J|A|C|S
ARTICLES

Published on Web 08/05/2009

Terminal Protection of Small-Molecule-Linked DNA for Sensitive Electrochemical Detection of Protein Binding via Selective Carbon Nanotube Assembly

Zhan Wu, Zhen Zhen, Jian-Hui Jiang,* Guo-Li Shen, and Ru-Qin Yu

State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China

Received May 11, 2009; E-mail: jianhuijiang@hnu.cn

Abstract: Small-molecule-linked DNA has emerged as a versatile tool for the interaction assay between small organic molecules and their protein receptors. We report herein the proof-of-principle of a terminal protection assay of small-molecule-linked DNA. This assay is based on our new finding that single-stranded DNA (ssDNA) terminally tethered to a small molecule is protected from the degradation by exonuclease I (Exo I) when the small molecule moiety is bound to its protein target. This finding translates the binding of small molecules to proteins into the presence of a specific DNA sequence, which enables us to probe the interaction between small organic molecules and their protein targets using various DNA sequence amplification and detection technologies. On the basis of selective assembly of single-walled carbon nanotubes (SWNTs) with surface-tethered small-molecule-linked ssDNA not protected by protein binding, a novel electrochemical strategy for terminal protection assay has been developed. Through detecting the redox signal mediated by SWNT assembly on a 16-mercaptohexadecanoic acid-blocked electrode, this strategy is able to ensure substantial signal amplification and a low background current. This strategy is demonstrated for quantitative analysis of the interaction of folate with a tumor biomarker of folate receptor (FR), and a detection limit of 3 pM FR is readily achieved with desirable specificity and sensitivity, indicating that the terminal protection assay can offer a promising platform for small molecule−protein interaction studies.

Introduction

The discovery of small-molecule ligands to proteins and protein receptors of small organic molecules is a central problem in chemistry, biology, and medicine.[1−3] Specifically, small organic compounds that bind to a particular protein with reasonable affinity and specificity offer invaluable probes to perturb the function of the proteins for chemical genetics studies[1] and track the location and concentration of the proteins for molecular diagnostics.[2] They are also potential starting points for drug development.[3] Typically, the identification of small-molecule ligands or protein receptors is performed by probing the affinity between the proteins and the small molecules with certain biomolecular interaction assay strategies, including biosensors,[4,5] affinity chromatography,[6,7] kinetic capillary electrophoresis,[8,9] fluorescence resonant energy transfer,[10,11] and protein-fragment complementation assay.[12,13] Notwithstanding the importance of these techniques, the need for costly instruments, specific signal reporters, or surface-based molecular immobilization can be a detriment. An attractive alternative to these methods is the use of DNA oligonucleotides tethered to protein-binding small molecules, considering the enormous capabilities of oligonucleotides in site-specific labeling,[14,15] as well as sequence-specific biobarcoding,[16,17] amplification,[18] and isolation.[19,20]

(1) Stockwell, B. R. *Nature* **2004**, *432*, 846–854.
(2) Gao, X. H.; Cui, Y. Y.; Levenson, R. M.; Chung, L. W. K.; Nie, S. M. *Nat. Biotechnol.* **2004**, *32*, 969–976.
(3) Michnick, S. W.; Ear, P. H.; Manderson, E. N.; Remy, I.; Stefan, E. *Nat. Rev. Drug Discovery* **2007**, *6*, 569–582.
(4) Cooper, M. A. *Nat. Rev. Drug Discovery* **2002**, *1*, 515–528.
(5) Kanoh, N.; Kyo, M.; Inamori, K.; Ando, A.; Asami, A.; Nakao, A.; Osada, H. *Anal. Chem.* **2006**, *78*, 2226–2230.
(6) Taunton, J.; Hassig, C. A.; Schreiber, S. L. *Science* **1996**, *272*, 408–411.
(7) Terstappen, G. C.; Schlupen, C.; Raggiaschi, R.; Gaviraghi, G. *Nat. Rev. Drug Discovery* **2007**, *6*, 891–903.
(8) Petrov, A.; Okhonin, V.; Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2005**, *127*, 17104–17110.
(9) Drabovich, A. P.; Berezovski, M. V.; Musheev, M. U.; Krylov, S. N. *Anal. Chem.* **2009**, *81*, 490–494.
(10) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906–918.
(11) Clapp, A. R.; Medintz, L. M.; Mauro, J. M.; Fisher, B. R.; Bawendi, M. G.; Mattoussi, H. *J. Am. Chem. Soc.* **2004**, *126*, 1301–1310.
(12) Huang, J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13396–13401.
(13) Kerppola, T. K. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 449–456.
(14) Huh, Y. S.; Lowe, A. J.; Strickland, A. D.; Batt, C. A.; Erickson, D. *J. Am. Chem. Soc.* **2009**, *131*, 2208–2213.
(15) Zhang, Y. L.; Huang, Y.; Jiang, J. H.; Shen, G. L.; Yu, R. Q. *J. Am. Chem. Soc.* **2007**, *129*, 15448–15449.
(16) Hill, H. D.; Mirkin, C. A. *Nat. Protoc.* **2006**, *1*, 324–336.
(17) Huang, Y.; Zhang, Y. L.; Xu, X. M.; Jiang, J. H.; Shen, G. L.; Yu, R. Q. *J. Am. Chem. Soc.* **2009**, *131*, 2478–2480.
(18) Schweitzer, B.; Kingsmore, S. *Curr. Opin. Biotechnol.* **2001**, *12*, 21–27.
(19) Xu, X. Y.; Georganopoulou, D. G.; Hill, H. D.; Mirkin, C. A. *Anal. Chem.* **2007**, *79*, 6650–6654.
(20) Miao, J. M.; Cao, Z. J.; Zhou, Y.; Lau, C. W.; Lu, J. Z. *Anal. Chem.* **2008**, *80*, 1606–1613.

