Chapter 7

Electrochemical DNA Biosensors: Protocols for Intercalator-Based Detection of Hybridization in Solution and at the Surface

Kagan Kerman, Mun'delanji Vestergaard, and Eiichi Tamiya

Summary

An electrochemical DNA biosensor is a device that utilizes the inherent ability of two complementary strands of nucleic acids to form a double helix. The specificity of this reaction, namely hybridization, is used in the detection of target DNA sequences with a view toward developing point-of-care devices. Since the early 1990s, great progress has been made in this field, but there are still numerous challenges to overcome. This chapter describes the components of an electrochemical DNA biosensor for researchers new to the field, paying particular attention to intercalator-based DNA biosensors. We will use a well-defined electro-active DNA intercalator Hoechst 33258, as our running example. Two of the most classic DNA sensing methods: solution-based and surface-immobilized methods are discussed, along with guiding notes that would help identify and overcome possible problems in a typical electrochemical DNA biosensor experiment.

Key words: Biosensor, Electrochemical, DNA, Intercalator, Hybridization, Hoechst 33258, Screen-printed electrode (SPE), Probe-modified electrode, Self-assembled DNA monolayer.

1. Introduction

A biosensor is a device that combines a biological component (*a recognition layer)* and a physico-chemical detector component (*a transducer)*. The transduction unit can be electrochemical, optical, piezoelectric, magnetic, or calorimetric *(1)*. Two groups of *recognition* molecules form the majority of biosensors: affinity-based and catalytic-based biosensors. Affinity-based biosensors are used to bind molecular species of interest, irreversibly and noncatalytically. Examples include antibodies, nucleic acids, and

Avraham Rasooly and Keith E. Herold (eds.), *Methods in Molecular Biology: Biosensors and Biodetection, Vol. 504*

DOI:10.1007/978-1-60327-569-9_7

hormone receptors. Catalytic-based sensors such as enzymes and microbiological cells recognize and bind a molecule of interest, followed by a catalyzed chemical conversion of that molecule to a product that is then detected.

Electrochemical biosensors enable fast, simple, and low-cost detection, mainly because there is no need for expensive signal transduction equipment since they give electronic signals directly. They exploit both catalytic and noncatalytic-based molecules for the recognition layer. The most popular examples of these two types of molecular recognition elements are enzymes and DNA. In this chapter, we will confine our attention to DNA-based biosensors. DNA is particularly well-suited for biosensing applications, because there is a robust and specific interaction of the base pairs between complementary strands. DNA biosensors convert the Watson–Crick base-pair recognition event into a readable analytical signal *(2–4)*.

A DNA biosensor is mainly designed in such a way that a distinction can be made between single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and rarely three-stranded DNA (triplexes), providing the basis for electrochemical detection of DNA hybridization. In this chapter, we will restrict our discussions to the most common forms (dsDNA and ssDNA). There are basically four different analytical techniques for electrochemical detection of DNA hybridization: monitoring of (a) electrochemical oxidation/reduction signals of metal nanoparticle-tagged oligonucleotides, (b) oxidation peak current of the electro-active DNA bases such as guanine and/or adenine, (c) enzymatically amplified electrochemical signal of a substrate in the presence of the enzyme-tagged hybrid, and (d) electrochemical signal of a label, which selectively binds with dsDNA/ssDNA *(2, 5)*. In electrochemistry, DNA labeling has been exploited widely, from indirect detection platforms that use chemical mediators such as polypyridyl complexes of Ru(II) and Os(II), through use of redox-active reporter molecules (ferrocene, gold nanoparticles, semiconductor nanostructures (quantum dots: CdS, ZnS, PbS), to DNA-mediated charge transport mechanisms that utilize redox-active electrostatic ($Ru(NH_3)_6^{3+}$) and intercalative ($Co(phen)_3^{3+}$) probe molecules *(5–12)*.

The interaction of DNA and a ligand, which can bind to DNA to form a larger complex, plays a major role in biological processes such as gene transcription and DNA replication. Intensive research on natural and synthetic ligands has revealed intercalative or nonintercalative mechanisms *(13–16)*. A large class of molecules intercalates in the space between two adjacent base pairs of dsDNA. These molecules mostly have a polycyclic aromatic ring with a planar structure, and are often known as nucleic acid stains. Electro-active DNA intercalators include ethidium bromide, methylene blue, and benzo[a]pyrene *(17, 18)*. **Figure 1** displays the chemical drawings of the representative intercalators

