Improved Sensitivity for the Electrochemical Biosensor with an Adjunct Probe

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Despite their promising applications in the biomedical research, the development of electrochemical biosensors with improved sensitivity and low detection limit has remained a great challenge. Here, we demonstrate a new approach to improve the sensitivity of the electrochemical biosensor by simply introducing an adjunct probe into its construction. This signal-on biosensor consists of a thiol-functionalized capture probe attached on the gold electrode surface, an electrochemical sign (methyl blue, MB)-modified reporter probe which is complementary to the capture probe, and an adjunct probe attached nearby the capture probe. The adjunct probe functions as a fixer to immobilize the element of reporter probe which is displaced by the target DNA and protein, increasing the chance of the dissociative reporter probe to collide with the electrode surface and facilitating the electron transfer. The biosensor with an adjunct probe exhibits improved sensitivity and a large dynamic range for DNA and the thrombin assay and can even distinguish 1-base mismatched target DNA. Importantly, the use of this biosensor is not limited to such and is viable for sensitive detection of numerous biomolecules, including RNA, proteins, and small molecules such as cocaine.

Nucleic acid analysis is becoming increasingly important in the diagnosis of hereditary diseases, detection of infectious agents, and forensic and paternity testing. Accordingly, the development of cost-effective biosensors capable of high-sensitive detection of nucleic acids has attracted much attention worldwide,[1−3] and great efforts have been put into developing a variety of such biosensors, including optical, piezoelectric, and electronic transduction-based biosensors.[4−6] Among them, electrochemical DNA (E-DNA) biosensors have been proved to be promising devices for the point-of-care diagnostics owing to their attractive features such as high sensitivity, fast response, and cost-effective requirements.[7−21] Most of the E-DNA biosensors are developed on the basis of an appropriate electrochemical-active indicator in combination with the DNA hybridization reaction,[7,16] and the "sandwich-type" architecture with a pair of probes, a capture and a reporter, is popularly employed.[17] The E-DNA biosensors include both signal-on biosensors and signal-off ones,[19−21] and the sensitivity of signal-on biosensors is usually superior to that of signal-off ones form a practical point of view. To improve the sensitivity of the E-DNA biosensors, different kinds of approaches have been developed, including the use of nanomaterial labels (such as carbon nanotubes,[22] gold and silver nanoparticles,[23,24] magnetic nanospheres,[25] and quantum dots[26]) and replacement of small redox molecules with some redox enzymes.[27] However, most of the previously reported E-DNA biosensors involve complicated structural design of the capture probes, complex pretreatment of electrodes, and

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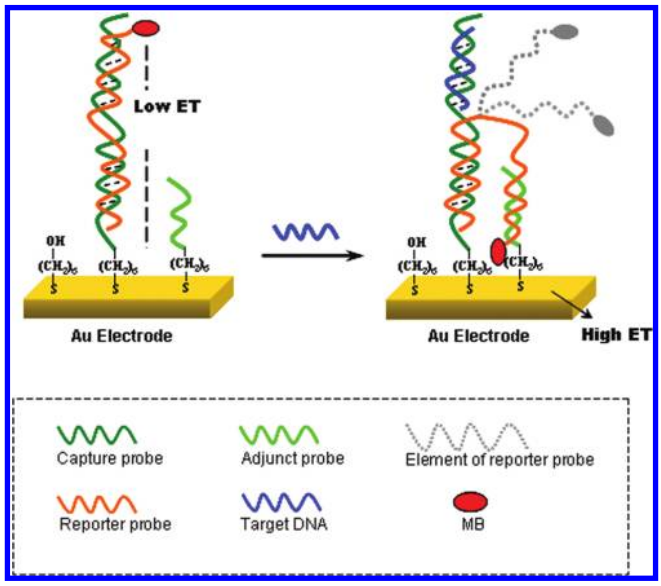
Scheme 1. Scheme for Sequence-Specific DNA Detection Using the E-DNA Biosensor with an Adjunct Probe



frequent interference from the detection environment.[28] Consequently, the development of E-DNA biosensors with improved sensitivity and low detection limit still remains a great challenge.