Small-molecule-linked DNA is emerging as a versatile tool for detecting the interaction between small organic molecules and their protein receptors. The oligonucleotides not only act as coding sequences for identifying the linked organic molecules but offer immediate signal amplification via polymerase chain reactions (PCR). Large collections of synthetic small molecules, individually linked to unique single-stranded DNA tags, then allow the selection of organic compounds with preferred protein binding specificity through the affinity-capture and PCR amplification cycles.[21,22] The unique self-assembly capacity of DNA also offers the possibility of constructing multivalent binding agents, which may represent an appealing avenue for the isolation of high-affinity small-molecule binders specific to the protein targets.[23−26] Despite of the proliferation of small-molecule-linked DNA as a smart module in highly sensitive selection of the synthetic compounds, it has rarely been implemented for the development of biosensor strategies.

Here we report the proof-of-principle of a terminal protection assay of small-molecule-linked DNA. This assay provides a promising strategy for specific, sensitive detection of the binding events of small molecules to their protein targets. The developed strategy is based on our new finding that single-stranded DNA (ssDNA) terminally tethered to a small molecule is protected from the degradation by exonuclease I (Exo I) when the small molecule moiety is bound to its protein target, as shown in Figure 1A. The protein-binding small molecules are covalently conjugated to ssDNA oligonucleotides of arbitrary sequences at the 3′ terminal. The small-molecule-linked ssDNA will be hydrolyzed successively into mononucleotides from the 3′ end by Exo I. When ssDNA is bound to the protein target through the small-molecule moiety, Exo I fails to catalyze the stepwise hydrolysis of the small-molecule-linked DNA, leaving the ssDNA intact. This terminal protection, in contrast to those mediated by the interaction of proteins with specific DNA sequences,[27−29] is sequence-independent with no interaction between DNA and proteins, because it merely involves the binding of the small molecule moiety to its protein target. Presumably, the terminal protection is attributed to steric hindrance of the bound protein molecule, which prevents Exo I from approaching and cleaving the phosphodiester bond adjacent to the 3′ terminus. Essentially, such terminal protection translates the binding of small molecules to proteins into the presence of specific DNA sequences, which enables us to probe the interaction between small organic molecules and their protein targets using various DNA sequence amplification and detection technologies. Therefore, the terminal protection is able to create a novel versatile platform for small molecule−protein interaction assay.

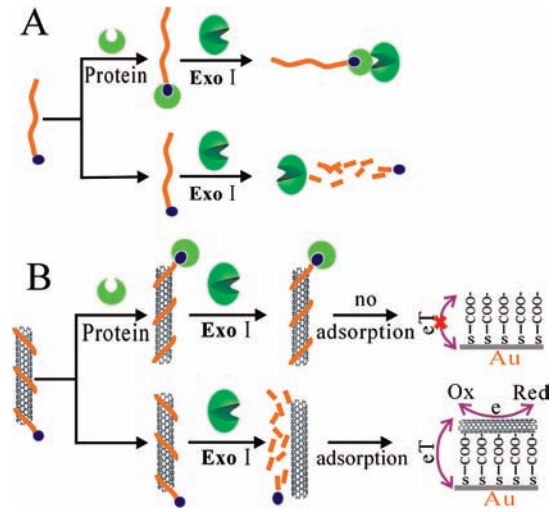

Figure 1. Terminal protection assay of small-molecule-linked ssDNA. Small-molecule-linked ssDNA is hydrolyzed successively into mononucleotides from the 3′ end by Exo I, while protected from the hydrolysis when the small molecule moiety is bound to its protein target (A). SWNT-wrapping ssDNA terminally tethered to small molecule is degraded by Exo I, rendering SWNTs assembled on MHA SAM that mediates electron transfer between electroactive species and the electrode. Protein binding of small-molecule-linked ssDNA prevents digestion of ssDNA, precluding adsorption of DNA-wrapped SWNTs on MHA SAM with no redox current generated (B).

In particular, the terminal protection assay, when coupled with certain unique properties of single-walled carbon nanotubes (SWNTs),[30−32] allows the development of a novel sensitive electrochemical biosensor strategy for detecting the binding of small molecules to proteins, as illustrated in Figure 1B. The biosensor utilizes gold electrodes for electrochemical readouts. The gold electrode is modified with a dense self-assembled monolayer (SAM) of COOH-terminated long chain alkanethiols such as 16-mercaptohexadecanoic acid (MHA). This hydrophobic SAM isolates the electrode from the aqueous solution; thus, the electron transfer between redox solutes and the electrode is blocked with no electrochemical signal detected. We then design to use the terminal protection to trigger a signal transduction cascade such that the isolating property of the MHA-blocked electrode can be modulated specifically by the binding event between ssDNA-linked small molecules and their protein targets. In the design, SWNTs are wrapped around by small-molecule-linked ssDNA through aromatic interactions between nucleotide bases and SWNT sidewalls, forming a DNA−SWNT complex stably dispersed in the solution.[30] The SWNT-wrapping ssDNA can still be digested stepwisely from the 3′ end by Exo I, producing SWNTs with no surface-tethered ssDNA. These "naked" SWNTs will be precipitated from the solution and assembled on the MHA SAM.[31] Because the SWNTs adsorbed on the isolating MHA SAM can mediate efficient electron transfer between the electrode and an electroactive species such as ferrocenecarboxylic acid (FcCOOH),

(21) Brenner, S.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5381–5383.
(22) Doyon, J. B.; Snyder, T. M.; Liu, D. R. *J. Am. Chem. Soc.* **2003**, *125*, 12372–12373.
(23) Melkko, S.; Scheuermann, J.; Dumelin, C. E.; Neri, D. *Nat. Biotechnol.* **2004**, *22*, 568–574.
(24) Sprinz, K. I.; Tagore, D. M.; Hamilton, A. D. *Bioorgan. Med. Chem. Lett.* **2005**, *15*, 3908–3911.
(25) Melkko, M.; Zhang, Y. X.; Dumelin, C. E.; Scheuermann, J.; Neri, D. *Angew. Chem., Int. Ed.* **2007**, *46*, 4671–4674.
(26) Rosenzweig, B. A.; Ross, N. T.; Tagore, D. M.; Jayawickramarajah, J.; Saraogi, I.; Hamilton, A. D. *J. Am. Chem. Soc.* **2009**, *131*, 5020–5021.
(27) Shalloway, D.; Kleinberger, T.; Livingdton, D. M. *Cell* **1980**, *20*, 411–422.
(28) Wang, J. K.; Li, T. X.; Guo, X. Y.; Lu, Z. H. *Nucleic Acids Res.* **2005**, *33*, 23–31.
(29) Xu, X. Y.; Zhao, Z.; Qin, L. D.; Wei, W.; Levine, J. E.; Mirkin, C. A. *Anal. Chem.* **2008**, *80*, 5616–5621.
(30) Zheng, M.; Jagota, A.; Semke, E.; Diner, B. A.; Mclean, R.; Ludtig, S.; Richardson, R. E.; Tassi, N. G. *Nat. Mater.* **2003**, *2*, 338–342.
(31) Wang, Y. H.; Maspoch, D.; Zou, S. J.; Schatz, G. C.; Smalley, R. E.; Mirkin, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 2026–2031.
(32) Mclean, R. S.; Huang, X.; Khripin, C.; Jagota, A.; Zheng, M. *Nano Lett.* **2006**, *6*, 55–60.

