Capture of p53 by Electrodes Modified with Consensus DNA Duplexes and Amplified Voltammetric Detection Using Ferrocene-Capped Gold Nanoparticle/Streptavidin Conjugates

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p53, a tumor suppressor protein and a transcription factor, is capable of inhibiting the growth of tumor cells by eliciting either cell-cycle arrest or apoptosis through a cascade of events. p53 binds sites within the promoters of several genes that conform to a sequence commonly defined as the consensus site. In more than 50% of cancer cases, the p53 gene has been found to be mutated and the p53 protein loses its ability to bind the consensus DNA. In this work, double-stranded (ds-) oligonucleotides (ODNs) containing the consensus site are immobilized onto gold electrodes to capture wild-type p53. The cysteine residues on the exterior of the p53 molecule were derivatized for the attachment of gold nanoparticle/streptavidin conjugates capped with multiple ferrocene carboxylic acid residues. Well-defined voltammetric peaks of high signal intensity were obtained, and p53 concentration as low as 2.2 pM was measured. The peak heights were found to be dependent on the surface density of the consensus ds-ODN, the sequence of the immobilized ODNs, and the p53 concentration. The sensitivity and amenability for real sample analysis of the method compared well with enzyme-linked immunosorbent assay (ELISA), and complements ELISA in that wild-type p53, instead of total p53 (wild-type and mutant p53) concentration, is measured. The method described herein is simple and selective and does not require the use of p53 antibodies.

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DNA. Thus, a critical measure of p53 function is the affinity of p53 for consensus DNA. It would be useful to determine the number of wild-type p53 proteins in both normal and cancer cells that are capable of binding consensus-site DNA. Such data may be used to create treatment modalities that capitalize on the presence of the wild-type p53 and the concentration variation.

The two common methods of measuring endogenous wild-type p53 are the electrophoretic mobility shift assay and p53 antibody-based enzyme-linked immunosorbant assay (ELISA). The former is simple but is semiquantitative at best, whereas the latter is sensitive but requires the use of p53 antibodies and measures only the total p53 (wild-type and mutant combined). Jagelská et al. developed a new ELISA technique for the analysis of p53/DNA binding. The strong binding between the wild-type p53 and negatively charged supercoiled DNA was studied via electrophoretic retardation of supercoiled DNA in agarose gels and scanning force microscopy. Several studies also detected p53 and its interaction with DNA in solution.

To our knowledge, utilizing the highly specific and strong DNA-binding property of p53 for detecting p53 concentration in a buffer solution and cell lysates has not been carried out at the solid/solution interface without the aid of p53 antibodies. In addition, the effects of surface parameters (e.g., surface density, sequence, and strand length) of immobilized DNA molecules on the extent of p53 binding have not been investigated. For clinical applications, it is highly desirable if a simple and durable method can be employed to measure p53 in cell lysates at a highly sensitive level.