Herein, we report the development of a signal-on E-DNA biosensor with improved sensitivity and large dynamic range by simply introducing an adjunct probe (Scheme 1). The adjunct probe attached nearby the capture probe functions as a fixer to immobilize the flexible electrochemical-sign-methylene-blue (MB)-labeled reporter probe which is displaced by the hybridization of target DNA with the capture probe, consequently increasing the chance of the dissociative reporter probe to collide with the electrode surface and facilitating the electron transfer. This E-DNA biosensor has significant advantages of simple fabrication process, signal-on detection, improved sensitivity, large dynamic range, and less interference from the detection environment. We demonstrate that this E-DNA biosensor might be further extended to the protein detection on the basis of the construction of aptamer-based biosensor for thrombin.

EXPERIMENTAL SECTION

Materials. All the probes and target oligonucleotides were obtained from Takara Biotechnology Company. The oligonucleotides were purified via C18 reversed-phase HPLC and polyacrylamide gel electrophoresis (PAGE). The sequences of probes and target oligonucleotides used for DNA detection were as follows: capture probe, 5′-HS-$(CH_2)_6$-AGA CAA GGA AAA TCC TTC CCC CCC CTG AAG TGG GTC GAA AA-3′; adjunct probe, 5′-AAT GAA GTG GGT CG-$(CH2)_3$-SH-3′; reporter probe, 5′-$(CH_2)_6$-MB-CGA CCC ACT TCA TTC CCC CCT TGA AGG ATT TTC CTT GTC T-3′; perfect matched target DNA, 5′-TTT TCG ACC CAC TTC A-3′; mismatched target DNA, 5′-TTT TCC ACC CAC TTC A-3′; mismatched adjunct probe, 5′-AAT GAA CTG GGT CG-$(CH_2)_3$-SH-3′. The sequences of probe and target oligonucleotides used for thrombin detection were as follows: capture probe, 5′-HS-$(CH_2)_6$-AGA CAA GGA AAA TCC TTC CCC CCC CGG TTG GTG TGG TTG G-3′; reporter probe, 5′-$(CH_2)_6$-MB-CCA ACC ACA CCA ACC CCC CCC CCT TGA AGG ATT TTC CTT GTC T-3′; adjunct probe, 5′-GGT TGG TGT GGT TGG-$(CH2)_3$-SH-3′. 6-Mercaptohexanol, Tris(2-carboxyethyl)phosphine hydrochloride, thrombin (lyophilized powder, 2000 units/mg), bovine plasma albumin (BSA), and lysozyme were purchased from Sigma-Aldrich, Inc. (U.S.A.) and used as received without further purification. The buffer solutions were as follows: the DNA immobilization buffer containing 200 mM Tris−HCl (pH 7.4, 25 °C) and 10 mM Tris−HCl (pH 7.4, 25 °C); the hybridization buffer containing 50 mM Tris−HCl, 100 mM NaCl, and 0.1% Tween 20 (pH 7.4, 25 °C); the washing buffer containing 100 mM phosphate buffer solution (PBS, pH 7.4, 25 °C) and 0.5% Tween 20; and the buffer for electrochemical measurements containing 10 mM PBS (pH 7.4, 25 °C) and 100 mM NaCl. All above solutions were prepared with Milli-Q water (18.2 MΩ·cm).

Preparation of the E-DNA Sensors. The E-DNA biosensor was fabricated by the modification of polycrystalline gold electrodes (2.0 mm diameter, CHI Co. Ltd., Shanghai, China) with the DNA probes according to the reported protocol.[29] Briefly, the gold electrode was first polished on microcloth with alumina suspensions (1.0, 0.3, and 0.05 mm in diameter) in sequence, and the residual alumina powder was removed by sonicating electrodes sequentially in Milli-Q water, ethanol, acetone, and Milli-Q water. The above electrode was further electrochemically cleaned by a series of oxidation and reduction cycling in 0.5 M NaOH, 0.5 M H_2SO_4, 0.01 M KCl, 0.1 M H_2SO_4, and 0.05 M H_2SO_4 and finally rinsed with Milli-Q water. After drying with nitrogen, the electrode was reacted with 0.5 μM capture probes and 5.0 μM Tris(2-carboxyethyl) phosphine hydrochloride in 200 mM Tris−HCl buffer (pH 7.4) for 16 h at 37 °C.[30] To immobilize the adjunct probes onto the electrode surface, the electrode was reacted with the solution containing 0.5 μM capture probes and the adjunct probes, and the density of adjunct probes on the electrode surface was modulated by varying the concentration of adjunct probes (0.01, 0.02, 0.05, 0.08, 0.1, and 0.12 μM). After being washed with Tris−HCl buffer, the capture probe/adjunct probe-modified electrode was treated with 1 μM 6-mercaptohexanol in 10 mM Tris−HCl buffer (pH 7.4) at 30 °C for 2 h to obtain well-aligned DNA monolayers on the electrode surface. The above electrode was further rinsed with Milli-Q water and treated with 0.5 μM reporter probe in the hybridization buffer (50 mM Tris−HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.4) at 40 °C for 4 h to yield capture/adjunct/reporter probe-functionalized electrode. This E-DNA biosensor might be stored in high salt phosphate buffer at 4 °C for at least 2 weeks.

