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Genosensor on gold films with enzymatic electrochemical detection of a SARS virus sequence

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21.1 Background

Biosensors, analytical devices incorporating a biological material (e.g., tissue, cell receptors, enzymes, antibodies, nucleic acids, etc.), a biologically derived material (e.g., recombinant antibodies, engineered proteins, aptamers, etc.), or a biomimic (e.g., synthetic receptors, biomimetic catalysts, combinatorial ligands, imprinted polymers, etc.) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic, or micromechanical [1], have become a very important area of Analytical Chemistry. The majority of current and future analytical requirements will be solved by the use of simple yet sensitive devices, able to be reshaped with new trends such as the integration with, e.g., smartphones [2]. Apart from the well-established enzymatic sensors, the selectivity of affinity interactions, such as those of antigen–antibody or nucleic acid hybridization, is exploited for the respective development of promising immune and nucleic acid assays. Nucleic acid detection is becoming relevant not only in the field of food analysis but also in clinical diagnosis. It is advantageous compared with immunoassay in cases of recent infection or underlying immunodeficiency. Moreover, it is very useful for treatment monitoring because elimination of nucleic acids coming from a pathogen indicates successful handling [3].

Electrochemical biosensors combine the sensitivity of electroanalytical methods with the inherent bioselectivity of the biological component [4]. In DNA sensors, several alternative ways of detecting DNA electrochemically (direct or indirectly) are possible. The intrinsic electroactivity of adenine and guanine can be the basis for direct measurement of nucleic acids
On the other hand, the use of indicator molecules that interact differentially with single- or double-stranded DNA has been widely exploited in indirect approaches. Bioconjugation is commonly not required because electrostatic or hydrophobic interactions take place easily. A different possibility for indirect detection is the use of enzymes, alternative that enhances assay sensitivity enormously because of their inherent amplification. Although enzymes can be directly conjugated to DNA strands, the use of a well-known affinity interaction such as avidin–biotin is an easy alternative. Horseradish peroxidase has been widely used as enzyme label as well as glucose oxidase and alkaline phosphatase (AP). AP (EC 3.1.3.1) is a hydrolase that converts orthophosphoric monoesters into alcohols and thus various phosphate esters can act as substrates. As the name suggests, they are more effective in alkaline media. 3-Indoxyl phosphate (3-IP) has been proposed as an adequate substrate for AP in immunoelectrochemical approaches [6]. Because of its satisfactory electrochemical behavior (see Chapters 5, 30, and 31), it has been used for the development of AP-based enzyme immunoassays. In this experiment, AP is used to detect DNA hybridization events on gold electrodes.

The surface of the genosensor is of paramount importance in the performance of the analytical device because it is where immobilization and transduction take place. Gold has always been an appropriate material for the electrochemical detection of substances, while also allowing different formats: disks, wires, films, etc. Thin-film (thickness in the nm-scale) gold electrodes can be produced by sputtering, a physical vapor deposition methodology in which gold is ejected from a target in a vacuum chamber onto a substrate. Gold also offers the relevant possibility of generating self-assembled DNA monolayers through thiol groups because of the strong binding between sulfur and gold, which may be considered almost covalent.

In this chapter, a DNA hybridization assay with enzymatic electrochemical detection (see Fig. 21.1) is carried out on a 100-nm sputtered gold film that allows working with small volumes. Reducing the cell volume has several advantages, the first being the decrease in the diffusion distances required for analytes to reach their surface-bound receptor partners. A simple, cheap, and easy-to-handle homemade device is presented allowing the performance of the hybridization procedure and sequential detection. Square wave voltammetry (SWV) (see Chapters 3 and 24) is employed for measuring in a fast and sensitive way.

FIGURE 21.1 Schematic illustration of the assay developed on this genosensor. 3-IP, 3-indoxyl phosphate; AP, alkaline phosphatase; St, streptavidin; IC, indigo carmine.
This experiment is adapted from Ref. [7]. The sequence chosen as target for the development of this genosensor corresponds to a gene that encodes the nucleocapsid protein (422 amino acids) of the SARS (severe acute respiratory syndrome) virus, specifically a short lysine-rich region that appears to be unique to SARS and suggestive of a nuclear localization signal [8]. A 30-mer oligonucleotide with bases comprised between numbers 29,218 and 29,247, both included, was chosen.

