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Oligonucleotide quartz crystal microbalance sensor for the microalgae Alexandrium minutum (Dinophyceae)

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Abstract: We report the immobilization on a gold surface of a 20-base DNA probe labeled with disulfide group and on the selective hybridization with the complementary 20-base DNA strand. The oligonucleotide probe is the complementary strand of a partial sequence of the gene encoding for a large ribosomal RNA sub-unit which is a coding sequence of Alexandrium minutum DNA, a microalgae that produces neurotoxins responsible for paralytic shellfish poisoning on European and Asian coasts. The kinetics of DNA probe immobilization and hybridization were monitored in situ by using a 27 MHz quartz crystal microbalance under controlled hydrodynamic conditions. The frequency of the setup is stable to within a few hertz, corresponding to the nanogram scale, for 3 h and makes it possible to follow frequency change from immobilization of the probe to hybridization of the complementary DNA target. This setup constitutes a biosensor, which is sensitive and selective, and the hybridization ratio between hybridized complementary DNA and immobilized DNA probes is 47%.

Keywords: Microbalance; Alexandrium minutum; DNA

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Abstract

We report the immobilization on a gold surface of a 20-base DNA probe labeled with disulfide groups and on the selective hybridization with the complementary 20-base DNA strand. The oligonucleotide probe is the complementary strand of a partial sequence of the gene encoding for a large ribosomal RNA sub-unit which is a coding sequence of *Alexandrium minutum* DNA, a microalgae that produces neurotoxins responsible for paralytic shellfish poisoning on European and Asian coasts. The kinetics of DNA probe immobilization and hybridization were monitored *in situ* by using a 27 MHz quartz crystal microbalance under controlled hydrodynamic conditions. The frequency of the set up is stable to within a few hertz, corresponding to the nanogram scale, for 3 hours and makes it possible to follow frequency change from immobilization of the probe to hybridization of the complementary target DNA. This setup constitutes a biosensor which is sensitive and selective, and the hybridization ratio between hybridized complementary DNA and immobilized DNA probes is 47%.

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1. Introduction

The microalgae *Alexandrium minutum* (-Halim, 1960; Balech, 1989; Bolch et al, 1991) produces neurotoxins which can be accumulated in shellfish and are responsible for damage to human health. This species recurrently forms toxic blooms in the northern part of the Brittany coast in France (Nezan et al, 1989; Belin, 1993; -Erard, 1990), southern Taiwan coast (Hwang et al, 1999), northern Adriatic waters (–Honsell, 1993) and New Zealand coasts (Chang et al, 1997). At present, algae identification relies on tedious microscope observations mostly performed by skilled taxonomists in laboratories. More recently real-time PCRs were performed to detect *Alexandrium minutum* from the Catalan coast (Galluzzi et al, 2004). Gravimetric sensors are useful for the design of sensitive and selective *in situ* biosensors (Bizet et al, 1997; Minunni et al 2003; Su et al, 1997) and appear as promising tools for the detection of toxic blooms in the framework of coastal water monitoring. We report in this work the immobilization on a gold surface of a 20-base DNA probe labeled with disulfide on a gold surface and the hybridization with the complementary 20-base DNA strand. The sequence of the oligonucleotide probe is a partial sequence of the gene encoding for the large ribosomal RNA sub-unit of *Alexandrium minutum* (Guillou, 2002).

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2. Experimental section

2.1. Chemical and biochemical reagents

H_2SO_4 (95%), H_2O_2 (30% in water), NaOH, HCl, $MgCl_2$, NaCl, 1 M 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid buffer (HEPES), 1 M tris-(hydroxymethyl)aminomethane buffer, sodium dodecyl sulfate (SDS), sodium citrate, casein, were from Sigma Aldrich. All chemicals reagents were of biochemical quality. DIG Oligonucleotide tailing kit was from Roche Diagnostic. Anti-DIG-alkaline phosphatase conjugate (150 mU/mL), nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (BCIP) were from Biorad. Water used in all experiments was deionized and double distilled. 5'-$H_3C(CH_2)_5$-S-S-$(CH_2)_6$-AGCACTGATGTGTAAGGGCT-3' (DNA-disulfide probe), 5'-AGCCCTTACACATCAGTGCT-3' (complementary DNA), phosphatase-5'-AGCCCTTACACATCAGTGCT-3' (complementary DNA-phosphatase) and 5'-CCTTGGTCCGTGTTTCAAGA-3' (non-complementary DNA) were from Eurogentec. The disulfide labeled oligonucleotide was synthesized by a crosslinked reaction (Atsushi et al, 1990). Purity of DNA chains was checked by chromatography, MALDI-TOF analysis and UV optical density measurements. Nylon transfer membranes, Nytran SuperCharge from Schleicher & Schuell, were used for control hybridization experiments.