The fabricating process of the aptamer-based biosensor was similar to that of the E-DNA biosensor. Briefly, the precleaned gold electrode was treated with 0.5 μM capture probe (aptamer) and 5 μM Tris(2-carboxyethyl) phosphine hydrochloride in 200 mM Tris−HCl buffer (pH 7.4) at 37 °C for 16 h. To immobilize the adjunct probe onto the electrode surface, the solution containing 0.5 μM capture probes and 0.08 μM adjunct probes was added to the electrode surface. After being washed with Tris−HCl buffer, the electrode was treated with 1 μM 6-mercaptohexanol in 10 mM

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Tris−HCl buffer (pH 7.4) at 30 °C for 2 h. After being rinsed with Milli-Q water, the electrode was modified with 0.5 μM reporter probes in the buffer (50 mM Tris−HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.4, 25 °C) at 45 °C for 4 h to yield the aptamer/adjunct/reporter probe-functionalized electrode.

Detection of Target DNA and Thrombin. After being washed with ice-cold washing buffer (100 mM PBS, pH 7.4, 25 °C, 0.5% Tween 20), the electrode was rinsed with the solution containing 10 mM PBS (pH 7.4, 25 °C) and 100 mM NaCl at least three times. All the electrochemical measurements were performed on the electrochemical workstation (CHI 660D, CH Instruments, Chenhua Corp., Shanghai, China) using square wave voltammetry (SWV) at an alternative frequency of 15 Hz and cyclic voltammograms (CV) at an scan rate of 50 $mV \cdot s^{-1}$ in a typical three-electrode system with a platinum electrode as the counter electrode, a Ag/AgCl electrode (saturated with KCl) as the reference electrode, and the functionalized electrode as the working electrode. The solution containing 10 mM PBS (pH 7.4) and 100 mM NaCl was used as the supporting electrolyte medium. To study the effect of adjunct probe density on the detection sensitivity, the adjunct probes with different concentration were used to modify the electrode surface. To detect the target DNA, the electrodes were incubated with target DNA solutions (125 μL in volume) in hybridization buffer (50 mM Tris−HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.4) at 45 °C for 5 h. To detect thrombin, the capture probe/reporter probe-modified electrode (with adjunct probe or without adjunct probe) was incubated with various concentrations of thrombin (125 μL in volume) in the buffer (50 mM Tris−HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.4) at 28 °C for 3 h. After the reaction with the targets, the electrode was rinsed with the washing buffer (100 mM PBS, 0.5% Tween 20, pH 7.4) and subjected to the measurement on the electrochemical workstation.

RESULTS AND DISCUSSION

Improved Sensitivity for DNA Detection with the Introduction of an Adjunct Probe. As shown in Scheme 1, the E-DNA biosensor was constructed by attaching a MB-modified reporter probe onto the thiol-terminated capture probe which was immobilized on the surface of gold electrode via gold−sulfur chemistry. In the absence of the target DNA, the double-strand DNA (dsDNA) consisting of a capture probe and a reporter probe blocked the MB from the electrode surface, generating a relatively low electrochemical signal. With the addition of a 16-base complementary target DNA, the hybridization of target DNA with the capture probe displaced the 12-base element from the reporter probe, producing a relatively dissociative MB-modified 22-base single-strand DNA (ssDNA). The flexible ssDNA allowed the MB to easily collide with the electrode surface, generating an enhanced electrochemical signal. However, due to the flexibility of MB-modified ssDNA, the MB might part from the electrode surface with a relatively long distance and an incomplete contact between them, consequently limiting the enhancement of electrochemical signals.