Table 1. Synthesized Oligonucleotides (5′→ 3′) Used in the Experiments[a]

probe **1**	GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT-NH-folate
probe **2**	GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT $GT-NH_2$
probe **3**	GT GT GT GT GT GT GT GT GT GT(-FITC) GT GT GT GT GT GT GT GT GT GT-NH-folate
probe **4**	GT GT GT GT GT GT GT GT GT GT(-FITC) GT GT GT GT GT GT GT GT GT $GT-NH_2$

[a] Probes **1** and **3** was obtained by conjugating probes **2** and **4**, respectively, with folate via the $-NH_2$ moiety at the 3′ terminus. The FITC label was conjugated to probes **3** and **4** via a modified nucleotide of T. Probes **1** and **2** were used in capillary electrophoresis and electrochemistry experiments as well as photography and scanning electron microscope characterization, and probes **3** and 4 were used in gel electrophoresis and fluorescence measurements.

a strong redox current is generated due to the signal amplification from a single SWNT to numerous electroactive molecules. In the presence of small-molecule-binding proteins, the SWNT-wrapping ssDNA is bound to the protein target through the small molecule moiety at the 3′ terminus, thus preventing the degradation of the ssDNA by Exo I. Because of strong electrostatic and hydration repulsions between the DNA−SWNTs and the negatively charged SAM, the SWNTs wrapped by the protected ssDNA will not be adsorbed on the MHA SAM,[32] keeping the MHA SAM isolating with no background current signal generated. As the electrochemical current decreases with increasing protein binding events, this biosensor indeed offers a cost-effective and quantitative approach for probing the interaction of small molecules with their protein targets. In this paper, we demonstrate that the electrochemical terminal protection assay can be exploited to detect the interaction of folate with its protein target, folate receptor (FR), a highly selective molecular biomarker associated with various tumors.[33−35] We also verify the quantitative nature of the developed strategy and characterize the limit of detection.

Experimental Section

Reagents and Materials. Streptavidin and Exo I were provided by New England Biolabs (Ipswich, MA). Purified SWNTs with an average diameter of ∼5 nm were purchased from Beijing Nachen Co. Ltd. (Beijing, China). FR, fluorescein isothiocyannate (FITC) monoclonal antibody, folate (FA), *N*-hydroxysulfosuccinimide (Sulfo-NHS), MHA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and FcCOOH were purchased from Sigma Aldrich Chemical Co. All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. The solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA) and had an electric resistance >18.3 MΩ. The oligonucleotides used in this work were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). The thermodynamic parameters of all oligonucleotides were calculated using bioinformatics software[36] (http://www.bioin-fo.rpi.edu/applications/), and no particular secondary structures were found for the synthesized oligonucleotide sequences. The sequences of the synthesized oligonucleotides are given in Table 1.

Labeling of Folate to NH_2-Modified Oligonucleotides. Folate was conjugated to the 3′ $-NH_2$ moiety of the oligonucleotides using the succinimide coupling (EDC-NHS) method.[37] Briefly, 0.5 mL of 20 μM DNA with the $-NH_2$ label at the 3′ end was mixed with 0.5 mL of 100 mM phosphate buffer (PB, pH 7.4) containing 10 mM folate, 1 mM EDC, and 5 mM Sulfo-NHS and incubated for 2 h at 37 °C in dark. The solution was then dialyzed against PB using a membrane with molecular weight cutoff of 1000 Da to remove excessive FA. The dialysis was performed for 3 days with shielding from light and changes of the fresh buffer every 4 h.

Electrophoresis Experiment. The capillary electrophoresis (CE) experiment was performed using a capillary electrophoresis system equipped with UV absorption detection (P/AGE MDQ, Beckman, Germany) under an applied potential of 15 kV using a quartz capillary with 75 μm diameter (total length, 50 cm; effective length, 30 cm) in 50 mM borate running buffer (pH 9.2). DNA samples were injected into the capillary using the pressure injection mode at 0.5 psi for 5 s and detected at 254 nm for 30 min. The temperature of the separation, 25 °C, was controlled by immersion of the capillary in a cooling liquid circulating in the cartridge. Between successive runs, the capillary was rinsed with ultrapure water and running buffer for 15 min, respectively.

The gel electrophoresis was performed using the DNA sample (10 μL per well) on a 3% agarose gel with the fluorescence stain ethidium bromide (0.5 μg/mL) in 50 mM Tris-borate running buffer (pH 9.2) containing 2 mM EDTA at 100 V for 1 h. After electrophoresis, the gel was visualized via fluorescence detection using a Tocan 240 gel imaging system (Shanghai Tocan Biotechnology Co.).

The digestion reaction was performed by adding Exo I (40 U) into in 20 μL reaction buffer containing 20 mM Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 50 mM NaCl, and 5 μM small-molecule-linked DNA probe or 5 μM small-molecule-linked DNA plus 5 μM protein target followed by incubation at 37 °C for 1 h. The digestion reaction was terminated through adding 20 mM EDTA. In the CE analysis, probe **1** or **2** was used in the digestion reaction. After the addition of adenosine (80 μM) as the internal standard, the resulting mixture was injected into the capillary for electrophoresis. In the gel electrophoresis experiment, probe **3** or **4** with FITC labels was used in the digestion reaction, and the product obtained (10 μL per well) was loaded on the gel for electrophoresis. The FITC labels were used to improve detection sensitivity of the ssDNA in the gel.