2.2. Buffers and solutions

The hybridization buffer for control experiments on nylon was 0.6 M NaCl, 60 mM sodium citrate, 1% blocking reagent (Roche), 0.1% sarkosyl (N-lauroylsarcosine), 0.02% SDS adjusted to pH 7.0. Post-hybridization washing solutions were prepared from 20X SSC (3M NaCl, 300 mM sodium citrate, pH 7.2) and 10% SDS. Tris Buffer for the color development solution was 0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5. The hybridization buffer for QCM experiments was 0.05 M HEPES 4 and 0.5 M NaCl adjusted to pH 7.43 with drops of 1 M NaOH, referred to as 'HEPES'.

2.3. Hybridization protocol on Nylon membrane

Nylon membrane: 50 µL of a 100 µM solution in sterile distilled water of the DNA-disulfide probe, of a non-disulfide-labeled same-sequence probe as positive control, and of a non-complementary probe as negative control were spotted on a positively charged nylon membrane (Sambrook et al., 1989) using a filtration manifold (Schleicher & Schuell). The membrane was let to dry and cross linked by U.V. exposition for 3 minutes. Prior to hybridization 2 µL of 50 µM complementary strand were labeled with Digoxygenin–dUTP according to the DIG Oligonucleotide tailing kit and diluted in 10 mL hybridization buffer (final concentration 10 nM). The hybridization steps were 30 minutes at 37°C for pre-hybridization, 2 hours at 37°C for hybridization and followed by stringency washing steps (2X5 minutes at room temperature in 2X SSC / 0.1% SDS, 2X15 minutes at 37 °C in 0.1X SSC / 0.1% SDS). DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (150 mU/mL) and the substrates NBT and BCIP, which give a light-blue precipitate. Just prior to use, 1 ml of 30mg/mL NBT and 1 mL of 15 mg/mL BCIP were mixed in 100 mL of Tris Buffer. The nylon membrane was immersed in the color development solution. BCIP and NBT are two colorless substrates which form a redox system. BCIP is oxidized by alkaline phosphatase to indigo by release of a phosphate group. In parallel NBT is reduced to diformazan. The reaction products form a water insoluble dark blue precipitate on the membrane.

2.~~3~~4. QCM apparatus

AT-cut planar quartz crystals 14 mm diameter with a 9 MHz nominal resonance frequency (Matel Fondhal France) were used. Two identical gold electrodes, 2000 Å thick and 5 mm in diameter, were deposited by evaporation techniques on both sides of crystals with a chromium underlayer. The gold side used in the experiments was cleaned with a 10 μL drop of 50% H_2SO_4, 50% H_2O_2 for 30 minutes and rinsed with deionized double distilled water. The resonators were connected with a silver conducting paste, through wires, to a BNC adaptator. A home-made oscillator was designed to drive the crystal at 27 MHz which corresponds to the third overtone of the quartz resonator. To improve the stability, all the electronic oscillator components where temperature-controlled by a Watlow heater current monitor with a stability better than 0.1 K. An experimental cell was developed: the crystal was mounted between two O-ring seals inserted in a plexiglass cell. Only one face of the quartz was in contact with the solutions. The cell volume was 50 μL. The apparatus included a P1 micropump (Pharmacia) to assure a 20 μL/min constant flow of the solutions. The experiments were performed at 22°C, the room temperature. The experimental QCM setup consisted of the 27 MHz QCM and a frequency counter PM 6685. The experimental setup was computer controlled by home-made software in C language.

3. Results

3.1. Hybridization on nylon

Hybridization experiments were first performed on nylon membrane to check the hybridization properties of the DNA-disulfide probe. Positive hybridizations were obtained with the DNA-disulfide probe and the non-disulfide-labeled same-sequence probe. With the non-complementary probe used as control hybridization was negative. Results of the colorimetric detection test are presented in figure 1.

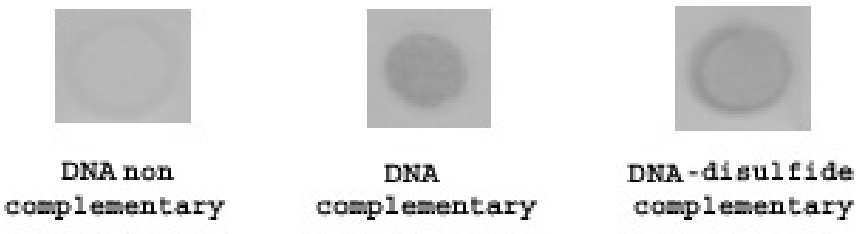

Fig. 1. Hybridization experiments on nylon

The hybridization properties of the DNA-disulfide probe were not changed by the 5'-end disulfide labeling.

3.2. Hybridization on quartz crystal microbalance

Immobilization of a DNA-disulfide probe, a selective hybridization test with a non-complementary DNA and hybridization with a complementary DNA chain were performed on the gold electrode of a quartz resonator by successive circulation of NaCl, HEPES and DNA solutions. The experimental conditions were already optimized: pH 7.4 and 0.5 M NaCl, the conditions of Zhou *et al.* (Zhou et al, 2001) who designed a 18-base DNA-disulfide probe gravimetric sensor. The frequency shift during circulation at a 50 μL/min flow rate of successive solutions in the quartz cell is presented in figure 2.

