transduced by means of DPV. The height of the guanine oxidation peak after baseline fitting was used as the analytical signal. The rapid detection procedure of the PCR amplicons consisted of the following steps.

Pretreatment of the Carbon Graphite Electrode (CGE). The CGE was pretreated by applying +1.40 V for 30 s in blank 0.50 mol/L acetate buffer solution (ABS) (pH, 4.8) containing 20 mmol/L NaCl.

PCR Amplicon Immobilization. The amplicons that have different characteristics were diluted with TBS, in the ratio of 1:100, then placed in a vial and the graphite leads were dipped into 100 μL of sample contained in vials for 40 min in order to immobilize amplicon onto the carbon lead surfaces by the wet adsorption method.[37,38] The same immobilization time provided the same stability of the signal obtained from sensor surface. The electrode was then rinsed with TBS for 5 s.

Voltammetric Transduction. After transfer of amplicon-modified CGE into the blank ABS solution, the oxidation signal of guanine was measured by using differential pulse voltammetry by scanning from +0.80 to +1.40 V. In each measurement, the length of the graphite lead was measured by a ruler and the volume of blank/DNA solution was optimized.

Control experiments were performed with a different amplicon that does not contain telomeric repeats.

RESULTS AND DISCUSSION

The telomeric repeats contain half the amount of guanine bases; hence, this important detail brings an advantage to our electrochemical assay to develop a label-free detection system as measuring guanine oxidation signal instead of using any indicator previously used in the literature for DNA analysis.[39−42]

A 1 μL aliquot of the amplified amplicon is simply diluted in the tris−HCl buffer solution and then interacted with the pretreated electrode in vials. Telomeric repeats are determined with the changes of the guanine oxidation signal at about +1.00 V.[32] DPV provides lower detection limits than the ones obtained by using potentiometric stripping analysis and square wave voltammetry.[43]

Figure 1 illustrates the different kind of PCR products on the electrode surface and their expected guanine oxidation signals. Both primer-dimer and heat inactivated negative controls contains only the 36 bp IC products and generate lower signals because of low guanine content in IC (Figure 1A). In some cases the heat

(37) Erdem, A.; Pividori, M. I.; Del Valle, M.; Alegret, S. *J. Electroanal. Chem.* **2004**, *567*, 29−37.
(38) Ozkan, D.; Erdem, A.; Kara, P.; Kerman, K.; Meric, B.; Hassmann, J.; Ozsoz, M. *Anal. Chem.* **2002**, *74*, 5931−5936.
(39) Erdem, A.; Kerman, K.; Meric, B.; Akarca, U. S.; Ozsoz, M. *Anal. Chim. Acta* **2000**, *422*, 139−149.
(40) Millan, K. M.; Mikkelsen, S. R. *Anal. Chem.* **1993**, *65*, 2317−2323.
(41) Erdem, A.; Kerman, K.; Meric, B.; Ozkan, D.; Kara, P.; Ozsoz, M. *Turk. J. Chem.* **2002**, *26*, 851−862.
(42) Kelley, S. O.; Barton, J. K. *Bioconjugate Chem.* **1997**, *8*, 31−37.
(43) Palecek, E.; Fojta, M. *Anal. Chem.* **1994**, *66*, 1566−1571.

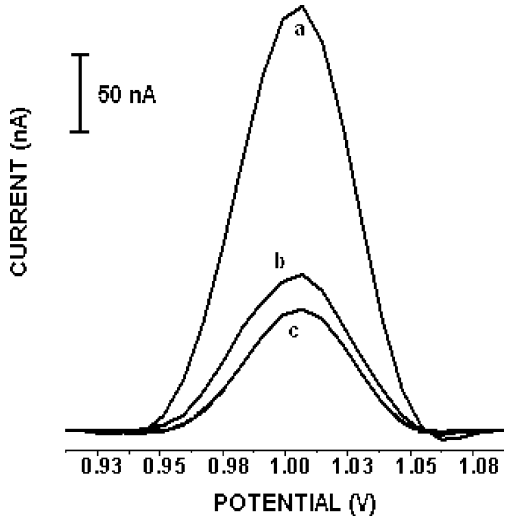

Figure 2. Voltammograms for the guanine oxidation signals of (c) primer-dimer negative control, (b) heat inactivated control, and (a) telomerase positive cell extracts.