Preparation of DNA−SWNT Complex. Two milliliters of aqueous solution containing 1 mg of purified SWNTs, 20 μM DNA oligonucleotides, and 0.1 M NaCl was sonicated in ice bath for 2 h under the power of ∼6 W using a probe-type sonicator. The resulting suspension was centrifuged at 12 000*g* for 70 min to remove possible SWNT aggregates. The supernatant was collected and recentrifuged under similar conditions, and the sediment was again discarded. The SWNTs in the supernatants were collected and filtered through a Millipore centrifugal filter with a molecular weight cutoff of 100 kDa (Billerica, MA) to remove excessive oligonucleotides not wrapping around the SWNTs. After washing with water 10 times, the DNA−SWNT complex was resuspended in ultrapure water. Such a suspension of SWNT−DNA complex, when stored at 4 °C, was found to be stable for weeks with no appreciable aggregates and precipitates.

Gold Electrode Treatment and Modification. Gold disk electrodes (99.99% polycrystalline, ∼2 mm diameter, CH Instrument Inc.) were treated with piraha solution (H_2O_2/H_2SO_4 1:3 in volume) for 2 h three times, polished on a microcloth (Buehler) with 0.05 μm γ-alumina suspension (CH Instrument Inc.) for 2 min, and again rinsed with ultrapure water and ethanol. The electrodes were then sonicated in ultrapure water for 5 min to remove adsorbed particles, rinsed thoroughly with ultrapure water, and dried under mild nitrogen stream. These gold electrodes were immersed into an ethanol solution of MHA (20 mM) for ∼24 h at 25 °C to allow the formation of a compact SAM. The electrodes modified with MHA SAM were then thoroughly rinsed using

(33) Kam, N. W. S.; O'Connell, M. J.; Wisdom, J. A.; Dai, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11600–11605.
(34) Henne, W. A.; Doomeweerd, D. D.; Lee, J.; Low, P. S.; Savran, C. *Anal. Chem.* **2006**, *78*, 4880–4884.
(35) Hemme, W. A.; Hartmann, L. C.; Low, P. S.; Savran, C. A. *J. Am. Chem. Soc.* **2007**, *129*, 15824–15829.
(36) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406–3415.
(37) Fahlman, R. P.; Sen, D. *J. Am. Chem. Soc.* **2002**, *124*, 4610–4616.

ethanol to remove MHA adsorbed on the electrode surface followed by drying under mild nitrogen stream. These gold electrodes could be used for assay of the next sample via polishing to remove the adsorbates followed by the aforementioned treatments.

Electrochemical Measurements of Terminal Protection Assay. A 2 μL aliquot of folate-linked DNA−SWNT complex was added into 20 μL of sample mixture containing 20 mM Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 50 mM NaCl, and FR sample of a given concentration (10 μL, final concentration ranging from 0 pM to 10 nM). The mixture was incubated at 37 °C for 20 min to allow complete interaction between the protein and the folate-linked DNA−SWNT complex. Then, 40 U of Exo I was added to the mixture and it was incubated at 37 °C for 30 min followed by the addition of 20 mM EDTA to terminate the reaction. The resulting solution was dropped on the surface of the MHA-modified electrode and incubated in a humid atmosphere at room temperature for 30 min. Subsequently, the electrode was thoroughly rinsed with ethanol and ultrapure water to remove SWNTs weakly adsorbed on the electrode surface.

All electrochemical measurements including differential pulse voltammetry (DPV), cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS) were performed at room temperature using a three-electrode system consisting of a KCl saturated calomel reference electrode (SCE), a platinum counter electrode, and the working electrode (the gold electrode with MHA modification). DPV and CV were executed on an Autolab PGSTAT12/FRA2 potentiostat/galvanostat system (Eco Chemie, Utrecht, Netherlands). EIS was carried out using a CH Instruments model 760C electrochemical analyzer (Shanghai, China). All electrochemical data were recorded in 20 mM phosphate buffer (pH 7.4) containing 0.1 M $KClO_4$ and 5 mM FcCOOH at room temperature. DPV was recorded within the potential range from 0 to 0.6 V (vs SCE) under modulation amplitude of 25 mV and a scan rate of 10 mV/s with a step potential of 1 mV. CV was performed within the potential range from −0.2 to 0.8 V using a step potential of 0.01 V at a scan rate of 100 mV/s. EIS was performed in the frequency range from 0.1 mHz to 100 kHz at a bias potential of 0.19 V (vs SCE) with a frequency modulation of 10 mV. The reported DPV curves were background-subtracted using the software GPES version 4.9.007 (Eco Chemie Utrecht, Netherlands) through extrapolation to the baseline in the regions far from the peaks.[38] All electrochemical measurements were repetitively performed four times except as otherwise stated. The results shown were the average of the four measurements with the error bars indicating the standard deviation (SD).

Results and Discussion

Electrophoresis Characterization of Terminal Protection. Figure 2 depicts capillary electrophoretograms of the DNA probe, $(GT)_{20}$, under the Exo I treatment. Because of the limited working concentrations and absorption coefficients at 254 nm of the proteins involved in the experiments, the peaks appearing in the electrophoretograms were exclusively arising from the DNA probe and its hydrolysis products. This allowed us to directly visualize the degradation status of the DNA probe. We observed a sharp peak for the folate-linked DNA probe **1** and two separate peaks for the Exo I-treated DNA, confirming a complete degradation of the folate-linked DNA sequence into two mononucleotides, GMP and TMP, by Exo I. After interacting with equimolar FR, the migration time of the folate-linked DNA increased slightly. This indicated that the folate moiety of DNA was bound to FR, a basic protein that would interact with exposed surface silanol groups on the capillary wall and exhibit a prolonged migration time. After extensive digestion

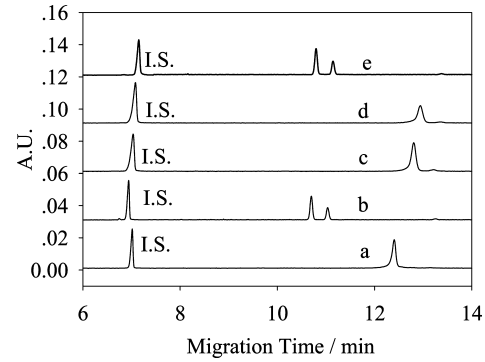

Figure 2. Electrophoretograms of 5 μM folate-labeled probe **1** (a), 5 μM probe **1** digested by 40 U of Exo I (b), 5 μM FR-bound probe **1** (c), 5 μM FR-bound probe **1** digested by 40 U of Exo I (d), and 5 μM probe **2** plus 5 μM FR digested by 40 U of Exo I (e). Internal standard is 80 μM adenosine.

