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Terminal Protection of Small Molecule-Linked DNA: A Versatile Biosensor Platform for Protein Binding and Gene Typing Assay

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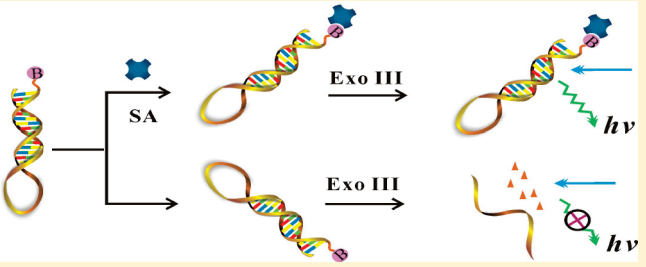
Supporting Information

ABSTRACT: Assays of small molecule−protein interactions are of tremendous importance in chemical genetics, molecular diagnostics, and drug development. This work reports a new finding of generalized terminal protection that small molecule-DNA chimeras are protected from degradation by various DNA exonucleases, when the small molecule moieties are bound to their protein targets. This generalization converts small molecule−protein interaction assays into the detection of DNA of various structures, affording a useful mechanism for the analytics of small molecules. On the basis of this mechanism, a label-free biosensor strategy has been developed for a homogeneous assay of protein−small molecule interactions based on the fluorescence staining detection. Also, a label-free SNP genotyping technique is proposed based on polymerase extension of a single nucleotide with a small molecule label. The developed techniques are demonstrated using a model protein−small molecule system of biotin/streptavidin and a model SNP system of human β-globin gene around the position of codon 39. The results revealed that the protein−small molecule interaction assay strategy shows dynamic responses in the concentration range from 0.5 to 100 nM with a detection limit of 0.1 nM, and the SNP typing technique gives dynamic responses in the concentration range from 0.1 to 200 nM with a detection limit of 0.02 nM. Besides desirable sensitivity, the developed strategies also offer high selectivity, excellent reproducibility, low cost, and simplified operations, implying that these techniques may hold considerable potential for molecular diagnostics and genomic research.



Molecular diagnostics and therapeutics would greatly benefit from novel techniques for detecting small molecule−protein interactions because of the tremendous importance of small molecules in chemical genetics, molecular diagnostics, and drug development.[1,2] Methods currently available for the assays of small molecules or their protein receptors include affinity chromatography,[3] kinetic capillary electrophoresis,[4,5] fluorescence resonant energy transfer,[6,7] fluorescence anisotropy,[8] a protein-fragment complementation assay,[9] and surface plasmon resonance (SPR).[10,11] Though these techniques have been demonstrated as useful tools for the assays, the need of costly instruments, specific labeling or immobilizing reagents, limited throughput, or susceptibility to nonspecific interactions may pose limitations in their applications. In the context, development of label-free homogeneous assay strategies that are highly specific, greatly robust, cost-efficient, easily automated, and scalable for parallel assays are still a central challenge in analytical chemistry.

The use of small molecule-linked DNA or small molecule-DNA chimeras may represent an ideal option for this analysis. The DNA part provides the chimeras with versatile ability for sequence-dependent separation, amplification and detection, and the protein-binding small molecule moiety offers the capacity of selective capturing the target proteins. It is demonstrated that such small molecule-DNA chimeras can be used as efficacious tools for the selection of protein-binding small molecules from a large chemical library.[12−14] Protein binding to small molecules in the chimeras may alter the dynamics of redox tags linked to the DNA. This facilitates the development of electrochemical sensors for protein binding assays.[15] We have recently reported that protein binding to small molecule in DNA-small molecule chimeras could protect the conjugated DNA from degradation by the 3′ single-strand specific exonuclease I (Exo I). This finding, which is called terminal protection, also forms a basis for constructing a sensitive biosensor for electrochemical detection of small molecule−protein interactions.[16] However, this electrochemical strategy may still suffer from the limitations of requiring deliberate modifications of electrode surfaces and careful control of assay conditions, increasing the difficulty in applying it for large-scale assays such as drug screening and molecular diagnostics.

In the present study, we report a generalized terminal protection mechanism for DNA-small molecule chimeras. This generalization converts small molecule−protein interaction assays into

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

Probe **1**	Biotin-GAC AAG ACA CTT GGA ATT CCA AGC GCG AAG TT TT C TTC GCG CTT GGA ATT CCA AGT GTC TTG TC
Probe **2**	GAA TTC CAA GCG CGA AG TTTT CTT CGC GCT TGG AAT TC-Biotin
Probe **3**	Biotin-CA CTG CCA AGA ATT CCA AGC GCG AAG TTT TTT-FITC
Probe **4**	FITC-TT CTT TTT CAC CAT TCT AAA GAA TAA CAG TAA TTT CTG GGT TAA GGT-Biotin
Probe **5**	GAA TTC CAA GCG CGA AGT TTT CTT CGC GCT TGG AAT TC-NH_2
Probe **6**	GAA TTC CAA GCG CGA AG TTTT CTT CGC GCT TGG AAT TC TTTTTT CAC CAT TCT AAA GA ATA ACA GTG ATA ATT TCT GGG TTA AGG
Probe **7**	GAG ATA TTG CTA TTG CCT TAA CCC AGA AAT TAT CAC TGT TAT TCT TTA GAA TGG TG CAA AGA GGC
Probe **8**	GAG ATA TTG CTA TTA CCT TAA CCC AGA AAT TAT CAC TGT TAT TCT TTA GAA TGG TG CAA AGA GGC
Probe **9**	CAG TTA TAG CAA TCA CGT TTA CGC AAC ACT TAG CAG AGT TGT ACT TCA GTA TCG TG CTA AGC AAC

