

Toward Detection of Harmful Algae Blooms by *in situ* Surface Plasmon Resonance Spectroscopy

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Abstract – Among marine algae species, *Alexandrium minutum* produces a phycotoxin called paralytic shellfish poisoning (PSP) that is introduced in the food chain through the ingestion of phytoplankton by shellfishes, and later by human consumers. Thus, *in situ* monitoring of *A. minutum* proliferation in coastal seawater is of great economical importance for marine resources exploitation. Here, we propose a rapid test for the detection of *A. minutum* by surface plasmon resonance spectroscopy. First, whole genomic DNA is extracted from the algae. Second, a 677 bp long portion of the 28S ribosomal DNA is amplified by PCR. Third, the PCR product is detected by surface plasmon resonance spectroscopy onto a DNA functionalized gold substrate.

Keywords – surface plasmon resonance; spectroscopy; *in situ*; algal detection; PCR

I. INTRODUCTION

Alexandrium minutum is a world-wide spread dinophyceae belonging to the harmful algae group ; this species causing “red-tides” during algal blooms produces phycotoxins named “paralytic shellfish poisoning” (PSP) under specific environmental conditions. Then, shellfishes ingest that phytoplankton and concentrate PSP in their glands. Human consumption of those infested shellfishes induces an acute intoxication, leading to several non specific neuronal and gastrointestinal symptoms [1]. Thus, as an important public health problem, public authorities need to control seawater in order to prevent human intoxications. Actually, as the mechanisms of induction for toxin production by *A. minutum* is unclear, a three step alert system has been established ; a regular numeration of *A. minutum* in seawater eventually followed by the AOAC reference mouse assay and finally a quantification of the PSP toxins.

Up to now, coastal laboratories monitor routinely *A. minutum* proliferation using a manual technique implying first a sampling of seawater, second a long time consuming decantation, and third a microscopic counting of phytoplankton in the sample. However, this technique suffers

from several limitations including the delay of sample treatment – 24 hours – and the need of very good skills in taxonomic identification of phytoplankton. Thus, *in situ* monitoring could avoid the need to return samples into the laboratory before analysis

In this context, development of marine *in situ* instrumentation associated with a rapid sensitive detection protocol is a promising emerging alternative for those routine assays [2, 3]. More, as *A. minutum* levels vary sharply temporally and spatially, *in situ* measurements would help to better forecast blooms apparition.

II. PROPOSED APPROACH

In this context, we have developed an assay based on DNA biosensors. As a transducer, we chose an optical method known as surface plasmon resonance (SPR) spectrometry [4] because of its marker free detection and thus limiting consumables for a future *in situ* autonomous sensor.

We adopted Krechtmann-Raether configuration for our SPR spectrometer as shown in Fig. 1 ; a polarized white light beam is reflected at the interface between a high refractive index glass (F2) prism and a 50 nm gold layer and then analyzed through a spectrometer coupled to a CCD camera.

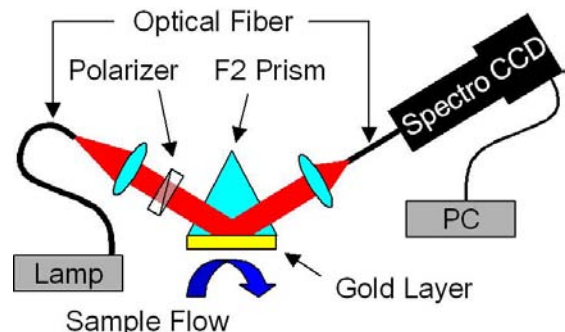


Figure 1. Schematic representation of our SPR spectrometer.

Briefly, the assay consisted first in a whole DNA extraction of *A. minutum* genome. In a second time, a single strand PCR was performed in order to amplify a specific sequence in the region coding for the 28S ribosomal subunit of *A. minutum*. Finally, the single stranded PCR product was assayed over the DNA biosensor.

In this study, we demonstrate in a first part the feasibility of that approach using our home-made SPR spectrometer with synthetic short length complementary oligonucleotides. In a second part, we detected single stranded DNA PCR product of *A. minutum*.

III. MATERIALS & METHODS

A. SPR spectrometer

As imaged in Fig. 1, a white light beam produced by a tungsten-halogen lamp (HL-2000, Mikropack) was guided through a multimodal optical fiber (ThorLabs), collimated (ThorLabs) and *p*-polarized (ThorLabs). Light beam was then reflected on a 50 nm thick gold interface of a F2 glass disk (Schott) optically coupled with an equilateral F2 prism (ThorLabs). Reflected beam was finally focused and guided through a multimodal optical fiber for subsequent spectrum analysis *via* a Vis-NIR axial spectrometric system (Horiba Jobin-Yvon) coupled to a CCD camera (ST3200ME, SBIG). Frame rate was set at 0.5 image/s.