Voltammetric techniques are simple, sensitive, and inexpensive to implement. Although cysteine residues on the p53 surface can be oxidized, thiols generally produce complex or ill-defined voltammetric peak(s). As a result, voltammetric detection of p53 binding to surface-confined DNA has not yet been achieved by direct measurement of cysteine thiol redox activity. Conceivably, the introduction of integrated nanoparticle/biomolecule conjugate systems should allow this binding to be detected due largely to the unique properties of nanoparticles, such as a high surface area and excellent biocompatibility. Electrochemical sensing platforms based on gold nanoparticle labels have been developed for sensitive detection of DNA hybridization and certain protein samples. To the best of our knowledge, other than one report on the detection of the binding event between Escherichia coli single-stranded (ss) DNA binding protein and DNA, the detection of p53 via its sequence-specific DNA binding has not been accomplished via voltammetric means. Our group has developed an amplified voltammetric technique to detect DNA hybridization at low levels by oxidation of ferrocene (Fc) groups covering gold nanoparticle/streptavidin conjugates. Because each conjugate is decorated with more than 100 ferrocenyl alkanethiol molecules, the electrochemical signals have been greatly amplified. This amplified voltammetric detection method has also been employed to detect small proteins and oligopeptides immobilized onto surfaces. As described in this paper, the use of DNA-modified electrodes to capture p53 and the follow-up amplification of the voltammetric signals using the gold nanoparticle/streptavidin conjugates afford the sensitivity and selectivity necessary for detecting p53 at the cellular concentration.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** N-Biotinoyl-N-[6-maleimidohexanoyl] hydrazide (biotin-Mi), 1-hexanethiol (HT), and gold nanoparticle/streptavidin conjugates were acquired from Sigma-Aldrich. 6-Ferrocenyl-1-hexanethiol was purchased from Dojindo Co. (Atlanta, GA). Oligonucleotides (ODNs) with their 5′ ends modified with hexylthiol groups and unmodified ODNs were purchased from Sangon Co., Ltd. (Shanghai, China). The 25-mer ODN has a sequence of 5′-HS(CH2)4-TTT TTA GAC ATG CCC AGA CAT GCC C3′. The sequences (mismatching sequences underlined) of three ODNs used to hybridize with the surface-confined ODN are the following: 5′-GGG CAT GTC TGG GCA TGT CT-3′ (fully complementary), 5′-GGG CAT GTC AGG GCA TGT CT-3′ (one mismatching base), and 5′-GA CA GAG ACT TAA CTA A′ (seven mismatching bases). The ODNs used to form the nonconsensus ds-ODN at the electrode have sequences of 5′-HS(CH2)4-TTT TTT TGG GCC GCA GTC CGA G-3′ and 5′-CTC GCC GCA CCT CGG CCG AC-3′, respectively. N-(2-Ethyl-ferrocene)maleimide (Fc-Mi) was synthesized according to literature procedures. The preparation and characterization of the Fc-capped gold nanoparticle/streptavidin conjugates have been reported previously. All stock solutions were prepared daily with deionized water treated with a water purification system (Simplicity 185, Millipore Corp.). Recombinant p53 samples were either purchased from BD Biosciences Pharmingen (San Diego, CA) or purified in-house with the procedure published by Makmura et al. The procedure for soluble cell lysis preparation from normal epithelial kidney cells and several types of cancer cells (colorectal, lung, liver, and stomach) is as follows: cells were washed three times with ice-cold phosphate-

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**References:**

buffered saline (PBS, 10 mM phosphate/137 mM NaCl/2.7 mM KCl, pH = 7.4). After decanting the PBS solution, cells were lysed in a cell dissociation solution on ice for 20 min. The lysis buffer comprised 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN3, 0.1% sodium dodecyl sulfate, 100 μg/mL phenylmethanesulfonyl fluoride, 1 μg/mL aprotinin, 1% Triton X-100, and 0.5% sodium deoxycholate. The lysed cells were then removed from the tube walls by a cell slicker and transferred to a centrifuge tube. After sonication for 30 s on ice, contents released from the cell were centrifuged at 4 °C at 12 000 rpm for 10 min. The supernatant was collected and immediately analyzed. Other reagents were all acquired from commercial sources with analytical purity and used as received.

**Instruments and Electrodes.** All electrochemical measurements were carried out with a CH 832 electrochemical workstation (CH Instruments, Austin, TX). The gold working electrodes (Bioanalytical Systems, Inc., West Lafayette, IN) have a diameter of 2 mm. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. Prior to each measurement, the gold electrodes were polished with alumina slurry down to 0.3 μm on a polishing cloth (Buehler, Lake Bluff, IL), followed by sonication in water and ethanol. The supporting electrolyte was a 0.1 M KClO4 solution.

**Procedures. 1. Immobilization of Thiolated ODNs onto Gold Electrodes.** Immobilization of the thiolated ODN was carried out by casting onto the electrode 10 μL of TE (10 mM Tris–HCl and 1.0 mM EDTA) solution containing 1.0 μM ODN for 12 h. This step was followed by washing the electrode thoroughly with water and soaking the electrode in an aqueous solution containing 0.1 mM HT for 5 min.

2. DNA Hybridization and Subsequent p53 Capture. To form ds-ODN at the electrode surface, 5.0 μL of TNE (TE + 0.1 M NaCl) comprising a predetermined concentration of ODN was cast onto the electrode premixed with an ODN/HT mixed self-assembled monolayer (SAM) and the hybridization reaction was allowed to proceed for 2 h at room temperature. After the surface had been carefully rinsed with TNE and water, 5 μL of PBS containing a given concentration of p53 was cast onto the electrode surface for 1 h. Throughout this work, the p53 concentration was reported based on the molecular weight of the p53 monomer. After the p53 capture step, the electrode was rinsed with the washing buffer and water to remove the unattached p53 molecules.