To improve the detection sensitivity, we introduced an adjunct probe nearby the capture probe. The adjunct probe was a thiol-terminated 14-base ssDNA which could hybrid with part of the MB-modified reporter probe. This adjunct probe might function a fixer to immobilize the dissociative MB onto the electrode surface (Scheme 1), increasing the chance of electrochemical sign MB to collide with the electrode surface. As shown in Figure 1A, with the addition of 0.2 nM target DNA, the electrochemical signal from the E-DNA biosensor without an adjunct probe increased by as much as 20.0% as compared to the control group, while the electrochemical signal from the E-DNA biosensor with an adjunct probe increased by as much as 84.0% as compared to the control group. As a result, the signal increase had been improved by as much as 3.2 times in the E-DNA biosensor with an adjunct probe compared to that without an adjunct probe. The improved sensitivity obtained by the E-DNA biosensor with an adjunct probe could be attributed to the immobilization of flexible MB-modified reporter probe onto the electrode surface by the hybridization of the adjunct probe with the reporter probe, which greatly shortened the distance between MB and the electrode and facilitated the electron transfer. This was confirmed by the measurement of CVs. As shown in Figure 1B, the E-DNA biosensor with an adjunct probe produced a higher CV signal than that without an adjunct probe. It was worth noting that the electrochemical signal from the E-DNA biosensor with an adjunct probe displayed a shift of up to 40 mV (Figure 1A-c) as compared to that without an adjunct probe (Figure 1A-b); this might result from the intercolation of MB within dsDNA, which would be present in the presence of an adjunct probe but not in its absence.[8,21]

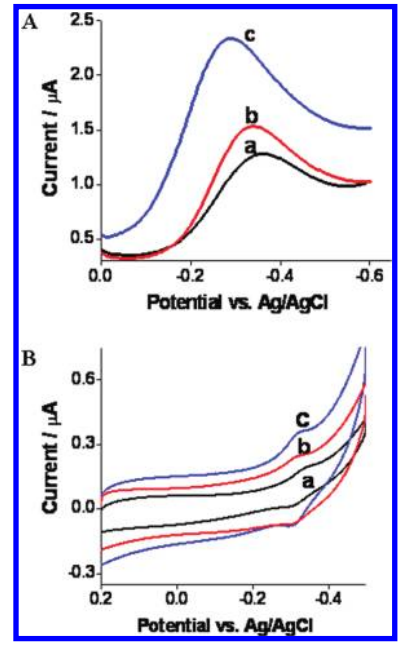

Figure 1. Improved sensitivity for the E-DNA biosensor with an adjunct probe. The electrochemical signals (SWV (A), CV (B)) obtained in buffer (a), capture probes only (b), and capture probes plus adjunct probes (c). The target DNA concentration was 0.2 nM in b and c. The electrode surface of the E-DNA biosensor was modified by 0.08 μM adjunct probe in c.

The introduction of an adjunct probe might lead to a much lower detection limit and large dynamic range for nucleic acid detection. Figure 2A,B shows the electrochemical signals in response to different concentrations of target DNA measured by square wave voltammetry. The measured detection limit of the E-DNA biosensor without an adjunct probe was only 40.0 pM (Figure 2C); However, the measured detection limit of the E-DNA biosensor with an adjunct probe might reach 2.0 pM (Figure 2D), which had been improved by as much as 20 times and was more

