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Short communication

Sensitive chemiluminescence detection of wild-type p53 protein captured by surface-confined consensus DNA duplexes

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ABSTRACT

A novel chemiluminescence (CL) biosensor for sensitive detection of wild-type p53 protein has been proposed. The wild-type p53 protein in solution was captured by highly specific consensus double-stranded (ds) oligonucleotides (ODNs) preimmobilized onto a gold plate. The cysteine residues on the exterior of the wild-type p53 molecules were then derivatized with *N*-biotinoyl-*N*′-(6-maleimidohexanoyl) hydrazide (biotin-Mi) for the attachment of streptavidin-horseradish peroxidase (SA-HRP) complex. The attached HRP molecules could catalyze the CL reaction between luminol and H_2O_2, producing an enhanced CL signal. The CL intensity was dependent on the surface coverage of the HRP molecules, which was related to the concentration of wild-type p53 protein. Under the optimal experimental conditions, the CL intensity increased linearly with the concentration of wild-type p53 protein from 0.01 to 0.5 nM. The detection limit was estimated to be 3.8 pM. The proposed method has been successfully utilized for the assay of wild-type p53 protein in normal and cancer cell lysates. The sensing protocol is sensitive, cost-effective, and holds great promise for clinical diagnosis.

1. Introduction

As "the guardian of the genome", p53 gene responds to the genotoxic stress and is capable of protecting the genome through DNA repair, cell cycle arrest, and even apoptosis (Lane, 1992; Levine, 1997). Loss of p53 gene activity or mutation of p53 gene thus leads to increased susceptibility to cancers (Hamroun et al., 2006; Koshland Jr., 1993; Soussi and Beroud, 2001). Typically, p53 protein contains three functional domains: the transactivation domain, the central DNA-binding domain, and the oligomerization domain (Iwabuchi et al., 1993; Rose et al., 2003; Stavridi et al., 2005; Wang et al., 1994). The oligomer of wild-type p53 protein (usually the tetramer) is capable of binding to the consensus double-stranded (ds) DNA with two or more copies of the 10 base pair half-site bearing a sequence of PuPuPuC(A/T)(T/A)GPyPyPy (where Pu and Py denote purines and pyrimidines, respectively) (El-Deiry et al., 1992; Wang et al., 1995; Wu et al., 1993). The binding of wild-type p53 protein to the consensus ds-DNA is highly specific (El-Deiry et al., 1992; Kern et al., 1991) and the mutated form with mutations deactivating p53 protein in the central DNA-binding domain (El-Deiry et al., 1993; Ko and Prives, 1996; Levine, 1997) has lost its ability to bind the consensus ds-DNA.

Thus far, a variety of techniques have been proposed for p53 protein assay, including but not limited to electrophoretic mobility shift assay (Adachi et al., 2005; Thornborrow et al., 2003), enzyme-linked immunosorbant assay (ELISA) (Gould et al., 2004; Jagelská et al., 2002), electrochemistry (Potesil et al., 2006; Wang et al., 2008; Yeo et al., 2009), and surface plasmon resonance (SPR) (Wang et al., 2009). Among them, the electrophoretic mobility shift assay is simple but is only semi-quantitative, whereas ELISA is sensitive but involves the p53 antibody conjugated to an enzyme, which is expensive and unstable. As an alternative, the consensus ds-DNA was used to strongly bind to the wild-type p53 protein (Paleček et al., 1997). The binding constant between wild-type p53 protein and PAb246, an antibody known to be specific to wild-type p53 protein, was reported to be $3.7 \times 10^8\ M^{-1}$, being only slightly larger than that of $1.1 \times 10^8\ M^{-1}$ between wild-type p53 protein and consensus ds-DNA (Wang et al., 2009). Moreover, the ODN strand can be chemically synthesized, while the antibody is generally produced in organism. On the other hand, many antibodies are temperature-sensitive, while the ODN strand is thermally stable and can undergo reversible denaturation (Kirby et al., 2004). Thus, relying on the specific binding of wild-type p53 protein to consensus ds-DNA, amplified voltammetric detection of wild-type p53 protein in normal and cancer cell lysates has been carried out by our group (Wang et al., 2008). In a later work, we have reported the simultaneous SPR detection of wild-type and

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mutated p53 protein in various cell lysates using a dual-channel SPR instrument (Wang et al., 2009).