[a] Probes **1**, **2**, **3**, and **4** were used in gel electrophoresis analysis for validating terminal protection of DNA−small molecule chimeras against double-strand or single-strand selective exonucleases. The FITC was labeled to probes **3** and **4** to allow the fluorescence detection of the ssDNA in the gel. Probe **2** was also used in the terminal protection based fluorescent assay of small molecule−protein interactions for SA. Probe **5** was used as the control in the terminal protection based fluorescent assay of small molecule−protein interactions for SA. Probe **6** was used in the terminal protection based fluorescent genotyping assay. Probe **7** was the wildtype DNA target. Probe **8** was the mutant DNA target. Probe **9** was a noncDNA target to probe **6**.

the detection of DNA of various structures, which affords a conceptually useful mechanism for the analytics of small molecules. On the basis of this mechanism, a novel label-free biosensor strategy for homogeneous assay of protein−small molecule interactions using terminal protection against Exo III have been developed based on fluorescence staining of double-stranded DNA. Furthermore, through extension of a single nucleotide with a small molecule label, which translates SNP detection of DNA target into the assay of protein−small molecule interactions, a novel label-free SNP genotyping technique is proposed by using the fluorescence DNA staining procedure. The developed techniques are demonstrated using a model protein−small molecule system of biotin/streptavidin and a model SNP system of human β-globin gene around the codon 39 position, a known biallelic (G > A) SNP highly associated with β-thalassemia.[17] Compared with existing protein-binding and nucleic acid assay technologies that commonly involve separation steps, surface-based operations or complicated modifications, our assay strategy is able to offer desirable sensitivity, high selectivity, increased throughput, low cost, and simplified operations.

EXPERIMENTAL SECTION

Reagents and Materials. Taq DNA polymerase, double-strand selective exonucleases (Exo III, Exo λ, and Exo T7), thermosensitive alkaline phosphatise, and single-strand selective exonucleases (Exo I, Exo RecJ, or Exo T) were purchased from New England Biolabs (Ipswich, MA). Biotoin-14-ddATP, Biotin-14-ddGTP, Biotin-14-ddCTP, and Biotin-14-ddUTP were obtained from PerkinElmer (Wellesley, MA). Streptavidin (SA), thrombin, folate receptor (FR), concanavalin A, folic acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (Sulfo-NHS), and monoclonal antibodies to fluorescein isothiocyannate (FITC) were from Sigma Aldrich Chemical Co. SYBR Green I, bovine serum albumin (BSA), human serum albumin (HSA) immunoglobulins G (IgG), and human serum were purchased from Dingguo Biotech. Co. (Beijing, China). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. All 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). Thermodynamic parameters and secondary structures of all oligonucleotides were calculated using bioinformatics software (http://www.bioin-fo.rpi.edu/applications/). The sequences of the synthesized oligonucleotides are given in Table 1.

Gel Electrophoresis Analysis of Terminal Protection. To verify terminal protection of hairpin-structured DNA probes **1** and **2**, respectively, against Exo T7, Exo λ, and Exo III, DNA samples were prepared by adding 5 μM DNA in 25 μL of buffer solutions supplied with the corresponding exonucleases. Each sample was incubated for 30 min in the presence or in the absence of target proteins followed by extensive digestion for 30 min with the corresponding exonuclease, Exo T7 (100 U), Exo λ (100 U), or Exo III (10 U). All reactions were performed at room temperature (~25 °C). The digestion reaction was terminated through heating at 85 °C for 15 min. The resultant mixture was analyzed using gel electrophoresis in 3% (w/w) agarose containing 1% (w/w) ethidium bromide. The gel was visualized using a Tocan 240 gel imaging system (Shanghai Tocan Biotechnology Company).

To test terminal protection of single-stranded DNA probes **3** or **4**, respectively, against Exo RecJ, Exo T, and Exo I, DNA samples were prepared by adding 5 μM DNA in 25 μL of buffer solutions supplied with the corresponding exonucleases. To make the DNA detectable in gel, both DNA probes were labeled with FITC at the ends opposite to the small molecule modifiers. Each sample was incubated for 30 min in the presence or in the absence target proteins followed by extensive digestion for 30 min with the corresponding exonuclease, Exo RecJ (2000 U), Exo T (500 U), or Exo I (10 U). Other reaction and analysis conditions were the same as those for the aforementioned hairpin-structured DNA probes.

Terminal Protection Based Fluorescence Assay of Biotin−SA Interaction. A 25 μL aliquot of reagent solution containing 100 nM biotin-linked hairpin probe **2** in 1× Exo III buffer (10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH

7.0) was added in a given SA sample (5 μL, final SA concentrations ranging from 0 to 100 nM). The mixture was incubated at room temperature for 10 min to allow complete interaction between SA and biotin. Then, 10 U Exo III was added in the mixture and incubated for 10 min to perform the digestion reaction. After digestion, the resulting solution was immediately subjected to fluorescence measurements. The fluorescence spectra were recorded at room temperature in a quartz cuvette on an F-7000 spectrofluorometer (Hitachi, Japan). The excitation wavelength was 490 nm, and the emission wavelengths were in the range from 510 to 650 nm with both excitation and emission slits of 5 nm.

