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Proximity Hybridization-Regulated Immunoassay for Cell Surface Protein and Protein-Overexpressing Cancer Cells via Electrochemiluminescence

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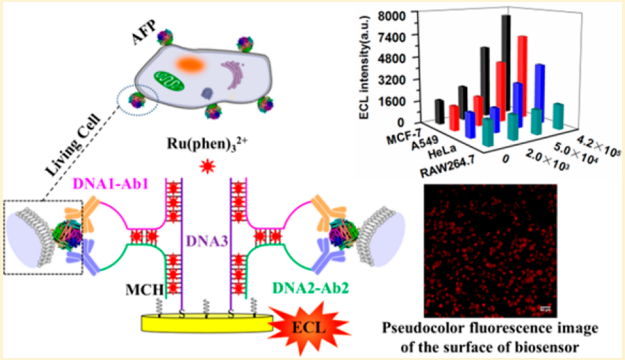
Supporting Information

ABSTRACT: A simple electrochemiluminescence (ECL) immunoassay based on a proximity hybridization-regulated strategy was developed for highly sensitive and specific detection of cell surface protein and protein-overexpressing cancer cells. A biosensor was fabricated by self-assembling a thiolated capture ss-DNA3 (partially hybridize with ss-DNA1 and ss-DNA2) and blocking with 6-mercapto-1-hexanol on a gold electrode surface. Target protein was simultaneously bound by two ss-DNA-tagged antibody probes (DNA1-Ab1 and DNA2-Ab2), while DNA1 and DNA2 were brought in sufficient proximity and hybridized with capture DNA3 on the surface of the biosensor. After ECL signal reagent $Ru(phen)_3^{2+}$ was intercalated into the hybridized ds-DNAs, ECL measurement was performed in the coreactant solution. A "signal on" proximity hybridization-regulated ECL immunoassay for alpha-fetoprotein (AFP) was developed. The ECL intensity increased with the increase of AFP concentration in the range of 0.05−20.0 ng/mL with a detection limit of 6.2 pg/mL. Moreover, the developed ECL method was successfully used to detect AFP-overexpressing cancer cells (MCF-7 cancer cells as model) with a detection limit of 620 cells/mL (~60 MCF-7 cells in 100 μL of cell suspension) and discriminate AFP expression on different types of the living cell surface. This work for the first time reports a proximity hybridization-regulated ECL immunoassay for the detection of the cell surface protein on a living cell surface with good specificity and sensitivity. This simple, specific, and sensitive strategy is greatly promising for the detection of proteins and specific cells.



INTRODUCTION

Quantitation of the cell surface proteins and discrimination of the protein-overexpressing cancer cells is crucial to the discovery of cancer drugs as well as understanding of the biological processes of cancer.[1,2] Immunoassay is one of the widely used methods for the detection of proteins because of the high specificity between antibody and antigen. A series of immunoassay methods with efficient specific, such as colorimetric,[3,4] fluorescence,[5] electrochemical,[6] and electrochemiluminescence (ECL)[7,8] immunoassays have been applied for protein analysis. However, the antigen/antibody could not be "amplified" like nucleic acids, and the concentrations of biomarkers protein were much lower in the real samples. Thus, design and development of highly sensitive and specific methods for the quantitation of proteins are required for clinical application.

The proximity hybridization assay (PHA), first reported by Landegren and co-workers in 2002,[9] is attracting more and more attention in the clinical diagnosis of proteins. In PHA, a protein-recognition event is converted into DNA detection.[10,11] The PHA with good performance has been applied for the detection of proteins, such as alpha-fetoprotein (AFP),[12,13] carcinoembryonic antigen (CEA),[14−17] total protein of nosema bombycis,[18] prostate-specific antigen,[19] thrombin,[20−24] platelet-derived growth factor BB,[25−27] insulin,[28] concanavalin A,[29] O-GlcNAcylated proteins,[30] cystatin C,[31] and prostasomes.[32] In the reported PHA, a number of DNA-based amplification strategies, such as polymerase chain reaction,[10,11,20,30] endonuclease-assisted recycling amplification,[12−16,19,31] localized rolling-circle amplification,[10,29] and nanoparticles- based amplification strategies,[17,18,24,28] have been employed to enhance the sensitivity. However, the involvement of ligase and polymerase in DNA amplifications and the complicated synthesis of nanoparticles in nanoparticle amplifications truly increased the cost and complexity of these detection methods.