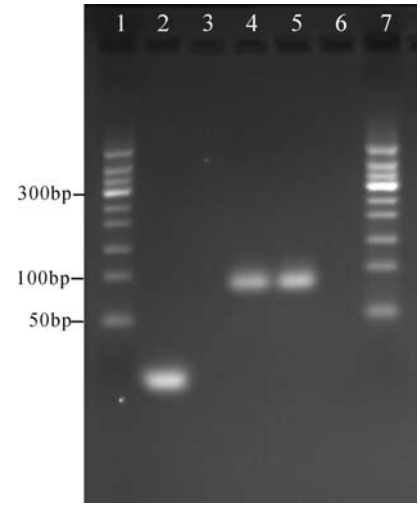

Figure 3. Agarose gel electrophoresis image for terminal protection assay: lane 1, DNA size marker; lane 2, 5 μM folate-labeled probe **3**; lane 3, 5 μM probe **3** digested by 40 U of Exo I; lane 4, 5 μM FR-bound probe **3**; lane 5, 5 μM FR-bound probe **1** digested by 40 U of Exo I; lane 6, 5 μM probe **4** plus 5 μM FR digested by 40 U of Exo I; lane 7, DNA size marker.

of the folate-linked DNA−FR complex using Exo I over 1 h, there was no noticeable change in the electrophoretic peak of the complex, clearly implying that hydrolysis of the folate-linked DNA by Exo I was prevented by the FR binding event. A control experiment of Exo I-catalyzed digestion was performed using the folate-free sequence, probe **2**, in the presence of equimolar FR. The electrophoretogram also gave two peaks for these two mononucleotides, indicators of completely hydrolyzed probe **2**. This gave immediate evidence that the terminal protection of DNA probes was specific to the folate-FR binding event rather than the binding of FR to the DNA sequence.

The terminal protection of the folate-linked DNA probe by FR was also verified using gel electrophoresis. Considering that degraded DNA probes would migrate out of the gel and only DNA oligonucleotides could be selectively stained by ethidium bromide, then we could merely visualize DNA probes **3** and **4**, besides the DNA markers on the fluorescence photograph of the gel. Thus, the appearance of visible bands in the gel could directly unravel the protection phenomenon of the DNA probes from Exo I digestion. As shown in Figure 3, bright bands were observed on the lanes where probe **3** was not subjected to Exo I treatment (lanes 2 and 4) or it was incubated with equimolar

(38) Steel, A. B.; Levicky, R. L.; Herne, T. M.; Tarlov, M. J. *Biophys. J.* **2000**, *79*, 975–981.

FR followed by Exo I hydrolysis (lane 5). This disclosed that the interaction with FR made probe **3** resistant to the digestion by Exo I, confirming the terminal protection of the probe.

Further CE and gel electrophoresis experiments were performed using other small molecule ligands and protein targets such as biotin and streptavidin, as well as FITC and its antibody (Supporting Information). The results revealed that the binding of small-molecule-linked DNA probes of different sequences to the corresponding protein targets through the small-molecule terminus were able to prevent the digestion of the ssDNA by Exo I. Therefore, we might infer that the terminal protection could be a common phenomenon for the small-molecule-linked DNA.

It is noteworthy that double-stranded DNA and ssDNA with its 3′ hydroxyl group blocked by a phosphoryl or acetyl group were reported to be resistant to digestion by Exo I at a low concentration (<0.27 U/μL).[39] These observations demonstrated the specificity of Exo I in attacking ssDNA at the 3′ hydroxyl with high efficiency. Our capillary and gel electrophoresis analysis revealed that the hydrolysis rate for ssDNA with 3′ small-molecule modification was substantially increased with high Exo I concentration (2 U/μL) to completely degrade the small-molecule-linked ssDNA. This implied that the cleavage activity of Exo I was substantially enhanced with increasing enzyme concentration. However, no concentration dependency was observed for the terminal protection in our experiments. Even with higher enzyme concentration (10 U/μL), the FR-bound folate-linked DNA was found to be still resistant to the digestion of Exo I. This might be due to the fact that binding of FR to the folate-linked DNA increased the steric hindrance so dramatically that the attack site of Exo I was totally blocked by the bound protein.

Electrochemical Terminal Protection Assay. Figure 4A depicts typical CV responses of the electrochemical terminal protection assay. No remarkable electrochemical peaks were obtained for FcCOOH at the MHA-assembled electrode. This indicated that the long-chain alkanethiol formed a densely packed isolating layer on the electrode that totally blocked the electron transfer between the electrode and the electroactive species. Addition of the folate-linked probe **1**−SWNT on the electrode had little effect on the CV responses, and no significant increase of the redox current was observed. This signified that the adsorption of DNA−SWNT on the MHA SAM was precluded because of strong electrostatic and hydration repulsion between the DNA−SWNT and the negatively charged SAM surface. This coincided with the observations reported previously[32] and implied a desirably low background signal for the developed strategy. We also found that, in the absence of probe **1**−SWNT, addition of Exo I solution on the electrode did not induce noticeable redox peaks in the CV curves. However, with Exo I digestion of the DNA−SWNT system, a couple of appreciable well-defined redox peaks appeared at 0.25 and 0.35 V, a characteristic electrochemical peak range of ferrocene derivatives. This conveyed that the isolating nature of the MHA SAM was altered by the reaction between Exo I and probe **1**−SWNT, hinting at the fact that SWNT-wrapping ssDNA could still be digested by Exo I. On adding 5 nM FR into the probe **1**−SWNT system followed by Exo I treatment, no significant redox peaks appeared in the CV curves, implying that binding of folate moiety to FR could protect the SWNT-wrapping probe **1** from degradation by Exo I. Additionally, we conducted a control experiment of Exo I digestion using the folate-free probe **2** in the presence of 5 nM FR, and large redox peaks were obtained in CV curves. This observation confirmed that the terminal protection of probe **1**−SWNT complex was specifically attributed to the binding of FR to the folate moiety tethered to probe **1**.