Terminal Protection Based Fluorescence SNP Typing Assay. A 12.5 μL aliquot of reagent solution containing 1 μM DNA probe **6**, 4 U *Taq* DNA polymerase, and one of four Biotin-14-ddNTP (10 μM) in 2× *Taq* DNA polymerase Buffer (40 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 2 mM DTT, pH 0.2 mM EDTA, 0.1% Triton X-100, pH 8.8) was added in 12.5 μL of target DNA sample of a given concentration (final target concentrations ranging from 0 to 200 nM). The mixture was subjected to a thermal cycling treatment to perform the single base extension reaction. Thermal cycling was carried out in a CFX96 real-time PCR detection system (Biorad, Germany) under the following condition: 20 cycles of 95 °C for 30 s, 66 °C for 45 s, and finally held at 4 °C in the thermal cycle. After the base extension reaction, 3 μL of SA (35 μM) was added into the reaction solution followed by incubation at room temperature for 10 min to allow complete interaction between SA and biotin. Then, 20 U Exo III and 20 U Exo I were added in the reaction mixture and incubated for 20 min to perform the digestion reaction. After digestion, the fluorescence response of the resulting solution was recorded at room temperature. The excitation wavelength was 490 nm, and the emission wavelengths were in the range from 510 to 650 nm with both excitation and emission slits of 5 nm.

For gel electrophoresis analysis, the base extension reaction was performed using 100 nM target DNA probe **7** and 2 μM DNA probe **6**. Other reaction conditions were the same as those for the given DNA samples.

Genomic Sample and PCR Amplification. Human genomic DNA was isolated from leukocytes in peripheral blood collected from patients with phenotypic β-thalassaemia using standard DNA extraction methods. PCR amplification was performed in 50 μL of 20 mM Tris-HCL (pH 8.8) with 10 mM $(NH_4)SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100 and 10 mM KCl, 250 μM dNTP, 250 μM forward and reverse primers (12.5 pmol for each primer), as well as ~20 ng of genomic DNA. The sequence of forward primer was 5′-CTT TCT TTC AGG GCA ATA AT-3′, and the sequence of the reverse primer was 5′-AAG AAA GCG AGC TTA GTG ATA-3′. Amplification was achieved by thermal cycling for 35 cycles at 95 °C for 30 s, 58 °C for 20 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min. In each amplification product, thermosensitive alkaline phosphatase (5 U) was added and incubated at room temperature for 30 min followed by inactivation of the phosphatase at 75 °C for 5 min. The resulting products were confirmed by an agarose gel assay and directly used for subsequent SNP typing without purification.

RESULTS AND DISCUSSION

Assessment of Generality of Terminal Protection from Exonuclease Digestion. We recently reported that the binding of a specific protein to a small molecule covalently attached to the 3′ terminus of a DNA strand could effectively prevent Exo I from digesting the DNA strand, presumably because the steric hindrance rendered from the protein−ligand interaction precluded the nuclease from freely accessing the 3′-terminus.[16] Although Exo I was the enzyme used in our previous investigation, we hypothesized that the terminal protection mechanism should work with other DNA exonucleases. To verify this, we tested several specially designed DNA probes (see Table 1 for their sequences) containing a biotin modifier at the 5′ or 3′ terminus for their abilities of acquiring protection against the digestion by some common DNA exonucleases in the presence of streptavidin (SA). The DNA digestion was analyzed using gel electrophoresis.

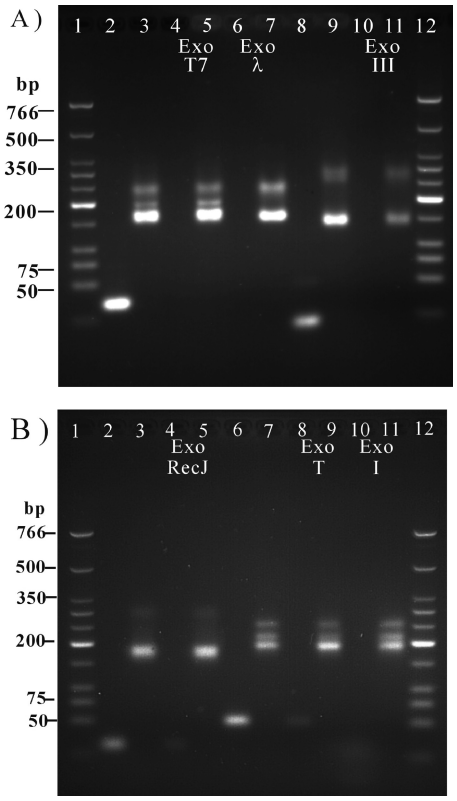


Figure 1. (A) Gel electrophoresis image for terminal protection against double-strand exonucleases. Lanes 1 and 12, DNA size marker; lane 2, biotin-linked probe **1**; lane 3, probe **1** plus SA; lane 4, probe **1** digested by Exo T7; lane 5, probe **1** plus SA digested by Exo T7; lane 6, probe **1** digested by Exo λ; lane 7, probe **1** plus SA digested by Exo λ; lane 8, biotin-linked probe **2**; lane 9, probe **2** plus SA; lane 10, probe **2** digested by Exo III; lane 11, probe **2** plus SA digested by Exo III. (B) Gel electrophoresis image for terminal protection against single-strand exonucleases. Lanes 1 and 12, DNA size marker; lane 2, biotin-linked probe **3**; lane 3, probe **3** plus SA; lane 4, probe **3** digested by Exo RecJ; lane 5, probe **3** plus SA digested by Exo RecJ; lane 6, biotin-linked probe **4**; lane 7, probe **4** plus SA; lane 8, probe **4** digested by Exo T; lane 9, probe **4** plus SA digested by Exo T; lane 10, probe **4** digested by Exo I; lane 11, probe **4** plus SA digested by Exo I.