Electrogenerated chemiluminescence (electrochemiluminescence, ECL) has becoming a powerful method due to its high sensitivity, low background signal, controllability, and simplified operation.[33−39] Recently, we developed a proximity hybridization-regulated ECL immunoassay for AFP, which was based on sensitization of gold nanoparticles and a target-induced quenching mechanism with one-step recognition, short

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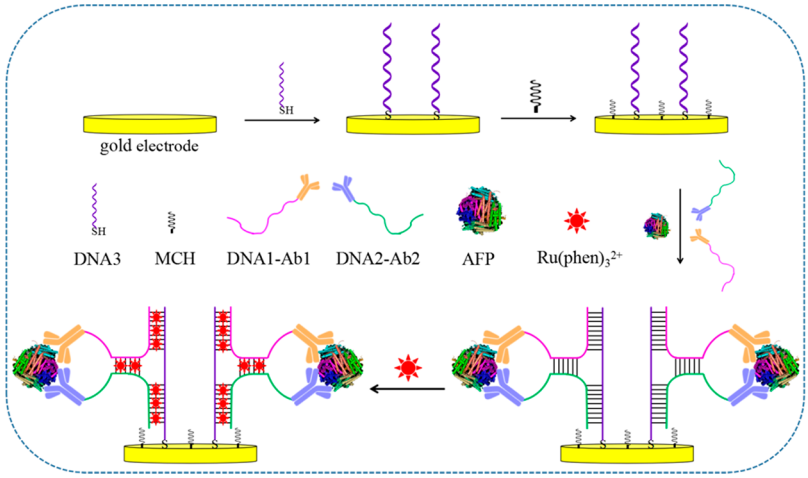

Figure 1. Scheme of the proximity hybridization-regulated ECL immunoassay for AFP.

detection time, and good accuracy.[40] In our previous work,[40] strong ECL emission from ECL reagent $Ru(bpy)_3^{2+}$, which was electrostatically adsorbed into the AuNPs/Nafion/glassy carbon electrode, was quenched by a ferrocene-labeled DNA, which was brought in sufficient proximity and hybridized with capture DNA on the surface of the biosensor under the assistance of a target protein and two ss-DNA-tagged antibody probes. This was a "signal off" method, and four ss-DNAs were required. Additionally, the detection limit is limited due to high ECL background. Hence, our efforts are continuously devoted to developing a signal-on assay-incorporated ECL method with proximity hybridization assay for sensitive and specific detection of proteins.

Herein, taking advantage of the ECL method with intercalation of ECL signal reagent (without ECL reagent label process) and PHA, we reported a simple proximity hybridization-regulated ECL immunoassay for highly sensitive and specific detection of cell surface protein and protein-overexpressing cancer cells for the first time. In this work, AFP, most extensively studied as a biomarker protein for the screening and diagnosis of hepatocellular carcinoma and yolk sac tumors,[41] was chosen as a model protein, while $Ru(phen)_3^{2+}$, which could efficiently intercalate into the ds-DNA grooves,[42] served as an ECL signal. The principle of the ECL immunoassay of AFP is depicted in Figure 1. Three single-strand DNAs (ss-DNA, Table S1), recorded as DNA1, DNA2, and capture DNA3, were designed according to ref 19 for the proximity hybridization-regulated ECL immunoassay. The biosensor was fabricated by self-assembling a thiolated capture DNA3 on the surface of a gold electrode and blocking with 6-mercapto-1-hexanol (MCH). In the presence of target AFP, AFP was simultaneously recognized by two ss-DNA-tagged antibody probes (DNA1-Ab1 and DNA2-Ab2), while DNA1 and DNA2 were brought in sufficient proximity and hybridized with capture DNA3 on the surface of the biosensor. After ECL signal reagent $Ru(phen)_3^{2+}$ was intercalated into the hybridized ds-DNAs including DNA3-DNA1, DNA1-DNA2, and DNA3-DNA2, ECL measurement was performed in a coreactant solution. A "signal on" proximity hybridization-regulated immunoassay for AFP was developed. Additionally, the developed method was evaluated by detecting AFP in clinical (serum) samples and AFP-overexpressing cancer cells (MCF-7 cancer cells as models) as well as discriminating AFP expression on different types of living cells.