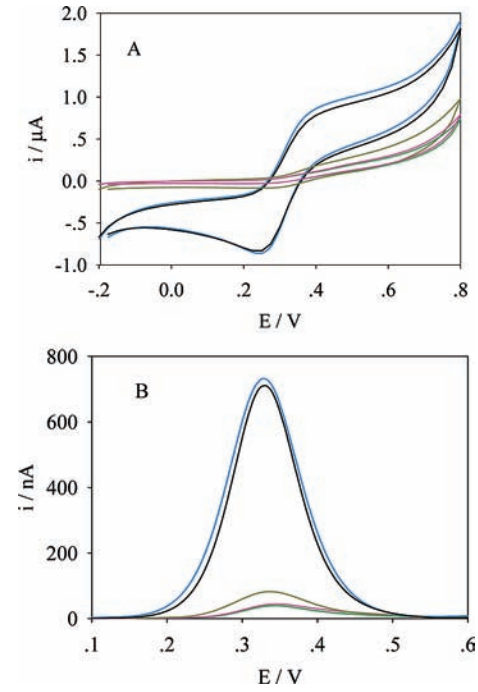

Figure 4. (A) CV responses of the electrochemical terminal protection assay for MHA-blocked electrode (green), probe **1**−SWNT on electrode (pink), Exo I-digested probe **1**−SWNT on electrode (blue), 5 nM FR-protected probe **1**−SWNT after Exo I digestion on electrode (yellow), and probe **2**−SWNT plus 5 nM FR after Exo I digestion on electrode (black). (B) Typical DPV responses of electrochemical terminal protection assay for MHA-blocked electrode (green), probe **1**−SWNT on electrode (pink), Exo I-digested probe **1**−SWNT on electrode (blue), 5 nM FR-protected probe **1**−SWNT after Exo I digestion on electrode (yellow), and probe **2**−SWNT plus 5 nM FR after Exo I digestion on electrode (black).

DPV signals gave better discrimination for the aforementioned findings, as shown in Figure 4B. The MHA-assembled electrode, even with the addition of DNA−SWNT, merely yielded a very small DPV peak, ~27 nA with a relative standard deviation (RSD) of 5.6% across five repetitive experiments. After Exo I treatment of probe **1**−SWNT and probe **2**−SWNT, strong peaks were observed at 0.32 V (~778 nA with a RSD of 3.9% across five repetitive experiments). After Exo I treatment of probe **1**−SWNT in the presence of 5 nM FR, we again obtained a small DPV peak, ~46 nA with a RSD of 6.1% across five repetitive experiments. This DPV signal only amounted to ~6% of the current response obtained in Exo I digestion of probe **1**−SWNT in the absence of FR, which clearly manifested that the electrochemical terminal protection assay offered desirable resolution (>16 fold) of the binding event between FR and the folate-linked DNA.

Additional control experiments were performed for electrochemical terminal protection assay using other proteins or matrices, such as bovine serum albumin (BSA), immunoglobulins G (IgG), carcinoembryonic antigen (CEA), α-fetoprotein (AFP), and human serum, as shown in Figure 5. It was revealed that these proteins and even human serum (10-fold diluted) had little effect on the Exo I hydrolysis reaction for the folate-linked

(39) Lehman, L. R.; Nussbaum, A. L. *J. Biol. Chem.* **1964**, *239*, 2628–2636.

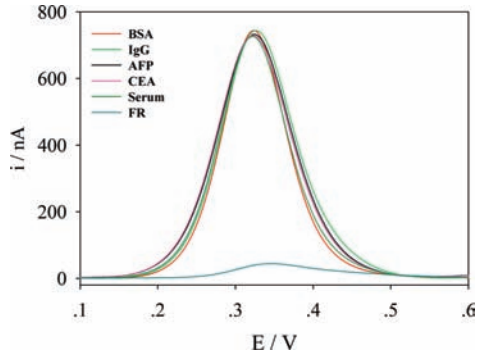

Figure 5. DPV responses of electrochemical terminal protection assay to different proteins (FR 5 nM; other proteins, 1 μM) and human serum (10-fold diluted).

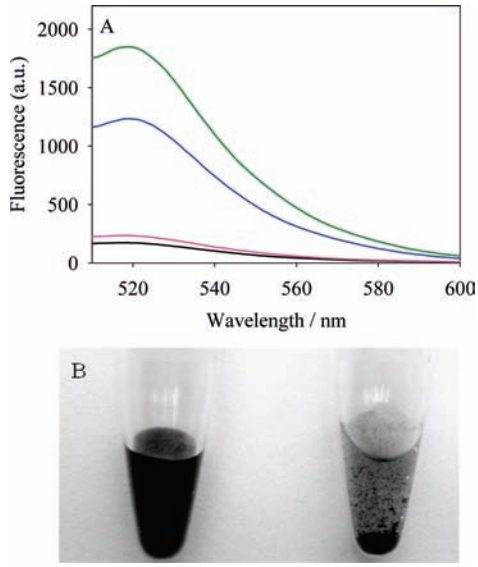

Figure 6. (A) Fluorescence emission spectra of 50 nM probe **3**-wrapped SWNTs digested by Exo I (green), 5 nM FR plus 50 nM probe **3**-wrapped SWNTs digested by Exo I (blue), 50 nM FR plus 50 nM probe **3**-wrapped SWNTs digested by Exo I (pink), and 50 nM probe **3**-wrapped SWNTs (blank). (B) Photographs of 50 nM FR plus 50 nM probe **3**-wrapped SWNTs digested by Exo I (left), and 50 nM probe **3**-wrapped SWNTs digested by Exo I (right).