3. Attachment of the Fc-Capped Gold Nanoparticle/Streptavidin Conjugates. To attach the Fc-capped gold nanoparticle/streptavidin conjugates onto the electrode surface covered with p53, the electrode was first allowed to react with 5 μL of 1.0 mM biotin-Mi dissolved in an acetic acid/NaOH mixture (pH 7.0) for 1 h. Upon rinsing with a copious amount of water, the electrode was exposed to 10 μL of Fc-capped gold nanoparticle/streptavidin conjugate solution for 1 h. For comparison, Fc-Mi was attached to the cysteine residues on the p53 molecules through a Michael-type electrophilic addition reaction.36

4. Electrochemical Detection. Once the Fc-capped gold nanoparticle/streptavidin conjugate was attached onto the electrode surface, the electrode was rinsed with water and transferred to a 0.1 M KClO4 solution and the p53 concentration was determined by scanning the electrode potential within the range of 0.0–0.6 V to oxidize the Fc groups on the nanoparticle conjugates.

5. ELISA. p53 pan ELISA kit was purchased from Adlitteram Diagnostic Laboratories (San Diego, CA). The ELISA assay, performed with a 96-well plate reader (The Sunrise Microplate, Tecan Trading AG, Switzerland), is based on a “sandwich immunoassay” protocol involving two monoclonal antibodies directed against p53. The capture antibody recognizes a conserved antigenic determinant of the p53 protein, and the detection antibody is specific for total p53 (wild-type and mutant forms) from different species. p53 concentration from the sample was calculated from a standard calibration curve (r² value = 0.9944) constructed using the standards included in the kit. ELISA tests were also performed on the recombinant p53 used for the electrochemical detection.

**RESULTS AND DISCUSSION**

Figure 1 illustrates the principle behind the p53 capture and detection via amplified voltammetric oxidation of Fc tags on the gold nanoparticle/streptavidin conjugates. The thiolated ss-ODN/
hexanethiol (HT) mixed SAM was first formed on the gold surface. Although the surface coverage of a thiolated ss-ODN is dependent on its length, the value has been reported to be approximately $2.6 \times 10^{12}$ mol/cm$^2$. The use of the mixed SAM of thiolated ODN and HT eliminates nonspecific adsorption of ss-ODN and orients the immobilized ODN molecules for more efficient DNA hybridization. Next, hybridization of the ss-ODN on the surface with its complementary ODN in solution was carried out. When exposing the resultant consensus ds-ODN-modified electrode to p53 in solution, p53/ODN complexes in various surface configurations may be produced. The most stable surface configuration is the binding of p53 tetramers onto the full binding sites of individual ds-DNA molecules, as shown in Figure 1a. Other possible surface binding configurations include insertion of a tetramer between two adjacent ds-ODN with the two dimers bound to the half-sites of the two ODN molecules or one dimer of a tetramer bound onto the half-site of a duplex with the other dimer unbound (Figure 1b). In the subsequent step, biotin-Mi was attached to the cysteine residues of p53. The five metal-free cysteine thiols, which are at the surface of the p53 molecule, are more likely to be tagged than the thiol groups buried within the protein. Finally, the Fc-capped gold nanoparticles/streptavidin conjugates were attached to the p53/ODN duplexes via biotin-streptavidin complexation. It is reported that the surface structure and elastic properties of DNA are crucial for p53 recognition and tumor suppression. The deformability of the DNA consensus site contributes significantly to the selective binding of p53, and DNA binding of the resultant p53/DNA complex is essential to its stability. Owing to the ODN elasticity and the relatively large mass of the nanoparticle conjugates, the Fc tags are positioned in close proximity to the electrode. Consequently, a facile electron-transfer reaction between the Fc tags and the electrode surface takes place. As described previously, each gold nanoparticle is capped with a large number of Fc molecules (127 ± 10 Fc molecules). Thus, the number of redox tags attached to the electrode will be much greater than the number of p53 molecules bound to the ds-ODN molecules. Consequently, the signal intensity is enhanced.