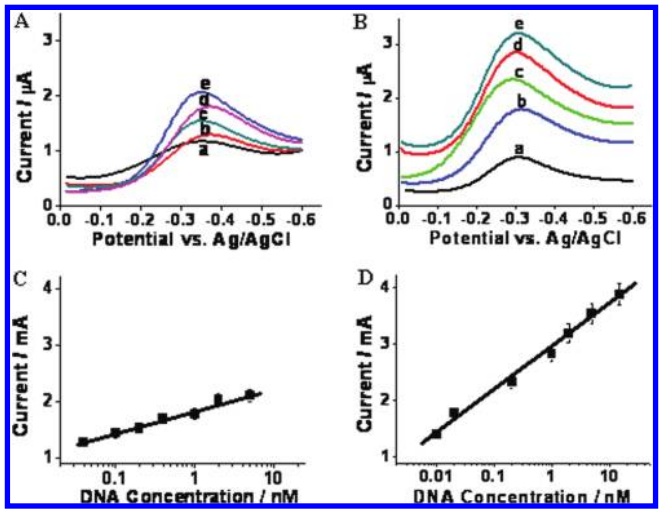

Figure 2. (A) Electrochemical signals from the E-DNA biosensor without an adjunct probe in response to different concentrations of perfect matched target DNA. The target DNA concentration shown in (A) was 0 (a), 0.04 nM (b), 0.2 nM (c), 1 nM (d), and 2 nM (e), separately. (B) The electrochemical signals from the E-DNA biosensor with an adjunct probe in response to different concentrations of perfect matched target DNA. The target DNA concentration shown in the (B) was 0 (a), 0.04 nM (b), 0.2 nM (c), 1 nM (d), and 2 nM (e), separately. (C) Variance of the redox current with the target DNA concentration measured by the E-DNA biosensor without an adjunct probe. (D) Variance of the redox current with the target DNA concentration measured by the E-DNA biosensor with an adjunct probe.

sensitive than the previously reported E-DNA biosensors.[31,32] The dynamic range of the E-DNA biosensor with an adjunct probe had also been extended by as much as 2 orders of magnitudes (Figure 2D) as compared to that without an adjunct probe (Figure 2C). The improved detection limit and the extended dynamic range obtained by the E-DNA biosensor with an adjunct probe might also be attributed to the function of an adjunct probe to immobilize the dissociative MB-modified reporter probe onto the electrode surface and to facilitate the electron transfer.

Sequence Specification for the Target DNA Detection. The introduction of an adjunct probe might also lead to excellent sequence specificity for target DNA detection and can distinguish even 1-base mismatched target DNA. In the presence of 0.2 nM mismatched target DNA, the electrochemical signal increased by 10.0% (Figure 3A-b) compared to that control group with only buffer (Figure 3A-a); while in the presence of 0.2 nM perfect matched target DNA, the signal increased by as much as 125.0% (Figure 3A-d), approximately 12 times the increase than that in the presence of 0.2 nM mismatched target DNA. Notably, even in the presence of 50.0 pM perfect matched target DNA, the signal increased by as much as 35.0% (Figure 3A-c), much higher than that in the presence of 0.2 nM mismatched target DNA (Figure 3A-b). In contrast, with the increase of the concentration of 1-base mismatched target DNA, no significant enhancement in the amperometric signal was observed (Figure 3B). These results demonstrated that the E-DNA biosensor with an adjunct probe exhibited excellent sequence specificity toward even 1-base mismatched target DNA.

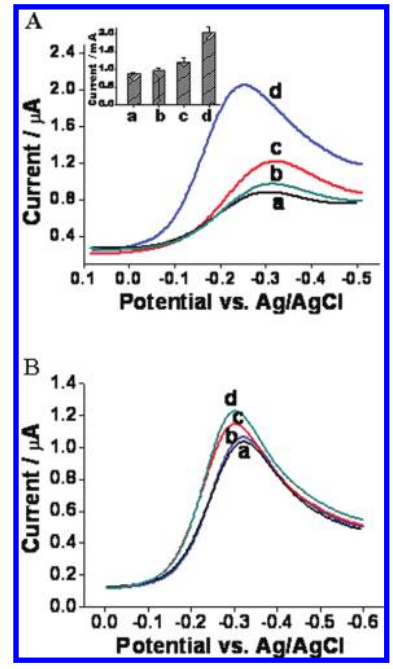

Figure 3. (A) Electrochemical signals from the E-DNA biosensor with an adjunct probe in response to buffer (a), 0.2 nM mismatched target DNA (b), 50.0 pM perfect matched target DNA (c), and 0.2 nM perfect matched target DNA (d). Inset: Comparison of electrochemical signals obtained above. (B) The electrochemical signals from the E-DNA biosensor with an adjunct probe in response to buffer (a), 0.04 nM (b), 0.2 nM (c), and 1 nM (d) mismatched target DNA.