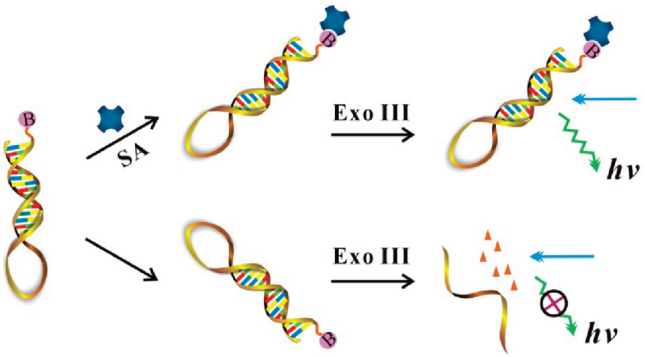
Figure 1 displays typical gel electrophoresis images of these biotin-DNA chimeras before and after degradation by DNA exonucleases. As shown in Figure 1A, it was observed that, after hairpin DNA probes **1** and **2** were digested by the corresponding 5′ or 3′ double-strand selective exonucleases (Exo T7, Exo λ, or Exo III), no bright bands were obtained on the gel (lanes 4, 6, and 10 in Figure 1A), which contrasted clearly with the intact probes

(Lanes 2 and 8 in Figure 1A). Note that only double-strand DNA could be stained selectively using ethidium bromide to generate strong fluorescence, while single-strand DNA and protein would not be stained to display fluorescence signal. We could infer that in the absence of SA, the double-stranded probes **1** and **2** were degraded by these double-strand exonucleases into single-stranded DNA residuals and nucleotides. Interestingly, after hairpin DNA probes **1** and **2** were digested by the exonucleases in the presence of SA, we still observed bright bands with obviously decreased migration shifts (lanes 5, 7, and 11 in Figure 1A). As decreased migration shifts were clear indicators for protein binding, these bright bands were actually arising from undegraded probes bound to protein target, suggesting terminal protection of these small molecule-linked DNA from double-strand selective exonucleases (Exo T7, Exo λ, and Exo III). Our hypothesis of terminal protection was also confirmed in the experiments with single-stranded DNA probes. To visualize single-strand DNA probes that could not be efficiently stained on the gel, we labeled each single-strand DNA probe with a fluorescence reporter at the end opposite to the biotin moiety. After single-stranded DNA probes **3** and **4** digested by 5′ or 3′ single-strand selective exonucleases (Exo RecJ, Exo T, and Exo I), we did not observe any bright bands on the gel, as shown in Figure 1B (lanes 4, 8, and 10). This suggested that in the absence of SA, the single-stranded probes **3** and **4** were degraded by the single-strand exonucleases. In contrast, with probes **3** and **4** incubated with SA followed by exonuclease digestion, bright bands with decreased migration shifts were obtained (lanes 5, 9, and 11 in Figure 1B), indicating terminal protection of small molecule-linked single-stranded DNA from single-strand selective exonucleases (Exo RecJ, Exo T, and Exo I).

Furthermore, we tested terminal protection using other small molecule ligands and protein receptors such as folate and its receptor FR (see Figure S1 in the Supporting Information) as well as fluorescein isothiocyannate (FITC) and its antibody (see Figure S2 in the Supporting Information). We also observed that the probes remained undegraded on binding the corresponding protein targets even after exonuclease digestion. The results were in good consistency with the aforementioned finding of terminal protection. Note that small molecule−protein interactions we investigated above typically displayed dissociation constants in the nanomolar range. For instance, the dissociation constant was ∼0.1 nM for folate and its receptor FR and was ∼10 nM for FITC and its antibody. Therefore, terminal protection of small molecule-linked DNA could be a general mechanism for interrogating the interactions of protein−small molecule pairs with nanomolar dissociation constants, and it is applicable to common double-strand or single-strand DNA exonucleases.

It is important to note that the system of terminal protection comprises two competitive reactions, digestion of small molecule-linked DNA by DNA exonucleases against binding of small molecules by protein targets. Terminal protection may occur only when small molecule−protein binding displays dominant kinetics over exonuclease digestion. For protein−small molecule pairs with dissociation constants up to the micromolar level, the rate of exonuclease digestion may be comparable to that for protein−small molecule binding. Then, terminal protection may not be achieved without thorough optimization of the digestion conditions. Despite the limit, terminal protection may still hold great potential for the analysis of small molecule−protein interactions because there have been lots of small-molecule antibodies or receptors as well as potent drugs with nanomolar dissociation constants. Moreover, with utilization of the photo cross-linking technology that allows covalent conjugation of small molecule-linked DNA to the interacting proteins,[18] terminal protection can be further extended beyond the assays of protein−small molecule pairs with varying dissociation constants (nanomolar to micromolar).