A complementary strand to the chosen SARS sequence is labeled with a thiol group and immobilized on the gold surface. The target (30-mer oligonucleotide with a sequence included in the SARS coronavirus) is conjugated to biotin and hybridized with the probe. Addition of AP-labeled streptavidin (St) allows enzymatic detection via the electrochemical signal of the product. The parameters affecting all assay steps are studied, and analytical characteristics, including selectivity, can be discussed.

### 21.2 Electrochemical cell

The electrochemical cell (see in more detail in Section 21.5.1) consists of 5 cm × 5 cm supports of a 0.125 mm thick polyimide substrate that are sputtered with gold (Emitech sputter coater model K550) and after delimiting areas, act as working electrodes. A miniaturized Ag/AgCl/saturated KCl electrode is employed as reference and a platinum wire as auxiliary electrode. All the three electrodes are connected with alligator clips to the potentiostat.

### 21.3 Chemicals and supplies

**Reagents**

- **Oligonucleotide buffer:** Tris-EDTA (TE) buffer pH 8 (0.1 M Tris-HCl buffer solution, 1 mM in EDTA).
- **Enzyme:** AP (conjugated to Streptavidin), AP-labeled St.
- **Substrate and product:** 3-IP and indigo carmine (IC).
- **IC stock solution:** 0.1 M H₂SO₄.
- **3-IP buffer:** 0.1 M Tris-HCl, 10 mM MgCl₂ pH 9.8.
- **St-AP buffer:** 0.1 M Tris-HCl, 1 mM MgCl₂ pH 7.2.
- **Hybridization buffer (2x SSC):** 30 mM sodium citrate buffer with 300 mM NaCl, pH 7 (Table 21.1).

**TABLE 21.1** Commercial DNA sequence, written from 5′ to 3′, and peptide sequence for the development of the assay.

| Description                     | Sequences (5′-3′)                              |
|---------------------------------|------------------------------------------------|
| Biotinylated target             | ACA-GAG-CCT-AAA-AAG-GAC-AAA-AAG-AAA-AAG-biotin |
| Biotinylated mismatched target  | ACA-GCG-CCT-AAA-AAC-GAC-AAA-AAG-AG-AAG-biotin  |
| Biotinylated and thiolated probe| Biotin-CTT-TTT-CTT-TTT-GTC-CTT-TTT-AGG-CTC-TGT–(CH₂)₅–SH |
- **Blocking proteins**: Bovine serum albumin (BSA, fraction V), biotin-labeled albumin, casein from sheep milk, and a gelatine derivative called “Perfect Block” in 0.1 M Tris-HCl pH 7.2.
- **Blocking agents**: Methanol, thiocystic acid, 1-hexanethiol, 6-mercapto-1-hexanol in ethanol.
- Saturated KCl solution.
- Milli-Q water is employed for preparing solutions.

**Materials**

- **Electrode fabrication**: Polyimide substrate, conductive wire, epoxy resin (CW2400), self-adhesive washers with 5 mm internal diameter (19.6 mm² internal area), crocodile clips, silver wire, platinum wire, syringe rubber piston, micropipette tip, insulating tape.
- **Apparatus**: Weighing scale, pH meter, and incubator.
- **Other materials**: 100-μL and 1-mL micropipettes with corresponding tips, 1.5-mL microcentrifuge tubes.

**21.4 Hazards**

Students are required to wear lab coat, appropriate gloves, and safety glasses. Special care should be taken when handling concentrated acids.

**21.5 Experimental procedures**

**21.5.1 Construction of the electrochemical cell**

The three-electrode potentiostatic system used for this experiment is schematized in Fig. 21.2. Working electrodes are made on 5 cm × 5 cm supports of a 0.125 mm thick polyimide substrate called Kapton HN. They are covered with gold by a sputtering process explained on Section 21.5.2. A conductor wire is attached to the center of one of the sides by means of an epoxy resin (CW2400) and it is cured at room temperature. The working area is limited by self-adhesive washers of 5 mm internal diameter. The gold film is placed on a support to which an alligator connection is fixed.