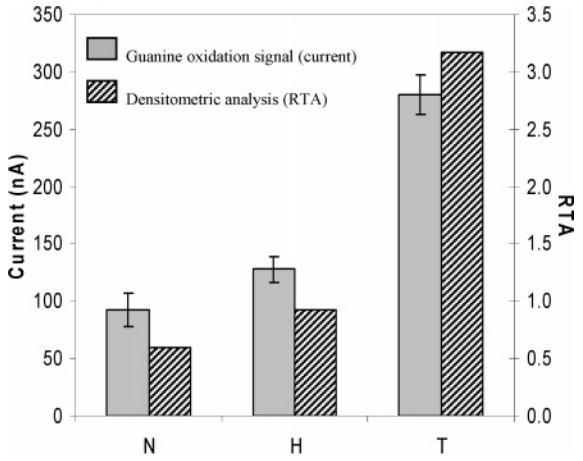

Figure 3. Histogram of (N) primer-dimer negative control, (H) heat inactivated control, and (T) telomerase positive cell extract detected by guanine oxidation signals and densitometric analysis of silver-stained gels. Mean current values and standard deviations were derived from three measurements. RTA values were calculated as described in the Experimental Section.

inactivated negative control gave slightly higher current signals than the primer-dimer negative control which might be due to inefficient inactivation.

It would be ideal to be able to use guanine free telomerase substrates and internal controls. This kind of approach would potentially decrease the detection limit since guanine oxidation signal would solely come from telomerase activity and not from other sources. However, telomerase activity from positive samples gave negative results when inosine substituted TS oligonucletides were used (data not shown). This might suggest that guanines in TS may play a critical role in telomerase elongation.

Figure 2 shows the differential pulse voltammogram for the oxidation signal of guanine obtained from telomeric repeats contained PCR (a), heat inactivated control (b), and primer-dimer negative control (c) immobilized on the CGE surface. The appearance of the highest guanine signal indicated the strong activity of telomeric repeats. An obvious decrease in the guanine peak was observed at the other PCR products-modified CGE