DNA−SWNT. This further verified that the protection of folate-linked DNA was specific to the FR binding event, implying high selectivity of terminal protection assay for probing the interaction between small molecules and their protein targets.

Characterization and Quantitative Nature of Electrochemical Terminal Protection Assay. To elucidate the underlying mechanisms of the aforementioned findings, thorough studies of Exo I reaction with the folate-linked DNA−SWNT were performed. Figure 6A depicts the spectra obtained in fluorescence investigation of the SWNT-wrapping folate-linked ssDNA, probe **3**, with a FITC tag. It was clear that, in the SWNT−DNA complex, the fluorescence of the FITC label was largely quenched because of energy transfer from the fluophore to SWNT.[40,41] However, the fluorescence was remarkably enhanced after Exo I-catalyzed degradation of the SWNT−DNA complex, evidencing that the FITC fluophors were dissociated from SWNT surface, and thus, SWNT-wrapping ssDNA was digested by Exo I. In contrast, no substantial fluorescence increase was achieved in Exo I digestion of SWNT−DNA when the folate-linked ssDNA was bound to FR, implying that the FITC fluophors were retained on SWNT surface. This also confirmed that the SWNT-wrapping folate-linked ssDNA was protected from Exo I digestion by the terminal binding of FR.

Visual observation of the folate-linked DNA−SWNT complex further disclosed that visible aggregates and precipitates appeared after Exo I treatment of the DNA−SWNT complex, while stable suspension was still retained for the FR-protected folate-linked DNA−SWNT under Exo I-catalyzed digestion (Figure 6B). These findings manifested that SWNT-wrapping ssDNA would be effectively degraded by Exo I with a concomitant precipitation of SWNTs from the aqueous solution, and the digestion could be almost completely prevented by FR binding to the terminal folate moiety, validating the terminal protection of small-molecule-linked DNA. It is noteworthy that the complete degradation of SWNT-wrapping ssDNA was obtained at high enzyme concentration (2 U/μL), which was different from the case for living cell studies. Actually, it was reported that DNA wrapped on SWNT surface could be stable over 24 h inside living cells,[33] indicating that CNT-wrapping DNA could be partially protected from enzyme digestion at cellular-like enzyme concentration.

To verify the assembly of SWNT selectively mediated by terminal protection, a scanning electron microscope (SEM) was utilized to closely inspect the assembly behaviors of the folate-linked DNA−SWNT on the MHA SAM, as shown in Figure 7. We observed that after Exo I digestion, the FR-protected folate-linked DNA−SWNT merely showed very unconspicuous adsorption on the SAM. In contrast, Exo I-catalyzed degradation of the SWNT-wrapping folate-linked DNA resulted in appreciable adsorbates of SWNTs on the MHA SAM surface. These findings testified that terminal protection of the folate-linked DNA by FR binding disabled the assembly of SWNTs on the MHA SAM, while the absence of a FR binding event allowed Exo I digestion of the SWNT-wrapping DNA followed by a spontaneous assembly of the "naked" SWNTs on the isolating MHA SAM.

Further electrochemical impedance spectroscopy (EIS) measurements were performed to interrogate the isolating properties of the MHA SAM in the terminal protection assay. Figure 8 depicts Nyquist plots obtained on the MHA-modified electrode. It was disclosed that the digestion of folate-linked DNA−SWNT by Exo I resulted in substantially decreased electrochemical impedance, while the FR-protected folate-linked DNA−SWNT merely induced slightly decreased impedance. Since Exo I digestion of folate-linked DNA−SWNT rendered "naked" SWNTs, this observation indeed revealed that the assembly of SWNTs on the MHA SAM could facilitate electron transfer between the electrode and FcCOOH. Combining the observations achieved in SEM and EIS, it was evident that the Exo I digestion of DNA−SWNT promoted the assembly of SWNTs on the MHA SAM surface, which then facilitated electron transfer between the electrode and FcCOOH with a redox current readily generated. On the other hand, the presence of FR protected DNA−SWNT from Exo I-catalyzed degradation, thus preventing the assembly of DNA-wrapped SWNTs on the MHA SAM with electron transfer on the electrode remaining blocked. This validated the putative mechanism for the developed electrochemical strategy of terminal protection assay.

(40) Kam, N. W. S.; Liu, Z.; Dai, H. J. *J. Am. Chem. Soc.* **2005**, *127*, 12492–12493.
(41) Yang, R. H.; Jin, J. Y.; Chen, Y.; Shao, N.; Kang, H. Z.; Xiao, Z. Y.; Tang, Z. W.; Wu, Y. R.; Zhu, Z.; Tan, W. H. *J. Am. Chem. Soc.* **2008**, *130*, 8351–8358.

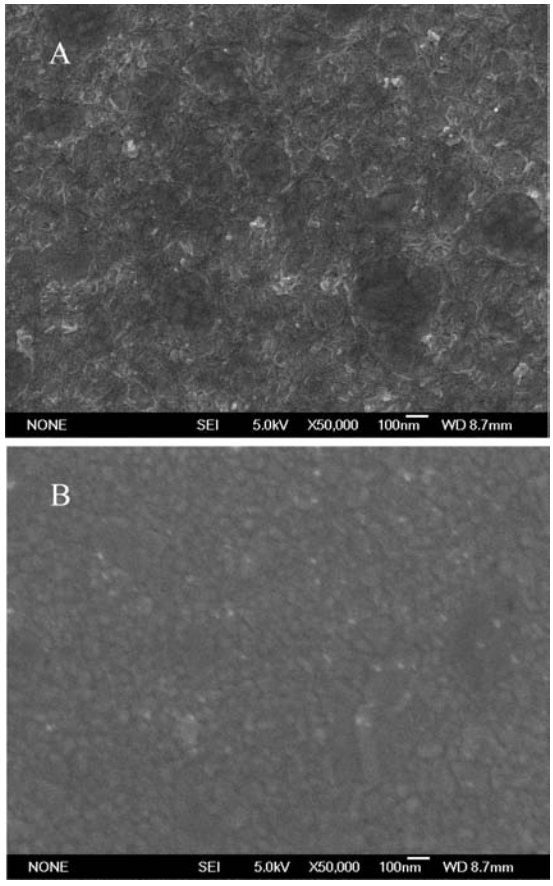

Figure 7. Typical SEM images for 50 nM probe **1**-wrapped SWNTs digested by Exo I (A) and 50 nM FR plus 50 nM probe **1**-wrapped SWNTs digested by Exo I (B).