Scheme 1. Terminal Protection Based Fluorescent Assay of Small Molecule−Protein Interactions[a]



[a] DNA probe is hydrolyzed from the 3′ end by Exo III, producing a single-stranded DNA not stained by SYBR Green I. When target protein is bound to small-molecule moiety, terminal protection prevents Exo III from degrading the DNA probe, leaving the double-structure intact, which is stained by SYBR Green I and with a strong fluorescence signal obtained.

On the basis of the generalization of terminal protection, small molecule−protein interaction assays can be converted to the detection of DNA of various structures. This conversion is conceptually useful, considering the tremendous importance of small-molecule analysis in chemistry and medicine[1,2] as well as the vast arsenal of signal transduction and amplification tactics for DNA assay.[19−21] In support of this concept, here we develop simple but sensitive, robust biosensor strategies for homogeneous assay of protein−small molecule interactions and SNP genotyping based on fluorescence staining of double-stranded DNA.

Design of Protein−Small Molecule Interaction Assay. The developed strategy for the protein−small molecule interaction assay relies on terminal protection of a double-stranded DNA probe against Exo III, a most common double-stranded exonuclease. This analytical principle is illustrated in Scheme 1. A DNA probe is designed to have a complementary sequence at two ends, which allows the probe to be self-assembled into a hairpin structure. The protein-binding small molecules are covalently conjugated to the DNA probe at the 3′ end. In the absence of target protein, the small molecule-linked DNA probe is hydrolyzed from the 3′ end by Exo III, successively liberating mononucleotides and finally producing a single-stranded DNA. Because single-stranded DNA cannot be stained with SYBR Green I, very weak fluorescence is obtained. On the other hand, when target protein is bound to the DNA probe through the small-molecule moiety, terminal protection prevents Exo III from degrading the hairpin DNA probe. This renders an intact double-structure, which can be stained by SYBR Green I with a strong fluorescence signal generated.

The developed strategy was demonstrated using a model system of SA and biotin commonly utilized for the small molecule−protein interaction assay.[15,22] Figure 2 depicts typical fluorescence responses of the terminal protection assay for this

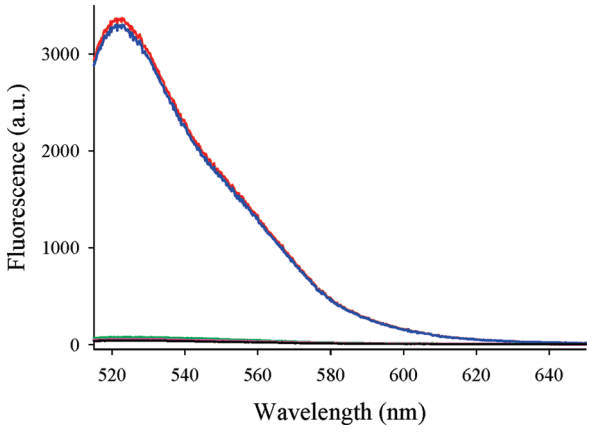


Figure 2. Typical fluorescence responses of small molecule−protein interaction assay. 100 nM probe **2** before digestion by Exo III (red); 100 nM probe **2** digested by Exo III (green); 100 nM probe **2** plus 75 nM SA digested by Exo III (blue); 100 nM probe **2** was plus 1 μM BSA digested by Exo III (black);100 nM probe **5** plus 75 nM SA digested by Exo III (pink).

system. Before the biotin-modified DNA probe **2** (100 nM) digested by Exo III, we observed a very strong fluorescence peak with a maximum intensity of ∼3200 at 522 nm in typical fluorescence spectra for double-stranded DNA stained with SYBR Green I. This suggested that DNA probe **2** was folded into a hairpin structure and stained effectively by SYBR Green I. After DNA probe **2** reacted with Exo III for 10 min, a diminished fluorescence response (fluorescence intensity ∼50 at 522 nm) was obtained, indicating that double-stranded structure of the DNA probe was degraded by Exo III. After DNA probe **2** was incubated with SA (75 nM) for 10 min followed by Exo III digestion, the fluorescence intensity (∼3200 at 522 nm) remained nearly equal to that for the intact probe, giving a high signal-to-background ratio of ∼64 (compared with fluorescence intensity for degraded probe). Two control experiments were performed, one using a nontarget protein (1 μM bovine serum albumin) instead of SA, and the other using probe **5**, a hairpin DNA without biotin modifier, instead of probe **2**. In contrast, no strong fluorescence readouts were obtained in these control experiments, implying that the terminal protection was specific to the interaction between biotin and SA.

Figure 3A displays the fluorescence spectra of terminal protection assay for SA of varying concentrations. Dynamically increased fluorescence peaks were observed in response to SA of increasing concentrations within the range from 0.5 to 100 nM. The peak intensity showed a linear correlation to SA concentration in the range from 0.5 to 37.5 nM, as shown in Figure 3B. The detection limit was estimated to be 0.1 nM according to the 3σ rule. It was also observed that the strategy exhibited excellent reproducibility due to its homogeneous assay format with simple operations. Relative standard deviations (RSDs) of fluorescence responses at 522 nm were 3.0%, 1.1%, 1.4%, and 1.2% in five repetitive assays of 0.5, 5, 25, and 50 nM SA. On the basis of the quantitative nature, the developed strategy could also be used for determining the affinity constant for a given small molecule−protein binding event or quantifying small molecules using a competitive assay format.