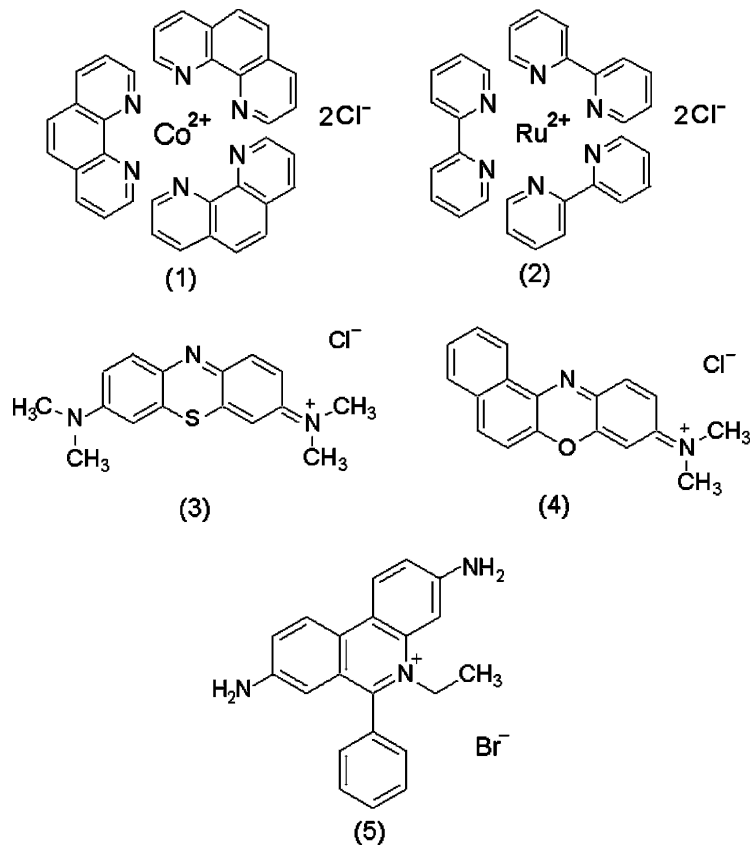

Fig. 1. Chemical drawings of the most-commonly used intercalators in the development of electrochemical DNA biosensors; (1) Cobalt(II)-Tris-(1,10-phenanthroline), (2) Ruthenium(II)-Tris-(1,10-bipyridine), (3) Methylene blue, (4) Meldola blue, (5) Ethidium bromide.

that have been commonly applied in electrochemical DNA biosensors. The fitting of an intercalator between DNA base pairs requires a separation of over 0.3 nm, inducing some local structural changes to the DNA strand such as unwinding of the double helix and lengthening of the DNA strand *(18)*. The small molecules, which noncovalently interact with DNA, are stabilized in binding to DNA through a series of weak interactions. They include π-stacking interactions associated with intercalation of aromatic heterocyclic groups between the base pairs, and hydrogen-bonding and van der Waals interactions of functionalities bound along the groove of the DNA helix. It would be valuable to understand quantitatively the contributions from the different modes to the stabilization of the bound complex at a certain DNA site. These structural modifications lead to the functional changes in DNA, often to the inhibition of transcription and replication processes. Therefore, the intercalators are also known as potent mutagens.

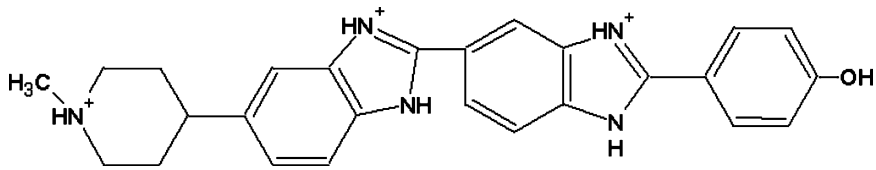

Fig. 2. Chemical drawing of Hoechst 33258.

In this chapter, we will use a DNA intercalator, Hoechst 33258 (2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi(1H-benzimidazole), **Fig. 2**) as our running example. Hoechst 33258 has an electrochemical oxidation potential at ~0.6 V (vs. Ag/AgCl) *(19–22)*. Its interaction with DNA results in the formation of aggregates, easily detected using atomic force microscopy (AFM) *(21, 22)*. Excess concentration of Hoechst 33258 induces DNA condensation. DNA can be analyzed either in solution and/or as an adsorbent on an electrode surface. We will provide protocols for both analytical situations.

For DNA detection process on the surface, basically, a single-stranded oligonucleotide (probe) is immobilized onto a transducer surface to recognize its complementary (target) DNA sequence, via hybridization, to form a DNA duplex (hybrid) on the electrode surface. This event is then converted into an analytical signal by a transducer. The immobilization of DNA probes on or near the electrode surface provides a suitable platform for highly integrated systems. However, an elegant surface chemistry is required for the preparation of probe modified electrodes.

For electrostatic immobilization of nucleic acids on the surface of a carbon electrode, an electrochemical pretreatment of the surface is required. For example, a carbon paste electrode (CPE) is activated by applying +1.7 V (vs. Ag/AgCl) for 1 min in 0.05 M phosphate buffer solution (pH 7.4) without stirring (*see* **Note 1**). Then, DNA is immobilized on the pretreated CPE by applying a potential of +0.5 V for 5 min in DNA containing 20 mM Tris–HCl (pH 7.0) with 200 rpm stirring (*see* **Note 2**). The positively-charged electrode surface attracts the negatively-charged phosphate backbone of DNA and loads them electrostatically onto the surface (**Fig. 3 A**, **B**). The electrode is then rinsed with a blank buffer solution (20 mM Tris–HCl) for 10 s. A similar procedure as above can be used for the immobilization of oligonucleotides on the surface of a carbon electrode.

The formation of self-assembled monolayer (SAM) of the thiolated nucleic acids on gold provides a very useful platform for the development of DNA sensors *(10, 11)*. However, researchers should pay particular attention to the pretreatment and cleaning of gold electrodes to avoid defects in the SAM. First, the gold electrodes must be sequentially polished using 6 and 1-μm