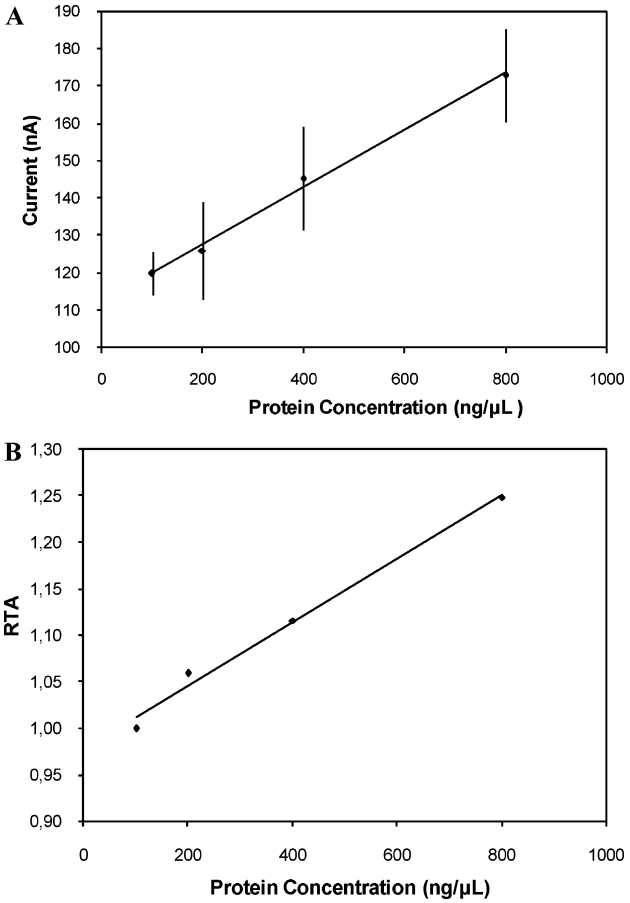

Figure 4. Detection of telomerase activity by (A) guanine oxidation signals or (B) densitometric analysis of silver-stained gels with increasing concentrations of the telomerase positive cell extract. Mean current values and standard deviations were derived from three measurements. RTA values were calculated as described in the Experimental Section.

surface (Figure 2b,c). A voltammetric signal is expected in all kinds of PCR products because of their guanine base contents.

Figure 3 shows the voltammetric (gray columns) and densitometric (lineated columns) analysis responses obtained from various PCR products as (N) primer-dimer negative control, (H) heat inactivated control, and (T) telomerase positive cell extract.

The DPV signals of guanine at the CGE were measured with each product of PCR; they are shown with error bars in Figure 3 (gray columns). Three repetitive measurements for voltammetric detection of telomeric repeats contained PCR products gave reproducible results as a mean guanine signal of 280 nA and a relative standard deviation (RSD) value of 6.2% ($n = 3$) by using a disposable sensor, CGE in 40 min immobilization time. A series of three subsequent measurements of guanine signal gave also reproducible results as a mean average value of 107 nA with a RSD of 16.0% for primer-dimer negative control (N) and an RSD of 8.4% for heat inactivated control (H). The significant correlation between guanine signals and RTA (Figure 3, lineated columns; $r = 0.989$) validates the use of guanine signals as a measure of telomerase activity.

Linearity and sensitivity of the electrochemical assay were comparable to that of the TRAP-silver staining assay. Telomerase activity could be detected in extracts containing as low as 100 ng/μL of protein, and protein concentration correlated significantly

with guanine oxidation signal ($r = 0.997$) as well as RTA ($r = 0.995$) in the 100−800 ng/μL range (Figure 4). Concentrations above 800 ng/μL resulted in a decrease in guanine signal as well as RTA levels (data not shown). This may be accounted for by an increased concentration of PCR inhibitors present in tumor cells.[10]

CONCLUSIONS

In this study we report the first label-free electrochemical assay for telomerase activity which is a rapid, user-friendly, inexpensive, and nonradioactive assay based on guanine oxidation signals. These features of the disposable assay make it a promising candidate for lab-on-a-chip technology.

In contrast to other detection techniques reported earlier in the literature, PCR products were simply immobilized on the carbon sensor surface in this study. We have exhibited that direct measurement of the samples can be realized using PCR-immobilized carbon lead and it effectively follows the guanine oxidation signal. It has also been demonstrated that PCR products can be detected sensitively based on the amount of guanine bases, with this less time-consuming, and cheaper electrochemical technique in comparison to other detection methodologies such as surface plasmon resonance (SPR) or quartz crystal microbalance (QCM) reported in the related literature. These optical and piezoelectrical biosensors are generally developed using expensive thiol-functionalized DNA sequences and gold sensor chip, and their long detection scheme contains self-assembled monolayers (SAMs) of nucleic acids adsorbed on the gold surface.[44]

The easy wet-adsorption immobilization method[37,38] has been demonstrated to be useful for monitoring the telomeric repeats on the CGE surface and label-free electrochemical detection eliminates the needs for external markers and long experimental procedures, and hence, results in a greatly simplified protocol. However, our assay still has the intrinsic limitations of the PCR based TRAP assay. To be used in routine analysis it is essential to develop an assay which does not depend on error prone PCR. Further studies are being done in our laboratory to eliminate PCR with a similar approach.

(44) Vikholm-Lundin, I.; Piskonen, R.; Albers, W. M. *Biosens. Bioelectron.* **2007**, 22 1323−1329.

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