Additional assays were performed to inspect the selectivity of the assay to other samples such as human serum albumin (1 μM), immunoglobulins G (1 μM), thrombin (1 μM), folate receptor

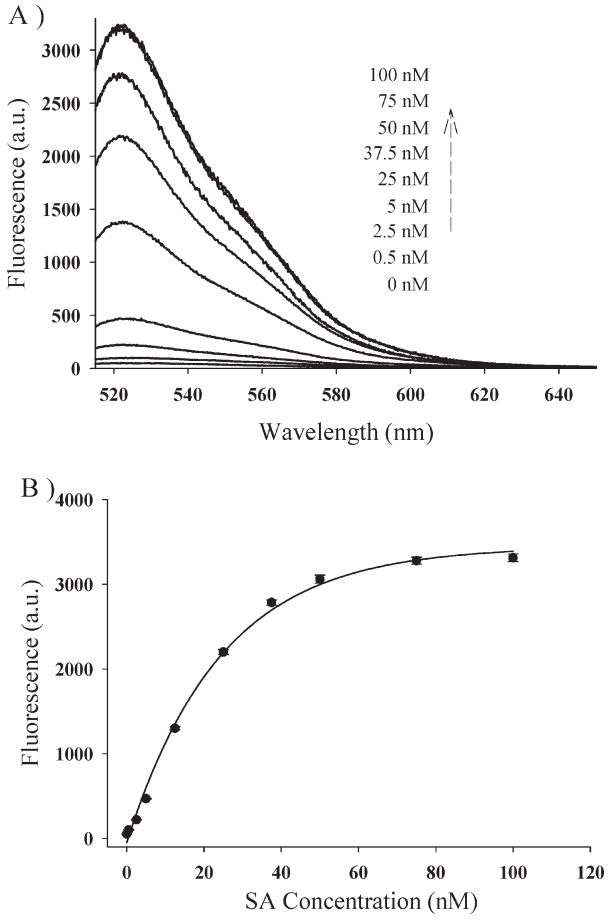


Figure 3. (A) Typical fluorescence spectral responses of the small molecule−protein interaction assay to SA of varying concentrations. (B) Fluorescence response at 522 nm of the small molecule−protein interaction assay versus SA concentration. Error bars are standard deviation (SD) across five repetitive experiments.

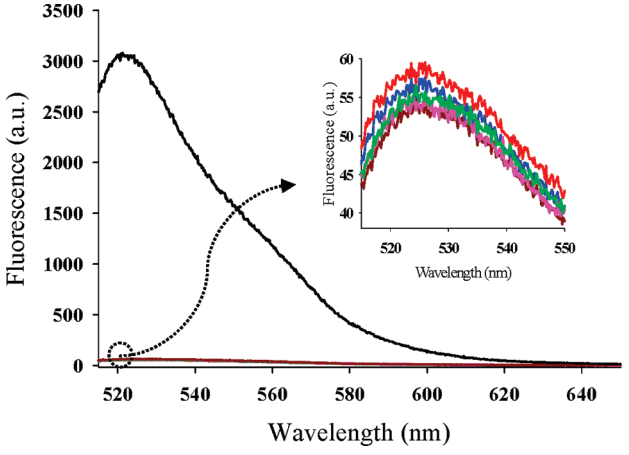


Figure 4. Fluorescence spectral responses of the small molecule−protein interaction assay to different samples: 75 nM SA (black), 1 μM human serum albumin (blue), 1 μM immunoglobulins G (red), 1 μM thrombin (green), 1 μM folate receptor (pink), 1 μM concanavalin A (dark red), and 10-fold diluted human serum (dark red).

(1 μM), concanavalin A (1 μM), and human serum (10-fold diluted), as shown in Figure 4. The results also revealed high

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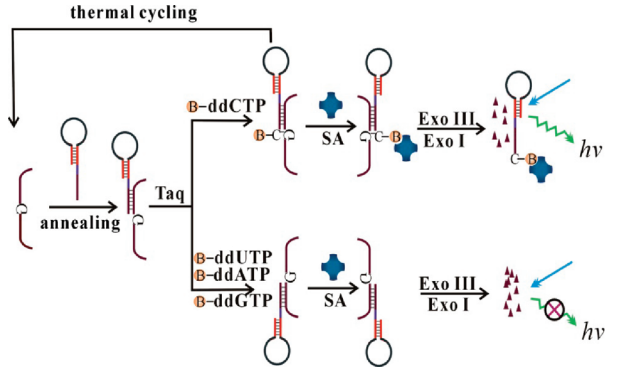
Scheme 2. Terminal Protection Based Fluorescent Genotyping Assay[a]



[a] DNA probe with 5′ hairpin structure and 3′ overhang primer is annealed to target DNA adjacent to the SNP base. Biotinylated nucleotide matching the SNP site is incorporated by Taq polymerase at the primer end. The biotin-linked DNA probe, when binding to SA, becomes protected against Exo I and Exo III with the intact hairpin structure stained by SYBR Green I and delivers strong fluorescence. Mismatched biotinylated nucleotides are not incorporated, and DNA probe remains unprotected and becomes degraded with no fluorescence staining detected.