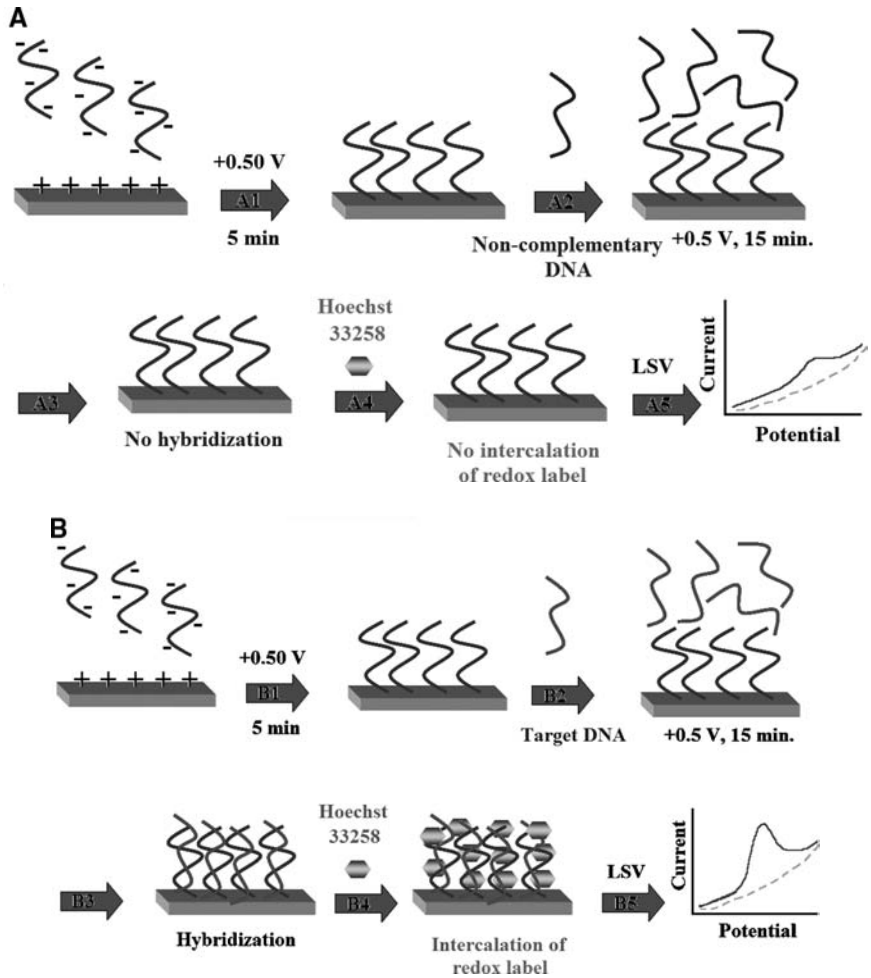

Fig. 3. (**A**) The electrochemical detection of hybridization on carbon electrode surface. Probe oligonucleotides are immobilized on the carbon surface with electrostatic attraction (**A1**), noncomplementary oligonucleotides are incubated with the probe-modified electrode (**A2**), no hybridization takes place between these two strands (**A3**), Hoechst 33258 is incubated with the probe-modified-electrode (**A4**), since the redox label cannot intercalate with the single-strand probe oligonucleotides on the surface, a small linear sweep voltammetric (LSV) current response is obtained (**A5**). The current intensity at the peak potential (~0.6 V) is recorded relative to the background response of the blank buffer solution (*dashed grey line*). (**B**) The electrochemical detection of hybridization on a carbon electrode surface. Probe oligonucleotides are negatively charged due to their phosphate backbone and are attracted onto the positively-charged carbon electrode surface (**B1**), target oligonucleotides are incubated with the probe-modified electrode (**B2**), hybridization takes place between the complementary strands resulting in the formation of a hybrid-modified electrode (**B3**), the intercalator, Hoechst 33258, is incubated with the hybrid-modified-electrode (**B4**), since Hoechst 33258 intercalates into the double-stranded hybrids on the surface, a high LSV current response is obtained (**B5**). The current intensity at the peak potential (~0.6 V) is recorded relative to the background response of the blank buffer solution (*dashed grey line*).

diamond pastes and 0.05-μm alumina on a polishing pad. After thorough rinsing, the electrodes are ultrasonically cleaned in distilled water for 1 min. The electrodes are then subjected to an electrochemical pretreatment step: cyclic voltammetry is applied in a 0.5 M H_2SO_4 solution from 0 to 1.7 V (vs. Ag/AgCl), until a

stable redox wave of Au oxidation (~1.2 V) and reduction (~0.8 V) is observed. Afterwards, the electrodes are rinsed with distilled water and immersed in the solution of the thiolated oligonucleotide for ~12 h at 4°C (*see* **Note 3**). Under these conditions, the sulfide-containing oligonucleotides are adsorbed to the gold surface involving a stable Au-S linkage. The electrodes are then washed with distilled water or preferably, a solution containing detergents (sodium dodecyl sulfate, Tween 20, PEG derivatives, etc.) to remove any nonspecifically adsorbed oligonucleotides.

As for the hybridization reactions on the surface, the electrode modified with the DNA probe is immersed in a hybridization buffer (*see* **Note 4**) containing the target oligonucleotide, which is complementary to the probe, for 1 h with moderate shaking or stirring (*see* **Note 5**), forming hybrids on the electrode surface. Afterwards, the intercalator (Hoechst 33258) is exposed to the electrode surface and incubated with the hybrids on the surface for 5 min. Following the incubation step, the electrode is washed to remove the excess intercalator, and an electrochemical measurement is taken using a suitable technique, such as linear sweep voltammetry (LSV), cyclic voltammetry (CV), and/or differential pulse voltammetry (DPV). The difference between the peak current responses obtained from the intercalator after its interaction with dsDNA and ssDNA provides the basis of this electrochemical detection system **(Fig. 3A, B)**.

If the probes are not immobilized on the surface, the preparation procedure becomes greatly simplified. However, the need for a suitable reaction chamber appears to be a drawback to the development of this system. A recent design, called a *DNA stick*, has been used in our laboratory for detecting DNA hybridization in solution with Hoechst 33258. Hoechst 33258 aggregates with the PCR amplified DNA in solution. These changes in the anodic current signal of Hoechst 33258 at ~0.6 V are monitored in the presence and absence of solution-phase DNA using a bare electrode. The mass transfer to the electrode surface is reduced with formation of the DNA-Hoechst 33258 aggregates, resulting in the reduced peak current intensity. Free Hoechst 33258 molecules can be transferred to the electrode surface more rapidly, and result in a high peak current intensity. Similar to the on-surface detection principle, the difference between the peak current signals obtained from the aggregated and free forms of Hoechst 33258 provides the basis of this detection system. The aggregation of DNA–Hoechst 33258 complex leads to a decrease in the voltammetric signal in proportion to the quantity of PCR-amplified DNA *(19–21)*.