The chemiluminescence (CL) technique is becoming increasingly important in various fields due to its extremely high sensitivity, wide linear range, rapidness, and cost-effectiveness. It has been widely utilized for glucose, DNA, and protein detection and for clinical assay (Lan et al., 2008; Pavlov et al., 2004; Previte et al., 2006). Typically, CL protocols rely on the specific activity of the labels, such as enzymes linked to the DNA probes (Li and He, 2009; Liu et al., 2005; Niazov et al., 2004; Pavlov et al., 2004; Zhang et al., 2008). To the best of our knowledge, little has been reported on the CL assay of p53 protein. Combining the enzyme activity and the specific recognition of wild-type p53 protein by the consensus DNA duplexes should improve the sensitivity of CL assay for wild-type p53 protein.

In this study, sensitive CL detection of wild-type p53 protein has been reported based on the surface-confined HRP-catalyzed luminol/H_2O_2 system. The detection limit of wild-type p53 protein was estimated to be 3.8 pM. The proposed method has been successfully utilized for assay of wild-type p53 protein in various cell lysates.

2. Material and methods

2.1. Materials and apparatus

Ethanolamine hydrochloride (EA), 11-mercaptoundecanoic acid (MUA), *N*-biotinoyl-*N*′-(6-maleimidohexanoyl) hydrazide (biotin-Mi), streptavidin-horseradish peroxidase (SA-HRP) complex, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ethylenediaminetetraacetic acid disodium salt (EDTA), dithiothreitol (DTT), luminol, H_2O_2, NaOH, KH_2PO_4, and K_2HPO_4 were acquired from Sigma (St. Louis, MO). Bovine serum album (BSA), human thrombin, and human immunoglobulin G (IgG) were from Beijing Dingguo Changsheng Biotech. Co., Ltd. (Beijing, China). Oligonucleotides (ODNs) were all purchased from Sangon Co., Ltd. (Shanghai, China). The sequences of 25-mer ODN probe with its 5′ end modified with amino groups and its complementary target to form the consensus ds-ODN are 5′-H_2N-$(CH_2)_6$-TTT TTA GAC ATG CCC AGA CAT GCC C-3′ and 5′-GGG CAT GTC TGG GCA TGT CT-3′. For forming the nonconsensus ds-ODN, the sequences of 25-mer ODN probe and its complementary target are 5′-H_2N-$(CH_2)_6$-TTT TTG TCG GCC GAG GTC GGC CGA G-3′ and 5′-CTC GGC CGA CCT CGG CCG AC-3′. Recombinant p53 protein samples were purchased from BD Biosciences Pharmingen (San Diego, CA). The preparation of normal and cancer cell lysates (Xiangya Hospital, Hunan, China) was according to previously published procedures (Wang et al., 2008). Highly pure gold plate was purchased from Gaoss Union Technology Co., Ltd. (Wuhan, China). All other reagents were of analytical grade and used as received. Ultra-pure water was prepared with a Milli-Q water purification system (Simplicity 185, Millipore Corp., Billerica, MA). The CL signals were measured with an IFFM-E flow injection chemiluminescence analyzer (Xi'an Remax Electronic Science-Tech. Co. Ltd., Xi'an, China) in which an IFFS-A luminometer was involved. All components in the flow system were connected with polytetrafluoroethylene (PTFE) tubes (0.8 mm i.d.).

2.2. Solution preparation

MUA was prepared with absolute ethanol. Biotin-Mi was dissolved in a mixed solution of acetic acid and NaOH (pH 7.0). SA-HRP solution was prepared with phosphate-buffered saline (PBS buffer, pH = 7.0, 10 mM phosphate/0.1 M NaCl). ODN probe and target were prepared with TE (10 mM Tris–HCl/1.0 mM EDTA, pH 7.0) and TNE buffer (TE buffer+0.1 M NaCl), respectively. Samples of p53 protein were diluted with PBS buffer containing 20 mM DTT. EDC/NHS solution was prepared by mixing 0.4 M EDC with 0.1 M NHS in water. EA was dissolved in water. The stock solution of luminol (1.0×10^{-3} M) was prepared by dissolving an appropriate amount of luminol in 20 mL of 0.1 M NaOH, followed by dilution to 1 L with water. Various luminol solutions were prepared by diluting the stock solution with water. H_2O_2 solution was freshly prepared with deionized water.