EXPERIMENTAL SECTION

ECL Measurements. Materials, apparatus, and synthesis of DNA-tagged antibodies (DNA1-Ab1, DNA2-Ab2)[43] as well as the fabrication of the biosensor are presented in the Supporting Information. The fabricated biosensor was immersed into 100 μL of 55 mM PB2 (0.055 M Na_2HPO_4, 0.055 M NaH_2PO_4, 150 mM NaCl, and 5 mM EDTA, pH 7.4) containing 13 nM DNA1-Ab1, 13 nM DNA2-Ab2, and a fixed concentration of AFP (serum sample or living cells, seen in Supporting Information) for 60 min at 37 °C. Then the resulted electrode was dipped into 100 μL of 50 μM $Ru(phen)_3^{2+}$ solution for 50 min. In each step, the electrode was washed thoroughly with 10 mM PB3 (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, pH 7.4). The bound biosensor as working electrode was transferred into an ECL cell containing 1 mL of 0.1 M PBS (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, and 0.1 M KCl, pH 7.4) and 50 mM tri-*n*-propylamine (TPA) and holding an Ag/AgCl (saturated KCl) electrode as reference electrode and a platinum wire as counter electrode. The ECL measurement was conducted by a triangular potential scan at 100 mV/s. AFP was quantified by the increased ECL intensity ($\Delta I = I_s - I_0$) at +0.9 V, in which I_0 is the blank ECL signal and I_s is the ECL signal in the presence of target.

RESULTS AND DISCUSSION

Characterization and Feasibility of the ECL Biosensor. Characterization of the synthesized DNA-Ab (DNA1-Ab1) was performed using UV−vis spectroscopy. Clearly, the absorption spectrum of DNA1-Ab1 showed a merged peak at 259 nm from a typical peak of DNA1 at 260 nm and antibody at 280 nm (Figure S-1), indicating that DNA1 was tagged with Ab1. On the basis of the absorbance ratio (260 vs 280 nm),[44] it was calculated to be four DNA1 on each antibody in DNA1-Ab1 (see the Supporting Information).

The biosensor fabricated with a thiolated DNA3 was characterized using X-ray photoelectron spectroscopy and electrochemical methods. The presence of the S 2p peak at 162.5 eV in the X-ray photoelectron spectrum of a thiolated DNA3 onto gold electrode was evidence of a thiol−gold bond (Figure S-2). The surface density of the capture DNA3 on the gold electrode was calculated to be 1.6×10^{12} molecule/cm^2 using cyclic voltammetry (Figure S-3).[45] Electrochemical impedance spectra of the biosensor in different steps showed that the charge-transfer resistance (R_{ct}) increased from 396.8 Ω

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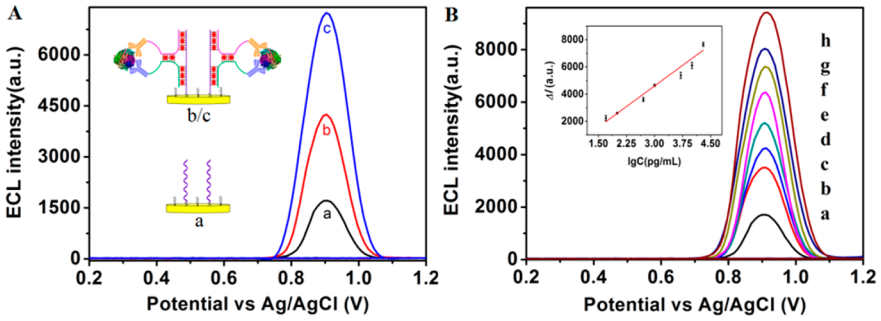

Figure 2. (A) ECL intensity vs potential profiles of the ECL biosensors before (a) and after reaction with 0.1 (b) and 5.0 ng/mL AFP (c). (B) ECL intensity vs potential profiles for the detection of AFP: (a) blank, (b) 0.05 ng/mL, (c) 0.1 ng/mL, (d) 0.5 ng/mL, (e) 1.0 ng/mL, (f) 5.0 ng/mL, (g) 10.0 ng/mL, and (h)20.0 ng/mL. (Inset) Calibration curve of AFP. Measurement solution: 0.1 M PBS containing 50 mM TPA (pH 7.4). Scan rate: 0.1 V/s.

at a bare gold electrode to 2700 Ω at the fabricated biosensor without MCH, then to 4739 Ω with blocking with MCH, and finally to 6380 Ω at biosensor bound with target AFP and both DNA1-Ab1 and DNA2-Ab2 (Figure S-4). All observations suggest that biosensor is successfully fabricated and bound with target AFP and both DNA1-Ab1 and DNA2-Ab2.