Over the past decade, great progress has been made in the field of electrochemical DNA biosensors, but there are still numerous challenges to overcome. Here we describe the components of electrochemical DNA biosensors and the important

steps in their application for the detection of hybridization, using an intercalator, for researchers new to the field.

2. Materials

2.1. Screen-Printed Electrode

Planar screen-printed electrodes (SPEs) were obtained from Biodevice Technology, Co. Japan. They are used once and discarded after each measurement. SPE consists of a carbon working electrode, a carbon counter electrode, and Ag/AgCl reference electrode (**Fig. 4 A**). Total length of the SPE is 11 mm, and the geometric working area is 2.64 mm^2. The SPEs should be kept in their plastic packages provided by the supplier, Biodevice Technology, Co. at room temperate, and cut away from their sheet when required. The SPEs are stable, unless they are damaged or used.

Instead of the SPEs, other commercially available electrodes can be used, for example a glassy carbon electrode (3.0 mm i.d.,

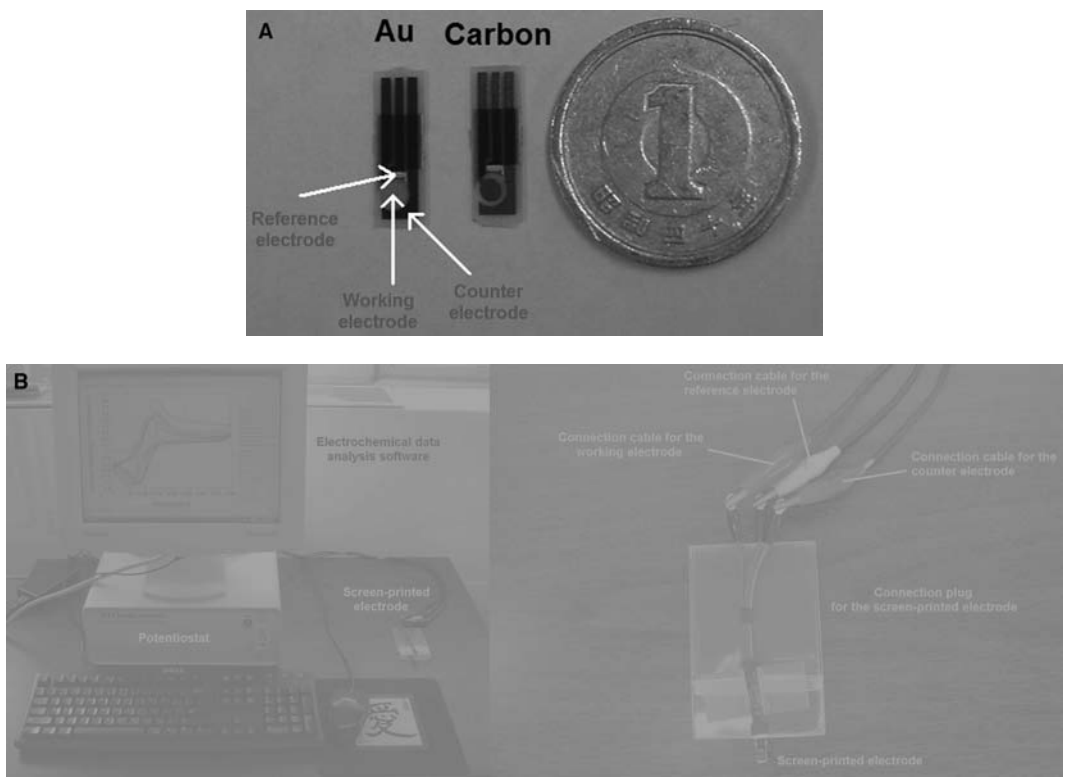

Fig. 4. (**A**) Photograph of screen-printed gold and carbon electrode with the three-electrode system including a carbon-based counter electrode and Ag/AgCl-based inner reference electrode. (**B**) Photograph of a typical electrochemical set-up with a computer-controlled potentiostat connected to a screen-printed electrode (SPE).

product # MF-2012, Bioanalytical System, Inc.), a gold electrode (1.6 mm i.d., product # MF-2014, Bioanalytical System, Inc.), and a carbon paste electrode (3.0 mm i.d., Teflon electrode body product # MF-2010, Bioanalytical System, Inc.). Ag/AgCl-based reference electrode can be used (Product # MF-2052, RE-5B, Bioanalytical System, Inc.). The auxiliary (counter) electrode can be made of platinum wire (6 cm, product # MW-4130, Bioanalytical System, Inc.). We would recommend VC-2 voltammetry cell with Teflon electrode holder (Product # MF-1052, Bioanalytical System, Inc.) in connection with the conventional electrochemical set-up.

2.2. Electrochemistry

Electrochemical measurements were performed using a potentiostat, for example Autolab PGSTAT 12 electrochemical analysis system (Eco Chemie, The Netherlands) in connection with General Purpose Electrochemical System (GPES) software, or CH Instruments 660 Electrochemical Workstation (Austin, TX). The electrochemical set-up consisted of a SPE system is as shown in **Fig. 4b**.

2.3. Spectrophotometry

UV-VIS DNA-protein analyzer (Shimadzu, Japan).

2.4. Hoechst 33258

Hoechst33258(2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl) -2,5′-bi (1H-benzimidazole) trihydrochloride, 2-[2-(4-Hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl) benzimidazole trihydrochloride, H33258) was purchased from Fluka BioChemika (Product # 14530). A stock solution is prepared by directly dissolving it in high purity water (ρ = 18.3 MΩ cm, Millipore, Bedford, MA, USA) and kept at 2–8°C in the dark (*see* **Note 6**). Hoechst 33258 solution (100 µM) is prepared in 50 mM phosphate buffer solution (PBS) including 100 mM NaCl (Sigma-Aldrich, Product # 71376) (pH 7.4). PBS is prepared by mixing 0.04 mol K_2HPO_4 (Sigma-Aldrich, Product # 60353) and 0.01 mol KH_2PO_4 (Sigma-Aldrich, Product # 60218).