Effect of Sequence and Density of the Adjunct Probes upon the Detection Sensitivity. To further understand the mechanism of improved sensitivity by the introduction of an adjunct probe, we investigated the effect of both sequence and density of the adjunct probes upon the detection sensitivity. Both 1-base mismatched and perfect matched adjunct probes were employed for this purpose. As shown in Figure 4A, in the presence of 0.2 nM perfect matched target DNA, the electrochemical signal from the E-DNA biosensor with a 1-base mismatched adjunct probe decreased by as much as 10.0% as compared to that without an adjunct probe. In contrast, the electrochemical signal from the E-DNA biosensor with a perfect matched adjunct probe improved by as much as 53.0% as compared to that without an adjunct probe (Figure 4A). These results demonstrated that the sequence specificity of the adjunct probes played a crucial role in the detection sensitivity of the E-DNA biosensor. The decrease of electrochemical signals in the E-DNA biosensor with a 1-base mismatched adjunct probe might result from its inability to hybrid with the flexible elements of reporter probe and the increase of steric hindrance on the electrode surface induced by the introduction of 1-base mismatched adjunct probes, which might adversely block the MB from colliding with the electrode surface.[33]

To study the effect of adjunct probe density upon the detection sensitivity, we modified the electrode surface with different concentration of perfect matched adjunct probes. Figure 4B shows the variance of electrochemical signal with the concentration of the adjunct probes in response to 0.2 nM perfect matched target DNA. The electrochemical signal increased as a result of the increase of adjunct probe concentration from 0.01 to 0.08 μM, but

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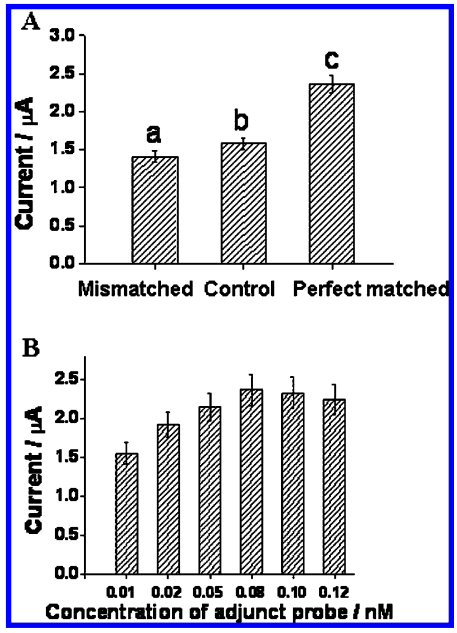

Figure 4. Effect of sequence and density of the adjunct probes upon the detection sensitivity. (A) Comparison of electrochemical signals obtained by the E-DNA biosensor with a 1-base mismatched adjunct probe (a), a perfect matched adjunct probe (c), and without an adjunct probe (b). The concentration of target DNA was 0.2 nM. (B) Variance of the electrochemical signal with the concentration of perfect matched adjunct probes.

beyond the concentration of 0.08 μM, the signal slowly decreased with the increase of adjunct probe concentration. The improvement of electrochemical signal with the increase of adjunct probe concentration could be easily explained by the function of the adjunct probes to immobilize more of the dissociative reporter probes onto the electrode surface and to facilitate the electron transfer. The decrease of electrochemical signal with the adjunct probe concentration beyond 0.08 μM might result from following two factors: (1) The high-concentration adjunct probes might weaken the density of the capture probes on the electrode surface due to the existence of competitive reactions between them with the electrode surface. (2) The high-concentration adjunct probes might increase the steric hindrance of the microenvironment, adversely preventing the dissociative reporter probes from colliding with the electrode surface.[33] These results demonstrated that the density of the adjunct probes played a critical role in the detection sensitivity of the E-DNA biosensor.