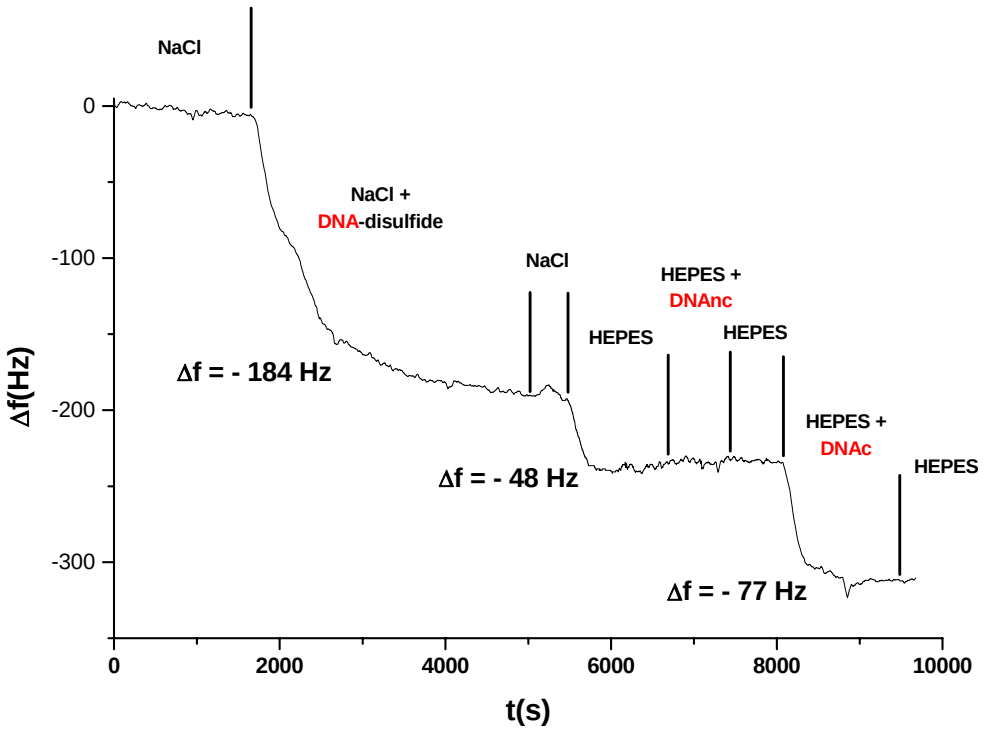

Fig. 2. Microbalance frequency change successive circulation of 20 μg/mL DNA-disulfide NaCl solution, 20 μg/mL non complementary DNA HEPES solution and 20 μg/mL complementary DNA HEPES solution

The first step, the immobilization of the DNA-disulfide probe, was performed by circulation of a 20 μg/mL DNA-disulfide solution. The frequency change observed is -184 Hz, as shown in figure 2. The coverage of the surface is estimated to be 66%, taking into account the experimental microbalance sensitivity (350 pg/Hz), the active surface of the QCM (0.2 cm^2), the molecular weight of the DNA-disulfide (6448 g/mol) and the average area of one molecule of adsorbed DNA-disulfide (2.2 nm^2) (Zhou et al, 2004). The frequency change of -48 Hz is attributed to the change of viscosity between 0.5 M NaCl and HEPES buffer. The second step that allows the selectivity to be checked was performed by circulation of 20 μg/mL non complementary DNA (DNAnc) solution in HEPES. There was no frequency change during the flowing of this solution (figure 2). This means that there is no hybridization or non-specific adsorption of the non-complementary DNA chain. The third and last step, hybridization, was performed by circulation of a 20 μg/mL complementary DNA (DNAc) solution in HEPES. The frequency change observed was -77 Hz (figure 2). The hybridization ratio of hybridizing complementary DNA versus immobilized DNA-disulfide probes is 45%, taking into account the molecular weight of the DNA-disulfide probe and of the complementary DNA (6055 g/mol). This experiment was reproductible, it was done 4 times; the mean value of the coverage is 67% (SD 3.1%) and the mean value of the hybridization ratio is 47% (SD 2.6%).

4. Conclusions

We have designed a selective and sensitive 20-base oligonucleotide gravimetric biosensor with a 47% hybridization ratio for a coding sequence of *Alexandrium minutum* DNA. The biosensor is stable at the nanogram scale for 3 hours, which allows us to

follow frequency change from immobilization of the probe to selective hybridization of the complementary target DNA. This is the first gravimetric biosensor for *Alexandrium minutum* and the first step towards the design of a biosensor for real-time coastal water monitoring of this harmful microalgae. We will present a complete thermodynamic and kinetic hybridization study in a subsequent full paper.

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