2.5. Deoxyribonucleic Acid

Deoxyribonucleic acid sodium salt from salmon testes (fsDNA) was purchased from Sigma (Product # D1626). Solution of natural fish sperm DNA prepared in MilliQ water gives UV absorbance ratios of ~1.8–1.9 at 260 and 280 nm (A_{260}/A_{280}), respectively, which indicates that DNA is sufficiently free of protein. Stock fsDNA is stored at 0–4°C and should be used within three days of preparation (*see* **Notes 7–9**).

2.6. Preparation of the Probe Solution

Incubate the probe/dithiothreitol (DTT; 0.1 M DTT in water) solution (purchased from Sigma-Aldrich, Product # 43816) at room temperature for 30 min, followed by removal of the DTT using a standard ethanol precipitation procedure. Determine the concentration of DNA solution and adjust it to 25 µM (make the

final buffer composition as 50 mM PBS at pH 7.4), and use it as the probe solution.

2.7. Preparation of Hybridization Buffer Solution

Hybridization buffer contains 20 mM Tris–HCl (Sigma-Aldrich, Product # T5941) and 500 mM NaCl (pH 7.0).

2.8. Preparation of Washing Solution

Washing solution contains 20 mM Tris–HCl with 300 mM NaCl and 0.1% sodium dodecyl sulphate (Sigma-Aldrich, Product # 71725) (pH 7.0).

3. Methods

3.1. Detection in Solution

Mix fsDNA solution and H33258 at a final concentration of 1 µM in 50 mM PBS and pipet an aliquot (20 µL) of this mixture on the gold or carbon SPE surface. Measure immediately using linear sweep voltammetry (LSV) after an equilibration time of 10 s, with a sample interval of 1 mV, and a scan rate of 0.1 V/s from 0 to 1 V (**Fig. 5**). Alternatively, cyclic voltammetry (CV) can be applied with a scan range between 0 and 1 V at a sweep rate of 0.1 V/s. For differential pulse voltammetry (DPV) measurements, the potential is scanned from 0 to 0.90 V with a step potential of 4 mV, pulse amplitude of 50 mV, and a pulse period of 0.20 s at a scan rate of 10 mV/s. The current height is recorded at the peak potential (~0.6 V) for analytical evaluation of the measurements. All measurements should be carried out at room temperature.

3.2. Detection at the Electrode Surface

Schematic representation of the hybridization detection using an electrochemical DNA biosensor using a SPE with a gold working electrode is shown in **Fig 6A**, **B**.

1. *Probe immobilization*. Pipet an aliquot (20 µL) of the probe solution onto the surface of gold SPE and spread it uniformly over the area of the three-electrode system. Incubate the SPE for about ~12 h at room temperature (**Fig. 6A1**, **B1**) and then flush with copious amounts of water to remove any nonspecifically adsorbed DNA probes. At the end of this step, a probe-modified electrode is obtained.
2. *Hybridization***.** Prepare the oligonucleotide (the target or noncomplementary) solutions of 20 µM in hybridization buffer. For each hybridization experiment, apply 20 µL of the sample to the probe-modified electrode surface (**Fig. 6A2**, **B2**). After 1 h of incubation at room temperature, rinse the electrode sequentially with the washing solution for 5 s and blank hybridization buffer for 5 s to remove any nonspecifically bound species. At the end of this washing step, a hybrid-modified

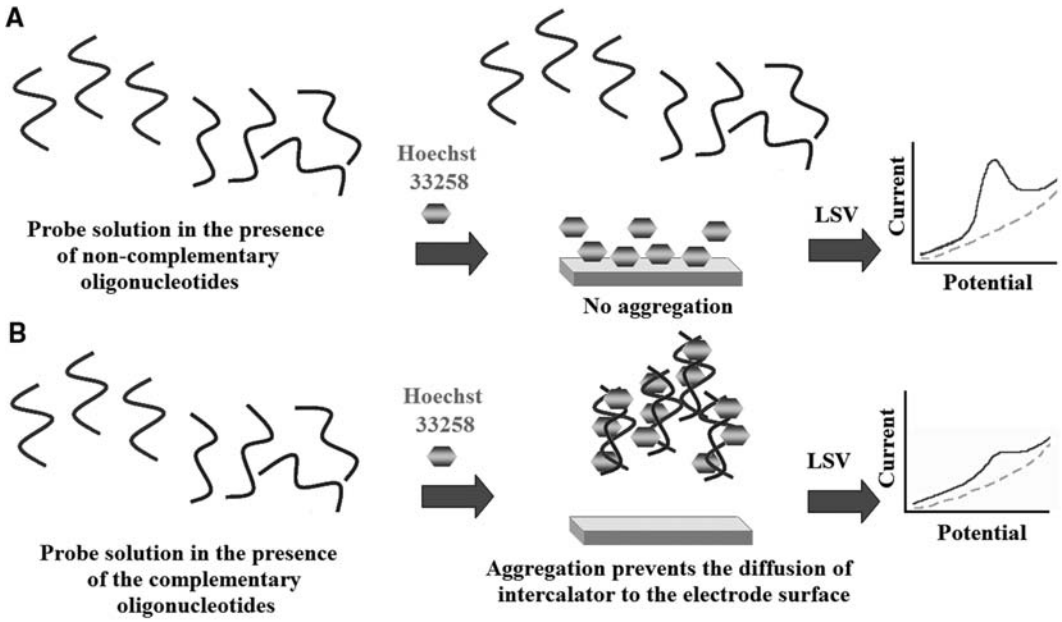

Fig. 5. The electrochemical detection of hybridization in solution. (**A**) Probe oligonucleotides do not hybridize with the noncomplementary ones and thus, the addition of the intercalator, Hoechst 33258, does not result in the formation of an aggregate. In the presence of single-stranded oligonucleotides, Hoechst 33258 can diffuse to the electrode surface and cause the formation of a high current response; (**B**) Probe oligonucleotides hybridizes with the complementary target oligonucleotide. An aggregate of hybrid duplexes together with the intercalator forms and prevents the access of the intercalator to the electrode surface. A small current response indicates the aggregation of hybrid duplexes with Hoechst 33258. The current intensity at the peak potential (~0.6 V) is recorded relative to the background response of the blank buffer solution (*dashed grey line*).

electrode is obtained (**Fig. 6B3**), but no hybridization would take place in the presence of noncomplementary oligonucleotides (**Fig. 6A3**).