Improved Sensitivity for Protein Detection with the Introduction of an Adjunct Probe. The E-DNA biosensor with an adjunct probe could be further extended to the construction of the aptamer-based biosensor for protein detection. To demonstrate the principle-of-concept, we developed an aptamer-based biosensor for thrombin (Figure 5A). The aptamer-based biosensor consisted of a specific aptamer for thrombin as a capture probe, the MB-functionalized ssDNA as a reporter probe, and a thiol-functionalized ssDNA attached nearby the capture probe as an adjunct probe. In the presence of thrombin, a section of the reporter probe might be displaced by the binding of thrombin with the aptamer (the capture probe). The element of reporter probe displaced was flexible and dissociative and had a chance to collide with the gold electrode to produce an electrochemical signal. The adjunct probes nearby the capture probes might bind

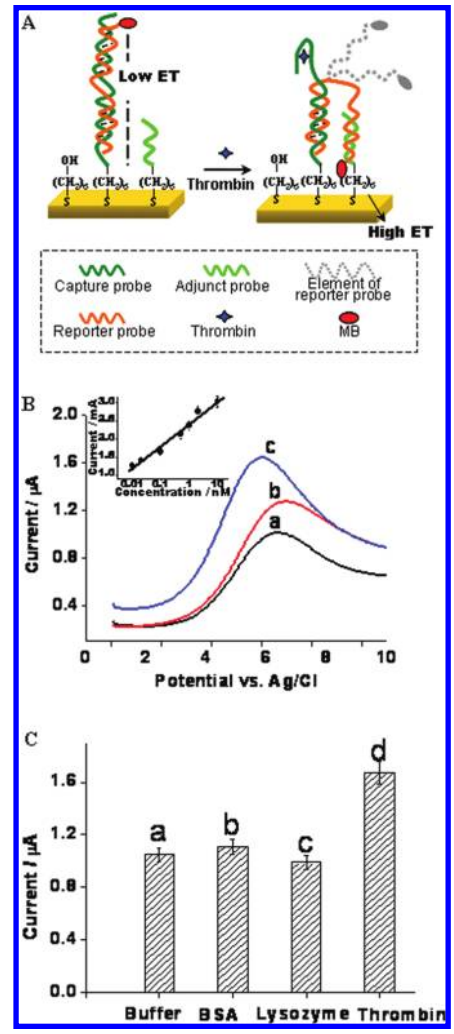

Figure 5. (A) Scheme for thrombin detection using the aptamer-based biosensor with an adjunct probe. (B) Electrochemical signal obtained in buffer (a), capture probes only (b), and capture probes plus adjunct probes (c). The concentration of thrombin was 0.1 nM in b and c. Inset of (B): variance of the redox current with the thrombin concentration. (C) Comparison of the electrochemical signals in response to buffer (a), 1.0 μM BSA (b), 1.0 μM lysozyme (c), and 0.1 nM thrombin (d).

the dissociative section of the reporter probes displaced by thrombin, increasing the chance of MB to collide with the gold electrode and thus facilitating the electron transfer. As shown in Figure 5B, with the addition of 0.1 nM thrombin, the signal increase improved by as much as 2.0 times in the aptamer-based biosensor with an adjunct probe compared to that without an adjunct probe. The measured detection limit of the biosensor without an adjunct probe was only 200.0 pM. In contrast, the measured detection limit of the biosensor with an adjunct probe might reach 20.0 pM (inset of Figure 5B), which had been improved by as much as 10 times and was more sensitive than the previously reported biosensors for thrombin,[34−36] indicative of the improved sensitivity for thrombin detection with the

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introduction of an adjunct probe. Moreover, the aptamer-based biosensor with an adjunct probe showed excellent specificity for protein detection. As shown in Figure 5C, even in the presence of a high-concentration interfering substance (1.0 μM BSA (Figure 5C-b) and 1.0 μM lysozyme (Figure 5C-c)), no significant difference in the peak current was observed as compared to the control group (Figure 5C-a). While in the presence of 0.1 nM thrombin (Figure 5C-d), the peak current significantly improved, suggesting the high specificity of the aptamer-based biosensor with an adjunct probe for protein detection.

CONCLUSIONS

In summary, we have developed an electrochemical biosensor capable of detecting sequence-specific target DNA and proteins with improved sensitivity and large dynamic range by simply introducing an adjunct probe. This adjunct probe might immobilize the element of reporter probes displaced by the target DNA and protein, increasing the chance of the dissociative reporter probe to collide with the electrode surface and facilitating the electron transfer. The biosensors with an adjunct probe have exhibited improved sensitivity and low detection limit for DNA and thrombin and can even distinguish 1-base mismatched target DNA. Importantly, the use of this biosensor is not limited to such and is viable for sensitive detection of numerous biomolecules, including RNA, proteins, and small molecule such as cocaine.[37]

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