Curve a in Figure 2 is a representative cyclic voltammogram (CV) collected at an electrode modified with consensus ds-ODN molecules for p53 binding and the subsequent attachment of the Fc-capped gold nanoparticle/streptavidin conjugates. Well-defined redox waves with a peak potential separation ($\Delta E_p$) of 27 mV were obtained. The voltammogram exhibits a characteristic diffusional "tailing" of the peaks, due to the repulsive interaction between the ferrocenium moieties confined at the electrode surface. For comparison, the same procedure was implemented with electrodes covered with nonconsensus ds-ODN (curve b). The current in curve b dropped to the background level, indicating that binding of p53 is greatly dependent on the sequence of the ds-ODN. When ss-DNA molecules interact with p53 tetramers, nonspecific binding could occur at the C-terminus of the p53 molecules. Thus, the absence of any discernible peaks in curve c, which was acquired at an electrode covered with ss-ODN, indicates that nonspecific interaction between p53 and ss-ODN is negligible. The control CV (curve d) was acquired at an electrode modified with the consensus ds-ODN (same as that for generating curve a) but without having exposed the electrode to the p53 solution. The featureless voltammogram in curve d indicates that the Fc-capped gold nanoparticle/streptavidin conjugates do not nonspecifically adsorb onto consensus ds-ODN-covered electrodes.

Previously, Glera et al. have used N-(2-ethyl-ferrocene)-maleimide (Fc-Mi) to tag cysteine residues on cytochrome P450 and obtained relatively reversible voltammograms. Kraatz has synthesized Fc conjugates of amino acids, peptides, and nucleic acids and utilized them for biomolecular interaction studies. We used Fc-Mi to tag small peptides preimmobilized onto electrode surfaces (e.g., glutathione) and demonstrated that facile electron-transfer reactions between the Fc groups and the substrate electrode could be achieved. However, for the case of p53

detection using consensus ds-ODN-modified electrodes, tagging p53 bound to the ODN duplexes with Fc-Mi did not result in a distinct voltammetric peak (curve b in Figure 3). We attribute the absence of distinguishable peaks to the long distance of the Fc tags on Fc-Mi from the electrode and the small number of Fc tags per p53 molecule. These factors hinder the electron-transfer reaction or produce immeasurable signals.

The formation of ODN duplexes and their surface coverage were found to also have a profound influence on the amount of p53 captured. The same ss-ODN used to generate curve a in Figure 2 was immobilized onto the electrode and hybridized with its complement to form consensus ds-ODN or hybridized with an ODN containing one or seven base(s) mismatching to the consensus sequence. The ODN-covered electrodes were exposed to a p53 solution, followed by the attachment of the Fc-capped gold nanoparticle/streptavidin conjugates. With only one base mutated, the oxidation peak current ($i_p$) decreased by approximately 80%. Since we conducted the DNA hybridization reaction at room temperature, it is likely that some duplexes have formed at the surface, but the surface density of ds-ODN decreased considerably. Consequently, there were less p53 tetramers adsorbed onto the electrode. The surface density of the ds-ODN was further decreased when the ss-ODN used for the hybridization contained seven mismatching bases, and the voltammetric signal was even smaller (data not shown).

We then studied the dependence of $i_p$ on the p53 concentration (Figure 4). The peak was still measurable even when as little as 2.2 pM of p53 was analyzed. The percent relative standard deviation (RSD) ranged from 11.9% to 1.9% for p53 concentrations between 2.2 pM and 56 nM. These RSD values are reasonable given that several steps are involved in the procedure and factors, such as DNA and p53 surface densities and p53/DNA surface binding configurations (cf., Figure 1), could affect the number of gold nanoparticle/streptavidin conjugates attached to the electrode. As can be seen from Figure 4, the surface coverage of the complex increases with the p53 concentration. At around 2.2 nM, the plot begins to plateau. This suggests that most of the ds-ODN molecules have been bound to p53 tetramers. As a result, the sites for p53 binding have decreased or the attachment of additional p53 tetramers onto the ds-ODN-covered surface has become sterically impeded. Note that the p53 detection level (2.2 pM) is remarkably low. It is about 5 times lower than that obtained by adsorptive stripping square wave voltammetry of purified and separated p53 through the oxidations of the p53 tyrosine and tryptophan residues. Measurements of recombinant p53 by ELISA showed a calibration curve with a slope 1.8 times greater than that in Figure 4 between 9.4 pM and 0.19 nM p53 and a detection level ca. 14 times lower than that obtained with our electrochemically based method. Our ELISA is consistent with other ELISA tests.