3. *Intercalator binding*. Put an aliquot (20 µL) of the intercalator solution onto the SPE surface (**Fig. 6A4**, **B4**). Then, incubate for 5 min at room temperature. Wash off the excess intercalator with blank PBS.
4. *Electrochemical measurement*. Measure the anodic peak current of the bound intercalator using LSV (**Fig. 6A5**, **B5**) after an equilibration time of 10 s, with a sample interval of 1 mV, and a scan rate of 0.1 V/s from 0 to 1 V (*see* **Note 10**). Alternatively, CV or DPV can be applied for the measurement as described in **Subheading 3.1**. The current height is recorded at the peak potential (~0.6 V) for analytical evaluation of the measurements.

Fig. 6. (continued) incubated with the probe-modified electrode (**B2**), hybridization takes place between the complementary strands resulting in the formation of a hybrid-modified electrode (**B3**), Hoechst 33258, is incubated with the hybrid-modified-electrode (**B4**), since Hoechst 33258 intercalates into the double-stranded hybrids on the surface, a high electrochemical current response is obtained (**B5**). The peak current intensity at the peak potential (~0.6 V) is recorded relative to the background response of the blank buffer solution (*dashed grey line*).

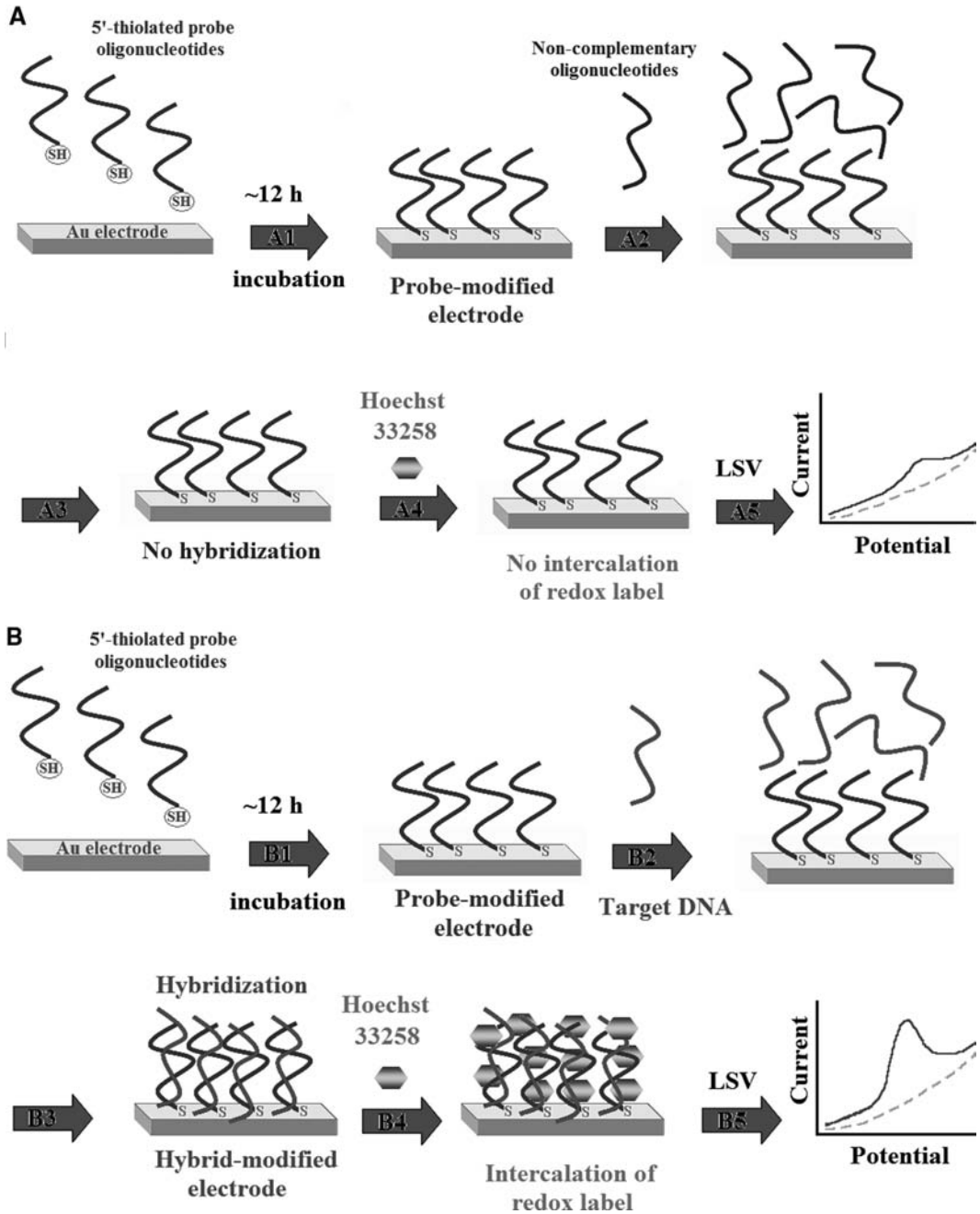

Fig. 6. (**A**) The electrochemical detection of hybridization on Au electrode surface. Probe oligonucleotides with 5′-thiol are incubated on the Au surface for 12 h. Strong covalent bond between thiols and Au surface results in the formation of the probe-modified electrode (**A1**), noncomplementary oligonucleotides are incubated with the probe-modified electrode (**A2**); however, no hybridization takes place between these two strands (**A3**), the redox label, Hoechst 33258, is incubated with the probe-modified-electrode (**A4**), since the redox label cannot intercalate with the single-strand probe oligonucleotides on the surface, a small electrochemical current response is obtained (**A5**). The current intensity at the peak potential (~0.6 V) is recorded relative to the background response of the blank buffer solution (*dashed grey line*). (**B**) The electrochemical detection of hybridization on Au electrode surface. Probe oligonucleotides with 5′-thiol are incubated on the Au surface for ~12 h for the preparation of the probe-modified electrode (**B1**), target oligonucleotides are