selectivity of the developed strategy. Such high specificity could be originated from the fact that terminal protection required relatively stable interaction between small molecules and target proteins. Because of the instability of nonspecific adsorption, nontarget proteins could not prevent degradation of DNA by exonucleases. This signified a prominent advantage of terminal protection that it had high resistance to nonspecific interactions over conventional small molecule−protein interaction assays such as fluorescence anisotropy[8] or surface plasmon resonance.[10,11]

In addition, the developed strategy was further validated using another system of folate and its receptor (see Figure S3 in the Supporting Information). It was observed that the strategy displayed fluorescence peaks in dynamic correlation to the FR concentrations in the range from 1 to 200 nM. The detection limit was estimated to be 0.4 nM according to the 3σ rule. Relative standard deviations (RSDs) of fluorescence responses at 522 nm were 1.9%, 2.1%, 2.1%, and 2.6% in five repetitive assays of 1, 25, 100, and 200 nM FA. These results confirmed the desirable performance of the developed strategy. Compared with other reported methods in detecting biotin and other small molecules,[15,22] the developed strategy displays slightly improved sensitivity, higher signal-to-background ratio, and enhanced resistance to nonspecific adsorption. Also, the homogeneous assay format allows this technique can be greatly robust, cost-efficient, readily automated, and scalable for parallel assays of hundreds of samples. In the setting, this strategy may hold potential as a versatile platform for molecular diagnostics, chemical biology, and drug developments.

Design of DNA Genotyping Based on Terminal Protection. Besides its potential in the protein−small molecule interaction assay, terminal protection may also find multiple uses in DNA analysis due to increasing significance in detecting the modifications of DNA with small molecules. In the context, this above-developed small molecule−protein interaction assay strategy is further adapted as a new technique for SNP genotyping.

SNP typing is to identify single base difference at a specific location of the genome.[23−25] Our SNP typing method is based on the extension of a single nucleotide with a small molecule

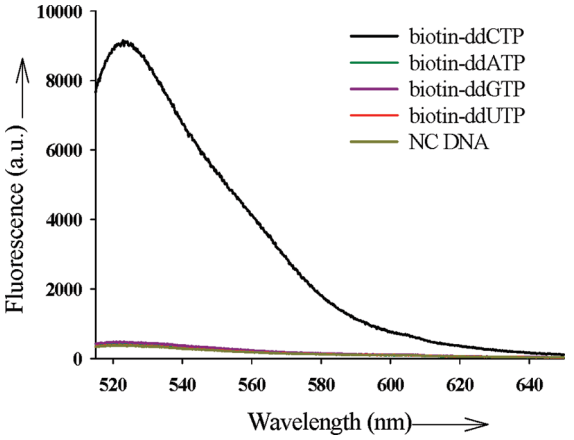


Figure 5. Typical fluorescence responses of genotyping assay to 100 nM wildtype DNA target 7 in the presence of one of four biotinylated nucleotides and to noncDNA target 9 (NC DNA).

label, thereby translating SNP detection into the protein−small molecule binding assay. This analytical principle is illustrated in Scheme 2. A DNA probe **6** is designed, in which near the 5′ end is a hairpin structured DNA sequence, and near the 3′ end is a primer that can be immediately annealed to target DNA upstream of the polymorphism site. After probe **6** hybridizes with target DNA adjacent to the SNP base, DNA polymerase can only incorporate the biotin-modified nucleotide complementary to the SNP base into the 3′ terminus of the primer. In the presence of the complementary biotin-modified nucleotide, probe **6** is extended by the biotin-modified nucleotide, yielding a biotin-linked DNA that can bind to SA and achieve terminal protection against Exo I and Exo III. Because the protected biotin-linked DNA has a double-stranded region stainable by SYBR Green I, a strong fluorescence signal is obtained. With biotinylated nucleotides noncomplementary to the SNP base, probe **6** cannot be extended, which cannot bind to SA and thus be completely degraded by Exo I and Exo III with no remarkable fluorescence generated. Therefore, on the basis of the identity of the added biotinylated nucleotide that enables the extension and terminal protection of DNA probe **6** as indicated by the strong fluorescent staining, we can define the genotype of the target DNA.

The SNP typing technique was tested using model DNA sequences of human β-globin gene around the codon 39 position, a known biallelic (G > A) SNP highly associated with β-thalassemia.[26] Figure 5 depicts typical fluorescence responses of this technique to 100 nM wildtype target **7** in the presence of one of four biotinylated nucleotides. With biotin-ddUTP, biotin-ddATP, or biotin-ddGTP added in the reaction mixture, very weak fluorescence peaks were observed (fluorescence intensity ~450 at 522 nm). In contrast, with biotin-ddCTP, we obtained a strong fluorescence peak (fluorescence intensity ~9000 at 522 nm) characteristic for double stranded DNA−SYBR Green I complex. This afforded a desirable signal-to-background ratio of ~20. Because DNA polymerase incorporated only the nucleotide complementary to the SNP base and then mediated terminal protection of DNA probe **6**, we could immediately identify the SNP base of the wildtype target as G, consistent with the genotype of the wildtype target. A control experiment was conducted using DNA sequence **9** different from the wildtype target. It was observed that even in the presence of biotin-ddCTP, we still obtained a very weak fluorescence signal. Note that