Figure 2A shows ECL responses of the biosensor to different concentrations of AFP. In the absence of AFP, a much lower ECL signal (1709, line a) is observed. This is attributed to the fact that negligible $Ru(phen)_3^{2+}$ is electrostatically interacted with capture DNA3 on the electrode. Additionally, without the assistance of target AFP, a sufficient proximity hybridization of the two probes (DNA1-Ab1 and DNA2-Ab2) with capture DNA3 barely occurred, so that $Ru(phen)_3^{2+}$ was scarcely intercalated into a much lower amount of formed ds-DNA, resulting in a much lower ECL signal. The ECL intensity increased from 4246 (line b) to 7338 (line c) as the concentration of AFP increased from 0.1 to 5.0 ng/mL. This is attributed to the fact that with the assistance of target AFP, a large amount of $Ru(phen)_3^{2+}$ is intercalated into a large amount of formed ds-DNA. With an increase of concentration of AFP, an amount of $Ru(phen)_3^{2+}$ intercalated into an increased amount of the formed ds-DNA was elevated. The ECL emission occurred at +0.90 V, attributed to the relaxation of $Ru(phen)_3^{2+*}$, which was electrochemically generated from TPA and $Ru(phen)_3^{2+}$.[36] These results indicate that the designed method can possibly be applied to detect the concentration of AFP.

In order to gain better insight into the proposed strategy based on a target assistant proximity hybridization and ECL signal reagent intercalation, the following experiments were performed including only one of two probes and ref DNA4 as a mimicking antibody. First, it was found that no obvious ECL intensity increased in the absence of one of DNA1-Ab1 (1942) and DNA2-Ab2 (2037) compared with that in the presence of both DNA1-Ab1 and DNA2-Ab2 in the tested system (Figure S-5), indicating that both DNA1-Ab1 and DNA2-Ab2 are required in the designed strategy. Second, PAGE analysis was performed to have insight into the hybridization of the DNAs. A ref DNA4 (sequence shown in Table S-1) was employed to mimic the function of the antibody,[19] which was checked using PAGE. In the PAGE images of different DNAs (Figure S-6), two clear bands (lanes 2 and 3) can be seen attributed to DNA1 or DNA2 and its duplex of self-hybridization, respectively, while one individual clear band (lanes 4 and 5) was observed for capture DNA3 and ref DNA4, respectively. No obvious hybridization among DNA1, DNA2, and DNA3 occurred (lane 6), while a migration band at 210 bp (lane 7) appeared for the mixture of DNA1, DNA2, capture DNA3, and ref DNA4. The difference between lane 7 and lanes 2−6 indicates that the cooperative complex of capture DNA3-DNA1-ref DNA4-DNA2 is formed. Additionally, a simulation of the Gibbs free energy (ΔG) of the hybridizations was carried out among the capture DNA3, DNA1, DNA2, and ref DNA4 using the computer program RNA Structure 4.5 (Figure S-7).[46] The ΔG is −10.8 kcal/mol for DNA3-DNA1, −10.6 kcal/mol for DNA3-DNA2, −5.5 kcal/mol for DNA1-DNA2, −25.3 kcal/mol for DNA4-DNA1, and −24.6 kcal/mol for DNA4-DNA2. This further supports that DNA1 and DNA2 were brought in sufficient proximity and hybridized with DNA3 under the assistance of ref DNA4.

Optimization Conditions. First, four single-strand DNA2 sequences with different complementary bases (4−10 bp) for DNA1, named DNA2-X (Table S1), were designed and checked in the designed ECL system for replacement of DNA2. It was found that the ECL intensity depended on the number of DNA2 complementary bases for DNA1, and a maximum signal-to-noise ratio was obtained for DNA2 with 6 bp complementary bases (Figure S-8). Thus, DNA2 was chosen as the optimized DNA sequence.

Furthermore, the incubation time between AFP and antibody, the intercalation time of $Ru(phen)_3^{2+}$ into dsDNA, and the concentration of TPA were optimized. An incubation time of 60 min (Figure S-9A), 50 min of intercalation time (Figure S-9B), and 50 mM TPA (Figures S-10 and S-11) were chosen in the following experiment.