4. Notes

1. The electrochemical pretreatment of a carbon paste electrode can be performed by applying high positive bias from +1.25 to +1.75 V for a short period of time. Time periods longer than 1 min are not usually recommended. As for glassy carbon electrodes, the application of a lower bias from 0.5 to 1 V for 1–3 min is recommended.
2. Electrostatic DNA immobilization on electrode surface may require longer accumulation time periods than 5 min. Adjustment of accumulation time periods according to the concentration of DNA sample and electrode surface is recommended. For example, the immobilization of a trace amount of DNA onto a carbon paste electrode with a large surface area would certainly require a long accumulation time, while stirring the DNA containing solution.
3. The formation of a SAM on gold electrode surface may take longer than 12 h due to the surface roughness and the concentration of the thiolated oligonucleotides. In certain cases, incubation periods of 5 days for the immobilization of 1 μM 23-mer oligonucleotides on 10 μm gold microelectrodes (i.d.) have been found as the optimum period.
4. *Our recommended hybridization buffers are as follows.* 30 mM sodium citrate including 300 mM NaCl (pH 7.0) and 20 mM Tris–HCl including 500 mM NaCl (pH 7.0).
5. The hybridization reaction may take longer than 1 h according to the surface area, the concentration of the oligonucleotides immobilized on the surface, and the concentration of the target strand in solution. For the detection of trace amounts of target DNA, a mass transport boost using a shaker or a stirrer is highly recommended.
6. Hoechst 33258 is a hazardous chemical and should be handled with care. During the experiments, personal preventive equipment should be worn with a breathing apparatus (a mask), safety goggles, and gloves to prevent contact with skin and eyes. If swallowed, mouth should be washed out with water. In case of skin contact, flush with copious amounts of water for at least 15 min and the contaminated clothing and shoes should be removed.
7. Sperm cells from salmon testes are a good source of non-mammalian DNA. The species of salmon used is Oncerhyncus keta. The %G-C content is ~41.2%. The melting temperature (T_m) is 87.5°C in 0.015 sodium citrate including 0.15 M NaCl. The lyophilized form can be stored at 2–8°C. The lyophilized DNA is assigned a shelf life of 5 years. DNA should be dissolved in

autoclaved or molecular biology grade MilliQ water (nuclease-free). The solution will need to be stirred for at least 2–4 h, at room temperature, to dissolve the DNA. The aliquots of the dissolved DNA solution should be kept at –20°C. Repeating cycles of freeze/thaw should be avoided. We recommend preparing aliquots and storing these.

8. Purity of DNA or RNA may be determined by measuring the A_{260}/A_{280} ratio. Buffered solutions may provide more accurate and reliable A_{260}/A_{280} ratios than water. TE buffer (10 mM Tris including 1 mM EDTA, pH 8) is highly recommended for the preparation of stock solutions. A_{260}/A_{280} ratios can be affected by pH and ionic strength. A lower pH will result in lower A_{260}/A_{280} ratios and reduced sensitivity to protein contamination. A_{260}/A_{280} measurements are expected to be 1.7–2.0 to indicate pure DNA or RNA. (Please note, the A_{260}/A_{280} ratio may not be an accurate measure of nucleic acid purity. This ratio was first used to detect nucleic acid contamination in protein preparations and as such, can be a poor indicator of nucleic acid quality. Alternatively, quality can be assessed by simply analyzing the DNA or RNA by agarose gel electrophoresis or by evaluating performance (for example, by PCR amplification)). Gently vortex DNA samples for 5 s and sonicate for 5 s. Prepare dilution tubes by adding 980 µL TE buffer to 1.5 mL Eppendorf tubes. Remove 20 µL DNA from each sample (gently pipet up and down two or three times before removing sample to mix) and mix with TE buffer to make a total volume of 1,000 µL, giving a 1:50 dilution. To prepare blank solution, take blank 1,000 µL TE buffer. Vortex all dilutions for 5 s and sonicate for 5 s. Set spectrophotometer program according to your machine. Rinse a clean, quartz cuvette twice with TE buffer, tap on bench paper to blot, and wipe with a tissue paper. Pipet blank solution into cuvette and read absorbances at 320, (background), 280, and 260 nm. Rinse cuvette twice. Pipet the first sample into the cuvette and read absorbances at 320, 280 and 260 nm. Continue by rinsing the cuvette twice and reading additional samples at 320, 280 and 260 nm. To calculate DNA concentration of each sample: $(A_{260}–A_{320}) \times 50$ (DNA extinction coefficient) × dilution factor (i.e., 1,000/20) × final sample volume = DNA yield in µg.

9. For the ethanol precipitation procedure, mix one volume of the probe/DTT solution thoroughly with 0.1 volume of 3 M sodium acetate (pH 5.6) and 3 volumes of ice-cold absolute ethanol. Keep at –80°C for 30 min and centrifuge at 14,000 × *g* for 15 min. Wash the DNA pellet with 1 mL of 95% ethanol, centrifuge again, dry, and resuspend in water.