2.3. Immobilization of the aminated ODN probe onto the gold plate

Prior to surface modification, the gold plates (30 mm × 3 mm × 0.1 mm) were pretreated with piranha solution (H_2SO_4:H_2O_2, 7:3, v/v) and then rinsed thoroughly with water. MUA self-assembled monolayers (SAMs) were formed by exposing one side of the gold plate to 40 μL of 10 mM MUA overnight. This step was followed by washing the plate with ethanol and water. The ODN probe was affixed onto the MUA-covered surface via incubating the plate in a mixed solution of 40 μL of EDC/NHS solution comprising 4.0 μM aminated ODN probe in a humid chamber for 2 h at room temperature. After the ODN probe attachment, the surface was rinsed with water to rid any nonspecifically adsorbed molecules and then dried under a stream of N_2. To block the unreacted sites, 40 μL of 0.1 M EA was cast onto the surface for 1 h.

2.4. ODN hybridization and subsequent capture of wild-type p53 protein

To form consensus ds-ODN, 20 μL of TNE buffer containing a predetermined concentration of target ODN was cast onto the capture probe-modified gold plate and the hybridization reaction was allowed to proceed for 2 h in a humid chamber at room temperature. The formation of nonconsensus ds-ODN is similar with that of consensus ds-ODN. After the surface was thoroughly rinsed with TNE buffer and water, 30 μL of PBS buffer comprising a given concentration of wild-type p53 protein was cast onto the gold plate for 1 h. Note that the concentration of wild-type p53 protein was based on the molecular weight of p53 monomer. After completion of the p53 protein capture step, the gold plate was rinsed with PBS buffer and water to remove loosely bound species.

2.5. Derivatization with biotin-Mi and subsequent attachment of SA-HRP complex

To attach SA-HRP complex, the wild-type p53 protein on the gold plate was first allowed to react with 30 μL of 1.0 mM biotin-Mi for 2 h at room temperature. Upon rinsing with a copious amount of water, 30 μL of 0.1 mg/mL SA-HRP was pipetted onto the biotin-Mi-covered plate and allowed to react for 1 h. After rinsing with PBS buffer and water, the gold plate was dried with N_2 and ready for use.

2.6. Flow injection CL assay

The gold plate with the assembled side directly facing the photo-multiplier tube (PMT) detector was located in a colorless glass tube which was pre-incorporated into the flow system. The mixed solution of luminol and H_2O_2 driven by pump 1 was first mixed with 0.05 M Tris–HCl buffer (pH 8.5) driven by pump 2 and then the final solution was delivered into the colorless glass tube where the CL reaction occurred. Catalyzed by the attached HRP molecules, the system of 4.0×10^{-4} M luminol and 5.0×10^{-3} M H_2O_2 produces an enhanced CL signal. The wild-type p53 protein concentration is determined based on the net CL intensity of

$\Delta I = I_0 - I$, where I_0 and I denote the CL intensity in the presence and absence of wild-type p53 protein, respectively.

3. Results and discussion

3.1. Principle of the CL assay for wild-type p53 protein

The schematic diagram for the CL assay of wild-type p53 protein was shown in Fig. 1. First, MUA was anchored onto the gold plate via the strong Au–S bond formation. This was followed by immobilization of 5′-aminated ODN capture probe onto the MUA SAMs through the widely used EDC/NHS chemistry (Huang et al., 2000). The gold plate was then treated with EA to block any remaining carboxyl groups of the SAMs (Susmel et al., 2003). Next, hybridization of the ODN probe with its complementary sequence was carried out. When exposing the resultant consensus ds-ODN-modified gold plate to wide-type p53 protein solution, the DNA/p53 complex was formed due to the high sequence specificity of the consensus ODN duplex toward wide-type p53 protein (El-Deiry et al., 1992; Kern et al., 1991). Subsequently, biotin-Mi was allowed to react with the DNA/p53 complex. Biotin-Mi was used for the derivatization of the cysteine residues on the wild-type p53 protein molecules through Michael-type electrophilic addition (Hare and Lee, 1989) and then for the SA-HRP complex attachment via the strong non-covalent binding between biotin and streptavidin (Samanta and Sarkar, 2011). The attached HRP molecules could catalyze the luminol–H_2O_2 system, producing strong CL signals. The enhanced CL was dependent on the quantity of HRP molecules, which was quantitatively related to the concentration of wide-type p53 protein.