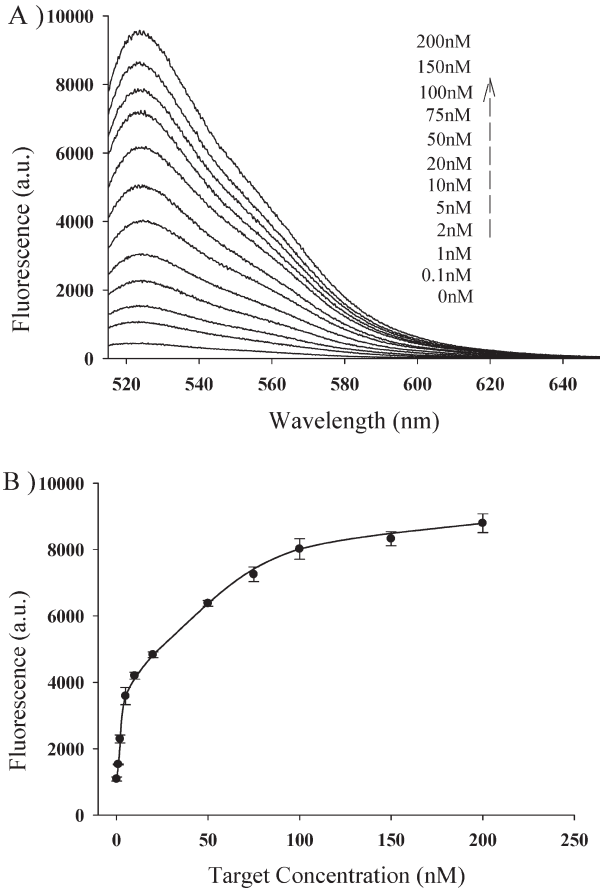


Figure 6. (A) Typical fluorescence responses of genotyping assay to wildtype DNA target **7** of varying concentrations with biotinylated ddCTP. (B) Fluorescence responses at 522 nm of the genotyping assay versus target DNA probe 7 concentrations. Error bars are SD across five repetitive experiments.

terminal protection only occurred when the DNA probe was extended in the presence of the DNA target and the matched biotinylated nucleotide, while genomic DNA and DNA probes were completely degraded by Exo I and Exo III. This observation actually implied that the developed technique was highly specific for target genes, and coexisting DNA sequences mismatching the primer region of probe **6** would not lead to substantial fluorescence background due to complete degradation. The assay of mutant DNA target **8** also confirmed this conclusion (see Figure S4 in the Supporting Information).

Quantitative Nature of DNA Genotying Based on Terminal Protection. Figure 6A displays fluorescence responses of the genotyping technique to wildtype target **7** of varying concentrations with the addition of biotinylated ddCTP. The fluorescence peaks were observed dynamically increased as increasing concentrations of wildtype target within the range from 0.1 to 200 nM with a detection limit estimated to be 0.02 nM, as shown in Figure 6B. Desirable reproducibility was also achieved in the SNP assay, and RSD of fluorescence readouts at 522 nm were 1.5%, 1.7%, 2.4%, and 3.2% in five repetitive assays of 0.1, 5, 50, and 200 nM wildtype targets. Such high stability, which was attributed to the choices of polymerase reaction, homogeneous format, simple assay operations, and the new terminal protection mechanism, might support the developed technique as an ideal alternative to existing SNP typing technologies.

The genotyping technique was further evaluated using human genomic DNA with SNP (G > A) at the codon 39 position in the β-globin gene. A total of 12 genomic samples were collected with the genotypes characterized by DNA sequence analysis. Polymerase chain reaction (PCR) was performed on these samples to obtain 423-base-pair amplicons. The PCR amplicons were confirmed by agarose gel assay (see Figure S5 in the Supporting Information) and subsequently utilized for SNP typing by using the developed technique. The results (see Figure S6 in the Supporting Information) revealed that the fluorescence response patterns could reliably signify the genotypes with favorable signal-to-background ratio, and the identified SNP types were in good agreement with the sequencing data. This suggested that the genotyping strategy might be suitable for genomic research and clinical diagnostics.

CONCLUSIONS

We reported a generalized discovery of terminal protection that small molecule-DNA chimeras were protected from degradation by various DNA exonucleases, 5′ or 3′-specific and single-strand or double-strand selective exonucleases, when the small molecule moieties were bound to their protein targets. This generalization converted small molecule−protein interaction assays into the detection of DNA of various structures, which afforded a conceptually powerful mechanism for the analytics of small molecules and DNA modifications with small molecules. As demonstrative examples, novel biosensor strategies for homogeneous assay of protein−small molecule interactions and SNP types were developed based on fluorescence staining of double-stranded DNA. These strategies were validated to exhibit superb robustness, high signal-to-background ratio, desirable sensitivity and selectivity, low cost, and simple operations. The combination of microtiter plate formats would facilitate the applications of these techniques in high-throughput studies. Further developments using multicolor dye−quencher based design could be devised, which should allow these strategies to be useful for multiplex assays. In view of these advantages, the generalized terminal protection mechanism and the developed biosensor strategies for the protein−small molecule interaction assay and SNP genotyping were expected to hold considerable potential for molecular diagnostics, genomic research, and drug development.

ASSOCIATED CONTENT

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

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