Analytical Performance. To illustrate the analytical performance of the proposed assay, the ECL responses and dynamic range toward AFP were examined (Figure 2B). The ECL intensity increased with the increase of AFP concentration in the range from 0.05 to 20.0 ng/mL. The linear regression equation was $\Delta I = 2020 \lg C - 1461$ (unit of C is pg/mL, R = 0.9864) with a detection limit (DL) of 6.2 pg/mL (~90 fM). By comparison of the reported methods for the detection of AFP (listed in Table S2), the ECL immunoassay developed in this work exhibits a larger linear range and excellent sensitivity. For example, the DL is lower than those of UV−vis or electrochemical methods and is comparable with nanoparticle amplification ECL methods (such as ECL immunoassay using CdTe nanocrystals (5.0 pg/mL).[47] The sensitivity of this method satisfactorily meets the requirement of the cutoff values of AFP in human serum (25 ng/mL).[48]

The reproducibility of the method developed in this work was satisfactory since the relative standard derivative was 1.3%

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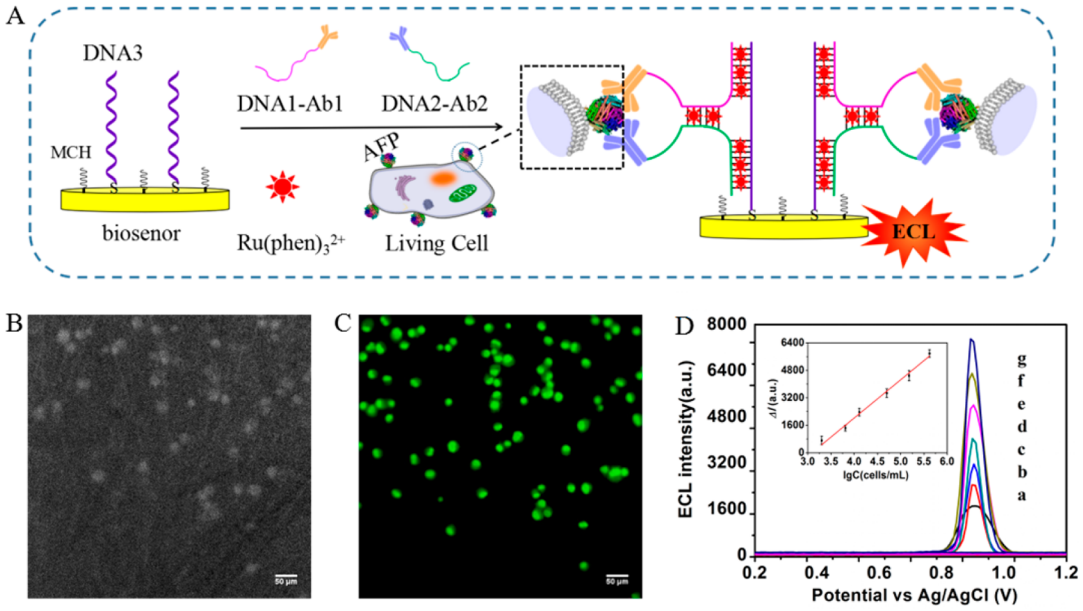

Figure 3. (A) Schematic illustration of the ECL immunoassay for living cells. (B) Bright-field optical microscopy image of MCF-7 cells captured on the surface of the biosensor. (C) Pseudocolor fluorescence image of AO-stained MCF-7 cells on the surface of the biosensor. (D) ECL intensity vs potential profiles of the ECL biosensor to different concentrations of MCF-7 cells: (a) 0, (b) 2.0×10^3, (c) 6.5×10^3, (d) 1.3×10^4, (e) 5.0×10^4, (f) 1.5×10^5, and (g) 4.2×10^5 cells/mL. (Inset) Calibration curve of MCF-7 cells.

for 1.0 ng/mL AFP (n = 5). The selectivity was checked by assaying 1.0 ng/mL AFP and 50.0 ng/mL interfering substances, including CEA, troponin I (TnI), human immunoglobulin (IgG), creative protein (CRP), and trypsin. No obvious change in the ECL intensity was obtained for the tested interfering substances, while a dramatic increase was found for AFP (Figure S-12). Good selectivity of this ECL method was evident. The clinical applicability of this ECL method was also carried out for three clinical serum samples. The results were agreeable with the CL values (Table S3), indicating that this ECL method can be applied to detect AFP in clinical tests.