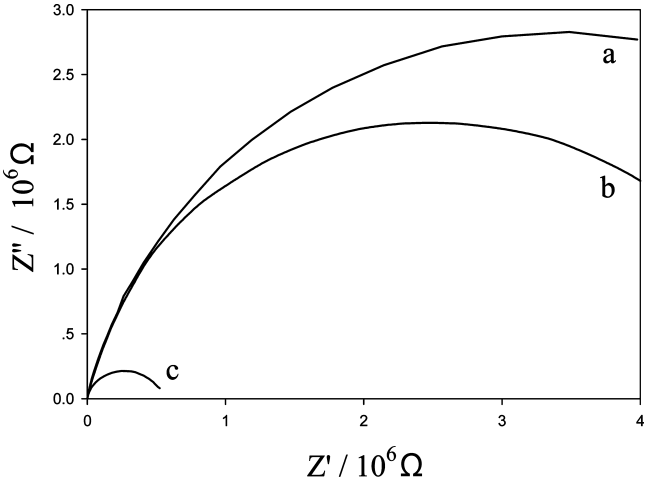

Figure 8. Nyquist plots obtained for MHA-modified electrode (a), 5 nM FR plus probe **1**−SWNTs digested by Exo I on electrode (b), and probe **1**−SWNTs digested by Exo I on electrode (c).

The terminal protection of small-molecule-linked DNA−SWNT was mediated by the binding of FR to the folate-linked DNA−SWNT, which was dependent upon the concentration of FR. We could anticipate a quantitative nature for the terminal protection assay. Figure 9 gives typical DPV responses of the terminal protection assay strategy to FR of varying concentrations. We observed dynamically decreased DPV peaks in response to FR of increasing concentrations within the range from 10 pM to 5.0 nM. A quasilinear correlation was obtained

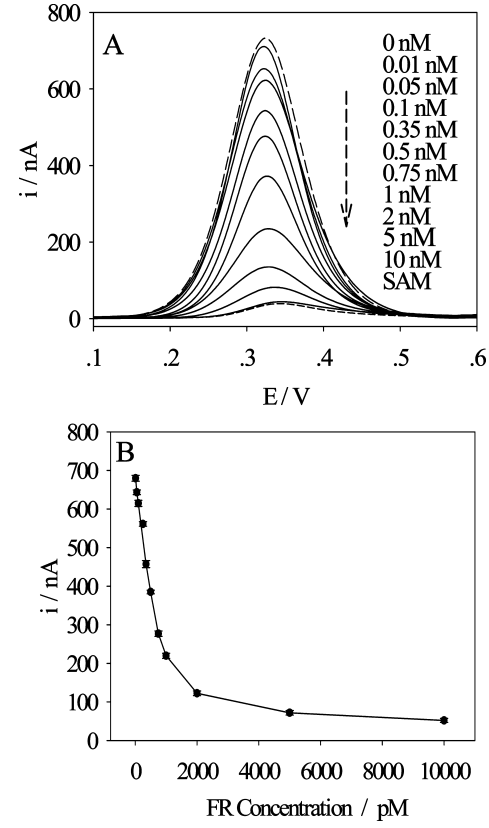

Figure 9. (A) Typical DPV responses of electrochemical terminal protection assay to FR of varying concentrations. (B) Corresponding DPV peak currents versus FR concentrations. Error bars are SD across four repetitive experiments.

in the concentration range from 10 pM to 1.0 nM with a readily achieved detection limit of 3 pM, which approached clinically relevant concentrations of FR.[34] This demonstrated that the terminal protection strategy could be used for quantitative analysis of the protein targets. On the basis of the quantitative nature, it could also be implemented for the determination of the affinity constants for different pairs of small molecule−protein targets or quantification of small molecules using a competitive assay format. In comparison with conventional small molecule−protein interaction assay such as the fluorescence anisotropy method, the developed strategy offered several prominent advantages: First, the transduction using SWNT assembly allowed enormous signal amplification, since a single SWNT mediated a redox current arising from numerous FcCOOH molecules. This furnished the developed strategy with superb sensitivity. Second, the strategy could be implemented using an electrode array format, thus enabling parallel assay of multiple samples. Third, electrochemical readouts offered the advantages of simple instrumentation, miniaturization, and excellent compatibility with assay of colored and viscous samples. Fourth, terminal protection was resistant to nonspecific binding events, thus imparting the strategy with high specificity. Therefore, it was expected that this strategy held potential in practical quantitative analysis of small molecule−protein interactions.

Conclusions

We have reported a new finding of terminal protection that small-molecule-linked ssDNA was prevented from Exo I-catalyzed degradation when the small molecule moiety is bound

to its protein target. A novel electrochemical strategy for terminal protection assay is then developed on the basis of selective assembly of SWNTs with surface-tethered small-molecule-linked ssDNA not protected by protein binding. This strategy offers a sensitive, specific, and efficient platform for quantitatively screening the small molecule−protein interactions. Because of its translating the small molecule−protein binding events into a DNA assay, the terminal protection assay is expected to provide a promising arsenal for signaling the interactions of small molecules with their protein targets via the use of versatile technologies for nucleic acid amplification and detection. With DNA sequences specifically coded for different small molecules, the technique also creates a new dimension for multiplex assay of multiple small molecule−protein interactions and rapid isolation of synthetic organic protein-binding ligands from libraries of small-molecule-linked ssDNA.

Acknowledgment. This work was supported by "973" National Key Basic Research Program (2007CB310500) and NSF (20875027, 20775023, 90817101) of China.

Supporting Information Available: Description of other experimental procedures and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

JA9038054