Finally, we explored the feasibility of the method for real sample analysis. As can be seen from Figure 5, well-defined voltammetric peaks were observed when the ds-ODN-modified electrodes were used to capture p53 from normal epithelial kidney cell lysates (curve a). We determined the p53 concentration level to be 1.22 ± 0.05 nM. When the same approach was extended to the analysis of the wild-type p53 in colorectal cancer cell lysates, a pair of much smaller and broader peaks was observed (curve b). The concentration of the wild-type p53 was found to be about 50 times lower (0.024 ± 0.001 nM), suggesting that the p53 gene had been severely mutated in these colorectal cancer cells. The absence of voltammetric signals acquired at the ODN-modified electrode without the ODN hybridization step indicates that the nonspecific interaction between p53 in real samples and a 25-mer ss-ODN is also negligible (curves c and d). To further establish the validity of this method for clinical applications, we conducted ELISA tests in parallel with this method for the analyses of five additional cancer cell lysate samples. Among the total six cancer cell-lines (three colorectal cancers, one lung cancer, one liver cancer, and one stomach cancer), the ELISA results showed that three cell-lines had significantly elevated total p53 concentrations (Table 1). This trend suggests that not all cancer cell-lines have abnormally high p53, an observation well noted in cancer research. Remarkably, the three cancer cell-lines assayed by

**Figure 3.** CVs acquired at electrodes covered with a 25-mer ODN and hexanethiol mixed SAM after hybridization with 10 nM 20-mer complementary ODN, p53 binding, derivatization of the cysteine residues with Fc-Mi (curve b) and biotin-Mi (curve a). For curve a, the biotin-Mi was reacted with the Fc-capped gold nanoparticle/streptavidin conjugates. The arrow indicates the initial scan direction. Other experimental conditions are the same as those in Figure 2.

**Figure 4.** Plot of the oxidation peak current ($i_p$) against the p53 concentration. Concentrations of p53 determined are 2.2 and 11 pM and 0.11, 1.1, 2.2, 3.4, and 5.6 nM. Other experimental conditions are the same as those in Figure 2. Each point was averaged from at least three replicates, and the relative standard deviations (RSDs) are shown as the error bars.

Our method all displayed substantially (50–182 times) lower wild-type p53 concentrations than that in the normal cell lysate (Tables 1 and 2). As shown by Table 1, the significantly higher total p53 concentration in cancer cell lysates, determined by the ELISA test, is predominantly contributed by the elevation of the mutant p53. When compared to the results obtained with our method, it is clear that the elevation of the mutant p53 concentration is accompanied by a precipitous decline of the wild-type p53 concentration. The data in Table 1 demonstrate that our method is highly complementary to ELISA, as both wild-type p53 and total p53 concentrations were measured in such a comparative study.

Our method is therefore amenable to the quantification of wild-type functional p53 levels in normal and cancer cells.

CONCLUSIONS

Detection of low levels of DNA-binding properties of p53 is helpful for deciding appropriate treatment modalities for cancer patients. Cancer cells that contain consensus-site binding capability may stand to benefit from therapies designed to boost endogenous p53 levels. On the other hand, cancer cells with mutant p53 may require therapies that replace p53 functions. In this work, sequence-specific binding between p53 molecules in solution and consensus ODN duplexes preimmobilized onto electrodes was used for detecting p53 at ultratrace levels. The small number of p53 molecules bound to the ODN duplexes, which do not yield measurable electrochemical signals by themselves or even when labeled with simple redox cross-linkers, were tagged with gold nanoparticles capped with a large number of Fc tags. Well-defined Fc voltammetric peaks, whose currents increase with both the p53 concentration and the number of consensus ds-ODN molecules (or ds-ODN surface density) at the surface, were observed. Analytical figures of merit (e.g., dynamic range, selectivity, and detection level) were evaluated through the analysis of different p53 concentrations using electrodes modified with various ds- and ss-ODNs. Voltammetric signals emanating from the Fc tags that report on the p53/DNA binding indicate that nonspecific interaction between p53 and ss-ODN is essentially negligible. The detection level (2.2 pM) is well below the p53 level in normal cells, as the analysis of a normal epithelial kidney cell lysate yielded a p53 concentration of 1.22 nM. The remarkable sensitivity of this method also facilitated the measurements of the level of consensus DNA Binding p53 present in cancer cell lysates. The markedly decreased concentration of the consensus DNA binding p53 indicates that the p53 gene had been mutated significantly in tumor cells. Such diagnoses are consistent with the ELISA results.

The method is highly selective for wild-type p53 and complementary to ELISA (which measures the total p53 concentration). Our method is cost-effective for both the p53 capture (ds-ODNs, instead of another antibody, are used) and detection (nanoparticle conjugates, instead of another antibody conjugated to an enzyme, are employed). Moreover, the method is capable of determining p53 from real samples without extensive sample pretreatment/separation or specialized instruments. It holds promise as a clinical protocol for assaying p53 DNA binding capacity in normal and cancer cells at sensitive levels.

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