10. The blank buffer solution for the measurement of the electrochemical responses should cover all three electrodes on the screen-printed electrode. An aliquot of 20–30 μL is usually enough for covering all three electrodes, but if there is a demand for working with less volume of solutions, a careful pipetting of 10 μL may also be successful.

References

1. Eggins, B. R. (ed.) (1997) *Biosensors: An introduction*, Wiley, New York.
2. Kerman, K., Kobayashi, M. and Tamiya, E. (2004) Recent trends in DNA biosensor technology. *Measur. Sci. Tech.* 15, R1–R11.
3. Murphy, L. (2006) Biosensors and bioelectrochemistry. *Curr. Opin. Chem. Biol.* 10, 177–184.
4. Soper, S.A., Brown, K., Ellington, A., Frazier, B., Garcia-Manero, G., Gau, V., Gutman, S.I., Hayes, D.F., Korte, B., Landers, J.L., Larson, D., Ligler, F., Majumdar, A., Mascini, M., Nolte, D., Rosenzweig, Z., Wang, J. and Wilson, D. (2006) Point-of-care biosensor systems for cancer diagnostics/prognostics. *Biosens. Bioelectron.* 21, 1932–1942
5. Kerman, K., Vestergaard, M., Nagatani, N., Takamura, Y. and Tamiya, E. (2006) Electrochemical genosensor based on peptide nucleic acid-mediated PCR and asymmetric PCR techniques: Electrostatic interactions with a metal cation. *Anal. Chem.* 78, 2182–2189
6. Kerman, K., Meric, B., Ozkan, D., Kara, P., Erdem, A. and Ozsoz, M. (2001) Electrochemical DNA biosensor for the determination of benzo[a]pyrene-DNA adducts. *Anal. Chim. Acta* 450, 45–52
7. Kerman, K., Ozkan, D., Kara, P., Meric, B., Gooding, J.J. and Ozsoz, M. (2002) Voltammetric determination of DNA hybridization using methylene blue and self-assembled alkanethiol monolayer on gold electrodes. *Anal. Chim. Acta* 462, 39–47
8. Kerman, K., Saito, M., Morita, Y., Takamura, Y., Ozsoz, M. and Tamiya, E. (2004) Electrochemical coding of single-nucleotide polymorphisms by monobase-modified gold nanoparticles. *Anal. Chem.* 76, 1877–1884
9. Kerman, K., Morita, Y., Takamura, Y., Ozsoz, M. and Tamiya, E. (2004) Modification of Escherichia coli single-stranded DNA binding protein with gold nanoparticles for electrochemical detection of DNA hybridization. *Anal. Chim. Acta* 510, 169–174
10. Li, X., Lee, J.S. and Kraatz, H.-B. (2006) Electrochemical detection of single-nucleotide mismatches using an electrode microarray. *Anal. Chem.* **78**, 6096–6101
11. Li, X., Song, H., Nakatani, K. and Kraatz, H.-B. (2007) Exploiting small molecule binding to DNA for the detection of single-nucleotide mismatches and their base environment. *Anal. Chem.* 79, 2552–2555
12. Kerman, K., Chikae, M., Yamamura, S. and Tamiya, E. (2007) Gold nanoparticle-based electrochemical detection of protein phosphorylation. *Anal. Chim. Acta* 588, 26–33
13. Pjura, P.E., Grzeskowiak, K. and Dickerson, R.E. (1987) Binding of Hoechst 33258 to the minor groove of B-DNA. *J. Mol. Biol.* 197, 257–271
14. Teng, M.K., Usman, N., Frederick, C.A. and Wang, A.H. (1988) The molecular structure of the complex of Hoechst 33258 and the DNA dodecamer. *Nucleic Acids Res.* 16, 2671–2690
15. de, C.T., Carrondo, M.A.A.F., Coll, M., Aymami, J., Wang, A.H.J., van der Marel, G.A., van Boom, J.H. and Rich, A. (1989) Binding of a Hoechst dye to d(CGCGATATCGCG) and its influence on the conformation of the DNA fragment. *Biochemistry* 28, 7849–7859
16. Tanious, F.A., Hamelbeg, D., Bailly, C., Czarny, A., Boykin, D.W. and Wilson, W.D. (2004) DNA sequence dependent monomer-dimer binding modulation of asymmetric benzimidazole derivatives. *J. Am. Chem. Soc.* 126, 143–153
17. Rohs, R., Sklenar, H., Lavery, R. and Roder, B. (2000) Methylene blue binding to DNA with alternating GC base sequence: a modeling study. *J. Am. Chem. Soc.* 122, 2860–2866
18. an, K.X., Shibue, T. and Gross, M.L. (2000) Non-covalent complexes between DNA-binding drugs and double-stranded oligodeoxynucleotides: a study by ESI ion-trap mass spectrometry. *J. Am. Chem. Soc.* 122, 300–307
19. Kobayashi, M., Kusakawa, T., Saito, M., Kaji, S., Oomura, M., Iwabuchi, S., Morita,

Y., Hasan, Q. and Tamiya, E. (2004) Electrochemical DNA quantification based on aggregation induced by Hoechst 33258. *Electrochem. Commun.* 6, 337–343

20. Chaumpluk, P., Chikae, M., Takamura, Y. and Tamiya, E. (2006) Novel electrochemical identification and semi quantification of bovine constituents in feedstuffs. *Sci. Tech. Adv. Mater.* 7, 263–269
21. Saito, M., Kobayashi, M., Iwabuchi, S., Morita, Y., Takamura, Y. and Tamiya, E. (2004) DNA condensation monitoring after interaction with Hoechst 33258 by atomic force microscopy and fluorescence spectroscopy. *J. Biochem.* 136, 813–823
22. Saito, M., Takamura, Y. and Tamiya, E. (2005) Nanoscale time-lapse AFM imaging in solution for DNA aggregation. *Nanobiotechmology* 1, 361–368