3.2. Optimization of the experimental conditions

To achieve high performance for wild-type p53 protein detection, the following experimental parameters, for example, the concentrations of luminol, H_2O_2 and SA-HRP, pH value of the buffer solution, flow rate, and the mixing tube length have been optimized.

Concentrations of luminol and H_2O_2 have a significant influence on the CL assay. We thus examined ΔI at luminol and H_2O_2 concentrations in the range from 1.0×10^{-4} to 1.0×10^{-3} mol/L and from 1.0×10^{-3} to 1.0×10^{-2} mol/L, respectively. We found that ΔI reached to a maximum value at luminol concentration of 4.0×10^{-4} mol/L or H_2O_2 concentration of 5.0×10^{-3} mol/L. Thus, 4.0×10^{-4} mol/L luminol and 5.0×10^{-3} mol/L H_2O_2 were chosen for wild-type p53 protein determination.

The amount of the attached SA-HRP complex exerts great influence on the CL response of luminol (4.0×10^{-4} mol/L) and H_2O_2 (5.0×10^{-3} mol/L). ΔI, the difference between the CL intensity in the presence and absence of wild-type p53 protein (75.5 nM), increased with the concentration of SA-HRP ranging from 0.01 to 0.1 mg/mL. Beyond 0.1 mg/mL, the curve began to level off, suggesting the saturated surface coverage of p53 protein on the gold plate. Thus, we fixed SA-HRP complex concentration at 0.1 mg/mL.

The CL intensity is largely dependent on the pH value of the detection solution (Tris–HCl buffer). After examining ΔI under different detection solutions with pH ranging from 7.0 to 10, we set the pH value at pH 8.5 due to the highest ΔI obtained. Furthermore, the optimized flow rate and mixing tube length were determined to be 1.5 mL/min and 12 cm, respectively.

3.3. Analytical performance of the CL assay for wild-type p53 protein

The capture of wild-type p53 protein by the surface-confined consensus ds-ODN duplexes is sequence-specific. As shown in Fig. 2A, a well-defined CL peak with high signal intensity was observed for the consensus ds-ODN duplexes (curve d), whereas the nonconsensus ds-ODN- (curve b) and single stranded (ss)-ODN- (curve c) covered gold plates yielded relatively weak CL signals. Without the wild-type p53 protein capture step, the CL response at the consensus ds-ODN-modified surface (curve a) was similar with that in curve b or c. These results indicate that nonspecific interaction between p53 protein and ss-ODN or nonconsensus ds-ODN was relatively weak. Thus, we used $\Delta I = I_0 - I$, where I_0 and I denote the CL intensity in the presence and absence of wild-type p53 protein, respectively, for background subtraction in wild-type p53 protein assay. To further illustrate the sequence specificity of the consensus ODN duplexes toward wild-type p53 protein, other proteins, such as BSA, thrombin, and IgG were attempted (Fig. 2B). Though the concentration of these proteins was 100-fold higher than that of wild-type p53 protein, ΔI remained only a little larger than the blank (no protein), but it was much smaller than that in the case of wild-type p53 protein. The sequence specificity thus provides the basis for wild-type p53 protein quantification.