ECL Assay of AFP on Living Cell Surface. Evaluation the average amount of AFP on cancer cells was helpful for clinical diagnosis and therapy of the cancers. Here, the developed ECL method was used to detect protein-overexpressing cancer cells. Figure 3A shows the scheme of ECL method for the detection of living cells. MCF-7 cells were first chosen as models because of their high levels of AFP expression. The cell-adhesion viability was examined to confirm whether the living cells were captured onto the electrode surface. The captured cells on the electrode surface were stained with acridine orange (AO), which is a widely used cell viability indicator. The cell adhesion captured on the surface of the biosensor could be clearly seen from the bright-field optical microscopy image (Figure 3B), and the strong pseudocolor fluorescence signals (Figure 3C) indicated that the MCF-7 cells were alive on the electrode surface after the capturing process. Figure 3D shows that the ECL intensity increased with increasing numbers of living MCF-7 cells, indicating the existence of antigens on the tested cell surface and more cells combined with the antibody-oligonucleotide probes. The ECL intensity increased with an increase of the numbers of MCF-7 cells in the range from 2.0×10^3 to 4.2×10^5 cells/mL, and the linear regression equation was $\Delta I = 2226 \lg C_{cell} - 6883$ (R = 0.9946). The DL was ~620 cells/mL for MCF-7 cells (S/N = 3), that is, a DL of ~60 MCF-7 cells in 100 μL of cell suspension is obtained, which is comparable with electrochemical methods (620[49] and 1000 cells/ mL[50]) and ECL methods using multiwalled carbon nanotubes[51] (800 cells/mL) or Au-$RuSiO_2$ nanoparticle[52] (600 cells/mL). However, the DL was higher than the need for quantification of circulating tumor cell (CTC).[53] In order to lower DL, the following problems should be considered. First is big size of the tested cells. When they were captured on the surface of the biosensor, the cells could hinder the electron transfer and diffusion of TPA to the electrode surface. This is supported by our ECL image with a significant spatial extension of the cell contour (Figure S-13, similar to the results in ref 54). Second is the length of DNA duplex designed in DNA3, DNA1, and DNA2 since the ECL signal reagent intercalated into the hybridized ds-DNA is dependent on the length of the DNA duplex.

This ECL biosensor is further applied to discriminate the AFP expression levels on living cells. MCF-7 cells, A549 cells, HeLa cells, and RAW264.7 cells were chosen as model cells since they have different AFP-expressing values. It was found that the ECL intensity increased for MCF-7 cells, A549 cells, and HeLa cells, and no obvious ECL change was observed for RAW264.7 cells (Figure S-14). Moreover, the average amount of AFP at the surface of tested cells was quantified by an increased ECL emission using the calibration plot of AFP in Figure 2B, suggesting that the sequence of the tested cells based on the average amount of AFP on the surface was MCF-7 cells (9.02 fg/cell) > A549 cells (1.80 fg/cell) > HeLa (0.22 fg/cell). Obviously, the AFP expression of MCF-7 cells is much higher than that of A549 cells and HeLa cells.[55,56] This suggests that the ECL biosensor can discriminate the AFP expression levels on living cells.

CONCLUSION

An ECL immunoassay, which involves proximity hybridization assay and ECL reagent intercalation into the hybridized ds-DNA of the capture DNA on the electrode with two antibodies

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tagged two ss-DNA sequences, has been presented for the detection of AFP and protein-overexpressing cancer cells. Compared with our previous ECL methods based on the target-induced quenching mechanism and reported methods based on DNA amplifications and nanoparticles amplifications, our present method demonstrates simplicity, high sensitivity, and selectivity. This is probably due to following three factors: (1) a label process is not required for the ECL signal reagent on the DNA sequences; (2) a large amount of the ECL signal reagent can be intercalated into the hybridized ds-DNA; (3) one target protein can be recognized by two antibody probes. Additionally, the developed method was applied to discriminate protein expression on different living cells. The current work demonstrates that the strategy is promising to develop ECL immunoassay and to discriminate biomarker proteins on different living cells. This strategy could be easily extended to detection other biomarker proteins and protein-overexpressing cancer cells for the discovery of cancer drugs and understanding of the biological processes of cancers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04359.

Experimental section, absorption spectra, different DNA probes, Nyquist plots of impedance spectra, XPS, CV, ECL profiles, PAGE image, optimized conditions, and analytical performances of the ECL biosensor (PDF)

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Notes

The authors declare no competing financial interest.

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