Reference and auxiliary electrodes are coupled in a micropipette tip. The reference electrode is an anodized silver wire introduced in the tip through a syringe rubber piston. In turn, the tip is filled with saturated KCl solution and contains a low-resistance liquid junction. The platinum wire acts as auxiliary electrode and it is fixed with insulating tape.

For measurement recording, the tip is fixed on an electrochemical cell Metrohm support, allowing horizontal and vertical movement.

**21.5.2 Gold sputtering**

The working electrodes made on polyimide supports are covered with gold by a sputtering process. The Kapton slide (5 cm × 5 cm) must be cleaned with ethanol and left drying. Once dried, the slide is covered with gold. Gold atoms are deposited (from the cathode) over the Kapton (placed on the anode) in a vacuum chamber filled with argon. The thickness
of the gold layer is controlled by means of the duration and intensity of the discharge (possible values are a 35 mA discharge applied for 220 s).

21.5.3 Hybridization assay

1. Deposit a volume of 5 μL of the thiolated DNA probe (in TE buffer pH 8) on the gold film (e.g., 1 μM in concentration).
2. Maintain at 37°C for 20 min or at 4°C for 12 h.
3. Wash with 0.1 M Tris-HCl buffer pH 7.2.
4. Add 15 μL of a 2% blocking agent solution (see Section 21.3.1, blocking agent in 0.1 M Tris-HCl pH 7.2 if it is a protein or ethanol if it is a thiol) and wait 10 min.
5. Clean it with 2x SSC buffer.
6. Add the biotinylated complementary strand (target) and wait 60 min at room temperature.
7. Wash the film with 0.1 M Tris-HCl, 10 mM MgCl₂ pH 7.2.
8. Add 20 μL of AP-labeled St at a 10⁻⁹ M concentration (in 0.1 M Tris-HCl, 1 mM MgCl₂ pH 7.2) and wait 60 min.
9. Wash the film with 0.1 M Tris-HCl, 10 mM MgCl₂ pH 9.8.
10. Add 20 μL of a 3 mM solution of 3-IP (in 0.1 M Tris-HCl, 10 mM MgCl₂ pH 9.8) and wait 10 min for its enzymatic hydrolysis.
11. Add, then, 5 μL of concentrated H₂SO₄ to stop the reaction.
12. Finally, add 5 μL of Milli-Q water and perform the electrochemical measurements.
21.5.4 Electrochemical measurement

The tip with the reference and auxiliary electrodes is introduced into the 30-μL drop (20 μL from the 3-IP solution plus 5 μL of the concentrated H₂SO₄ and 5 μL of Milli-Q water) deposited on the gold film. A potential of −0.35 V is applied for 30 s before scanning the potential between −0.15 and +0.3 V following a square wave format with frequency of 50 Hz and amplitude of 50 mV. Measure the peak current, which is the analytical signal that can be correlated with the concentration.

21.5.5 Effect of evaporation

Evaporation seems to be a critical condition in the immobilization of SH-DNA. A more rigorous study (step 1–2 of Section 21.5.3) could be done. Using a drop of 5 μL and a strand concentration of 1 μM, different immobilization times can be employed at room temperature (22°C approximately), 37 and 47°C. Note down when evaporation occurred and compare the signals obtained. Select the best immobilization conditions.

21.5.6 Surface blocking

Blocking the surface is one of the most important steps to minimize and control nonspecific adsorptions. Two main types of agents can be considered: proteins and sulfur-containing compounds. The signal/background ratio, and therefore the blocking capacity, should be studied for each compound.

Proteins such as albumin are common blocking agents in bioanalysis and adsorb well onto gold surfaces. Evaluate the nonspecific signal obtained after blocking (step 4 in Section 21.5.3) with aqueous solutions of BSA, casein, and a gelatine derivative called Perfect Block.

The other group of blocking agents are compounds that contain sulfur atoms and therefore present a high affinity for gold. Ethanolic solutions of thioctic acid, 6-mercapto-1-hexanol, and 1-hexanethiol could be tested. It is very interesting to note the effect of these agents on both capacitive and faradaic currents.