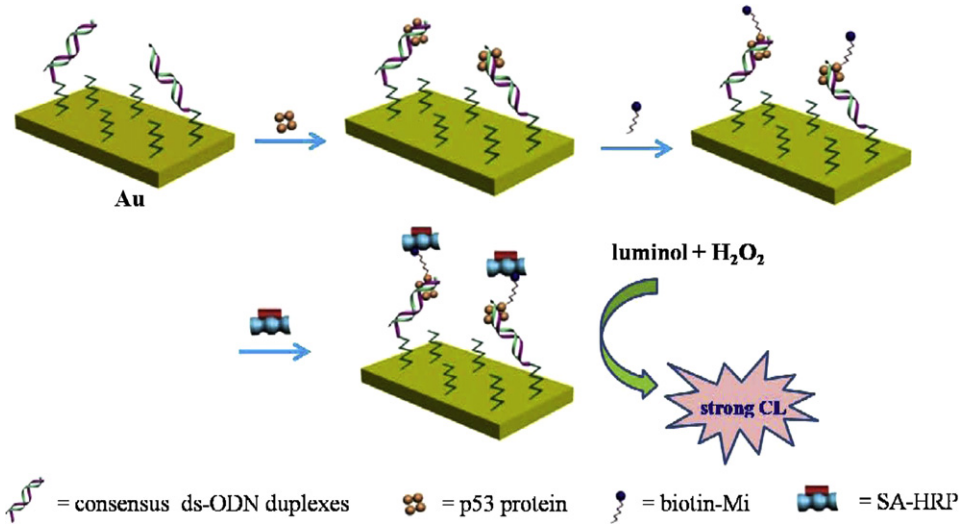

Fig. 1. Schematic representation of the chemiluminescence detection of wild-type p53 protein. The capture of wild-type p53 protein by the consensus ds-ODN duplexes was followed by the derivatization of the protein sulphydryl groups with biotin-Mi and the attachment of SA-HRP complex.

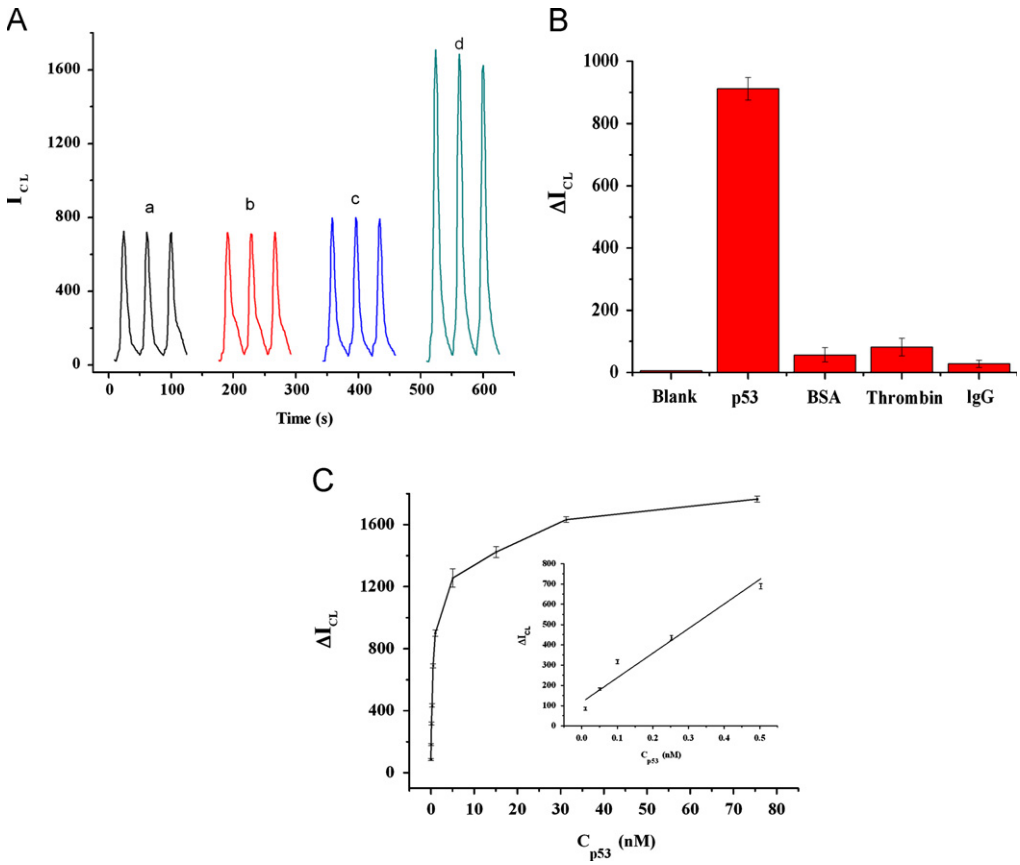

Fig. 2. (**A**) CL intensities of various ODN-modified gold plates. (a) Consensus ds-ODN without wild-type p53 protein, (b) nonconsensus ds-ODN, (c) ss-ODN, (d) consensus ds-ODN. The concentration of wild-type p53 protein used is 1.0 nM. The capture of wild-type p53 protein was followed by derivatization with biotin-Mi and attachment of SA-HRP. (**B**) CL assay specificity. The concentration of wild-type p53 protein is 1.0 nM, while that of BSA, thrombin, and IgG is 100 nM. (**C**) Dependence of ΔI on the concentration of wild-type p53 protein. The concentrations of wild-type p53 protein determined are 0.01, 0.05, 0.10, 0.25, 0.50, 1.00, 5.03, 15.1, 31.3, and 75.5 nM. The inset shows the linear portion of the curve with wild-type p53 protein concentrations ranging from 0.01 to 0.50 nM. Each point was averaged from at least three replicates and the standard deviations are shown as the error bars.

The CL intensity was dependent on the concentration of wild-type p53 protein. As shown in Fig. 2C, ΔI increased with wild-type p53 protein concentration from 0.01 to 5.03 nM and beyond 5.03 nM the curve began to level off. The plateau beyond 5.03 nM was resulted from the saturated binding sites of the surface-confined DNA duplexes for wild-type p53 protein. A linear relationship between 0.01 and 0.50 nM was obtained with a linear regression equation expressed as $\Delta I = 116.8 + 1210\ C_{p53}$ (nM) ($R^2 = 0.964$, the inset of Fig. 2C). The relative standard deviation (RSD) for 0.05 nM of p53 protein was 9.6% at the same gold plate ($n = 3$) and was 15.9% at six different gold plates. Such values are quite reasonable since several steps are involved in the procedure and several factors, such as DNA and p53 protein surface densities may affect the CL detection. The detection limit (3σ) of the method was estimated to be 3.8 pM. Compared with ELISA assay (Jagelská et al., 2002), the proposed method used highly specific consensus ds-ODN to capture the wild-type p53 protein rather than the more expensive and thermally unstable p53 antibody. In comparison with SPR instrument (Wang et al., 2009), the CL analyzer is much cheaper. Our method also obviated the need to synthesize the ferrocene-capped gold nanoparticle/streptavidin conjugates involved in the amplified electrochemical detection (Wang et al., 2008). The method described herein combines the specific recognition of wild-type p53 protein by the consensus DNA duplexes and the catalytic activity of the incorporated enzyme toward the luminol–H_2O_2 system, thus exhibiting higher sensitivity.

The gold plate could be used repeatedly several times, thereby providing the possibility of the method to replicate sample measurements. After determination, the gold plate was soaked in 0.1 M NaOH for 5 min, followed by rinsing the surface with 50% ethanol to desorb the target ODN, wild-type p53 protein, biotin-Mi and SA-HRP complex. The regenerated surface was then re-hybridized with target ODN in TNE buffer, followed by the attachment of wild-type p53 protein, biotin-Mi and SA-HRP complex. The three regeneration/assay cycles yielded a RSD of 11.8% at 0.50 nM wild-type p53 protein.

Table 1
CL assay for wild-type p53 in normal and cancer cell lysates.

Cell lysates	Wild-type p53 (nM)[a]
Normal embryonic kidney cells (HEK-293T)	1.95 ± 0.038
Glioma cells (U251)	0.33 ± 0.018
Astrocytic glioma cell (SF767)	0.20 ± 0.011

[a] Mean $\pm$ standard deviation ($n = 3$).

Finally, the feasibility of the method for analysis of wild-type p53 protein in one normal and two cancer cell lysates has been demonstrated. Prior to detection, the lysates were diluted to the appropriate concentration. As summarized in Table 1, U251 and SF767 (cancer cell lysates) both exhibited lower levels of wild-type p53 protein than HEK-293T (normal cell lysate), indicating that the p53 gene had been mutated significantly in cancer cells. The above results are consistent with our previous reports by electrochemistry and SPR (Wang et al., 2008, 2009). Thus, the proposed method is capable of discriminating between normal

and cancer cells and quantifying wild-type p53 protein in a sensitive manner.

4. Conclusions

A novel and sensitive CL assay for wild-type p53 protein has been demonstrated. The enhanced CL intensity was dependent on the quantity of the attached HRP molecules, which was quantitatively related to the concentration of wild-type p53 protein captured by the consensus ds-ODN-covered gold plate. A detection limit of 3.8 pM was achieved, being comparable with that by the amplified voltammetric detection of wild-type p53 protein using ferrocene-capped gold nanoparticle/streptavidin conjugates and a little lower than that by adsorptive stripping voltammetry and surface plasmon resonance detection using consensus ODN duplexes specific to wild-type p53 protein. The sensing protocol has successfully been applied to analysis of wild-type p53 protein in normal and cancer cell lysates. The method described herein is sensitive and does not involve the p53 antibody and extensive sample pretreatment. The CL assay of wild-type p53 protein may find potential applications in clinical diagnosis.

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