21.5.7 Analytical characteristics

A calibration curve for the complementary strand (c-DNA) can be performed under optimized conditions, employing a 1-μM solution of SH-DNA for immobilization. As an example, concentrations in the 0.1–10 nM interval could be tested (step 6 in Section 21.5.3).

SWV measurements for different concentrations are included in Fig. 21.3. Plot the values of the peak current for different concentrations and fit the data to a regression curve. Take into account that it can be nonlinear and fit into logarithmic or a different model. Determine the dynamic range, sensitivity, and limit of detection of the assay.

Precision studies of the platform could be made by obtaining measurements with the genosensor on different working areas, polyimide substrates, days, or groups. Evaluate the results through the value of the relative standard deviation of the peak current.
21.6 Lab report

Write a lab report following the typical scheme of a scientific article, including a brief introduction, experimental part (materials, equipment, and protocols), results and discussion, and conclusions. The following points should be borne in mind:

1. In the introduction, explain the purpose of the experiment and do a short review of the methods described to tackle this problem.
2. Protocols must be suitably detailed including schemes preferentially where appropriate and the necessary calculations.
3. Discuss the main variables that influence the analytical signals and include figures with representative raw data and results presented in tables and graphs for optimization studies.
4. Include graphs for the calibration curve, discussing the values obtained for the figures of merit. Pay attention to the significant figures in each case.
5. Indicate and comment in case the results obtained in terms of selectivity (see additional note number 8) and precision.
6. Discuss the incidences during the course of the experiment.

21.7 Additional notes

1. Oligonucleotide solution aliquots must be prepared and maintained at −20°C and working solutions must be conserved at 4°C.
2. IC solutions can be employed to know the electrochemical behavior of the enzymatic product (and the analytical signal). They must be protected from light and kept refrigerated at 4°C. Working solutions must be prepared daily.
3. 3-IP solutions must be prepared daily and kept at 4°C, protected from light.
4. AP-labeled St aliquots must be prepared and maintained at −20°C; working solutions are conserved at 4°C.
5. A drop of 5 μL and a strand concentration of 1 μM are employed in the immobilization steps (step 1 in Section 21.5.1), but both variables could be varied to study their influence.

FIGURE 21.3 Response curves for background (nonspecific adsorptions) and several concentrations of biotinylated target (from a to e).
6. The blocking of the active surface remaining after immobilization is very important in bioassays. Different agents could be checked, as commented in Section 24.5.2. The influence of different concentrations could also be evaluated.

7. SWV is employed for measurement because it is a fast and sensitive electrochemical technique. However, cyclic voltammetry or differential pulse voltammetry could also be evaluated. In particular, cyclic voltammetry should be made initially to know the electrochemical behavior and processes of 3-IP.

8. Selectivity of the genosensor can be studied by evaluating the signal of a, e.g., 3-base mismatch strand: 5'‐ACA‐GCG‐CCT‐AAA‐AAC‐GAC‐AAA‐AAG‐AG‐AAG‐3'‐biotin. Mismatches are located in base numbers 5, 15, and 26. It is also biotinylated at the 3'‐end to allow hybridization detection by interaction with AP-labeled St. Adding agents that increase stringency (e.g., 50% of formamide) should be considered.

9. The sensitivity could be improved using different conditions (drop volume, time of the different steps, buffer composition, etc. [7]). Students are encouraged to discuss and evaluate the different variables.

10. In this case, a proof of concept of a biosensor able to detect SARS DNA is presented. The target is labeled with biotin. Then, in a real assay, DNA would have to be amplified using biotinylated primers. Alternatively, a sandwich format (thiolated capture probe–target–biotinylated detection probe) should be employed.

### 21.8 Assessment and discussion questions

1. Indicate clearly all the steps of the procedure. The use of a scheme is encouraged.
2. Why a DNA strand is functionalized with a thiol group?
3. What is the role of the blocking agent? Enumerate the different possibilities.
4. What is the aim of employing the biotin–avidin interaction here?
5. Explain how the analytical signal is obtained, especially in what concerns to the electrochemical technique employed.

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