Signals were then processed by Visual Basic home-made software interface. The latter calculates the prediction of the plasmonic dip position employing the centroid algorithm. Sensorgrams were finally analyzed and aligned with Origin software.

The microfluidic system was composed of 0.03 inch. ID Teflon tubing (Upchurch Scientific) for input / output and a 10 mm peak circular interaction chamber specially designed for F2 glass substrates. Waterproofness of the chamber was done by a O-ring joint (Cluzeau Info Labo). Liquid circulation was made by a peristaltic pump (Meredos) with a 1.02 mm ID Tygon tubing (Bioblock Scientific).

B. Chemicals & oligonucleotides

All chemicals were purchased at Sigma-Aldrich. Thiolated, phosphorylated and non-modified oligonucleotides were purchased at Eurogentec, dissolved in sterile deionized water and conserved at -20°C until use. Oligonucleotides figuring in Table 1 were all quantified spectrophotometrically before injection inside the microfluidic system.

C. Whole DNA extraction from *A. minutum* culture

A. minutum strain AM89BM (Bay of Morlaix, France) came from the IFREMER Centre de Brest collection. The inoculum was cultured in f/2 Guillard and Ryther medium [5] and grown axenically at $18 \pm 1^{\circ}\text{C}$ under cool-white fluorescence light, at a photon flux of $150 \text{ mE}/(\text{m}^2.\text{s})$ with 14:10 LD photoperiods.

Whole DNA extraction protocol followed CTAB methodology. 100 mL of culture was harvested and centrifuged 5 min at 6000 g. The pellet containing cells was

then mixed in 2X CTAB buffer (CTAB 2 %; Tris-HCl 100 mM; 2-mercaptoethanol 0,2 %; SDS 1 %; proteinase K $184 \mu\text{g.mL}^{-1}$) and incubated at 65°C for 90 minutes. Then, aqueous phase was purified after centrifugation (15 min, 12 000 g) in 3 steps using alternatively chloroform / isoamyl (24:1), phenol / chloroform / isoamyl (12:12:1), and chloroform / isoamyl (24:1) as extraction solvent mixes. DNA was precipitated from aqueous phase by adding frozen isopropanol (-20°C) overnight and centrifugation (20 minutes, 10000 g, 4°C). Pellet was purified with Wash Solution (ethanol 75 %; ammonium acetate 3 mM) and centrifuged (5 min, 10000 g). Finally, pellet was washed 2 times with ethanol (70 %) and dried. Extracted DNA was dissolved in sterile water and stored at -20°C until use.

D. Obtention of ssDNA PCR product

PCR primers were designed to amplify a specific sequence (677 bp) of *A. minutum* genome coding for ribosomal RNA of the 28S subunit. Primers sequences were the following ones : 5'-TGCCAGCACTGATGTGTAAGGGCTT-3' (ARC) as forward primer and P-5'-ACCCGCTGAATTTAAGCATA-3' (P-D1R) as 5' phosphorylated reverse primer.

50 ng of the previous extracted DNA was brought in a medium that contained : MgCl_2 1.5 mM, 0.2 mM of each dNTP, Taq polymerase 0.04 U/ μL (Red Goldstart, Eurogentec), PCR buffer 1 X (Eurogentec) and 0.5 μM of each primer. PCR was carried out on a thermal cycler (Mastercycler, Eppendorf) with the following profile : 94°C for 5 min (initial denaturation), followed by 40 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (hybridization), 72°C for 1 min 30 s (elongation), and 72°C for 7 min (final elongation). PCR products were analyzed by agarose gel electrophoresis, purified with Qiaquick PCR purification kit (Qiagen) and quantified with a Nanodrop spectrophotometer (Thermo Scientific).

5' phosphorylated PCR strand was subsequently digested at 37°C during 15 min by lambda exonuclease 0.25 U/ μL (Epicentre). Finally, ssDNA was purified with Qiaquick PCR purification kit and quantified with a Nanodrop spectrophotometer (Thermo Scientific).

E. Injection protocol

Before injection of biological molecules, air redemption from the microfluidic circuitry was achieved with an injection of ethanol. Whether for immobilization or target sequence detection, DNA strands were diluted in an hybridization buffer (50 mM HEPES; 0.5 M NaCl) just before injection. For all experiments, reactions had taken place at room temperature (22°C) and flow rate was set at 60 $\mu\text{L}/\text{min}$. In order to minimize reactive consumption, the microfluidic circuitry was set in a closed configuration after 1 000 s of injection for thiol immobilization and ssPCR product hybridization. Regeneration step was achieved with a 0.3 M NaOH aqueous solution.

IV. RESULTS

A. Sensor sensitivity and accuracy

Sensor sensitivity was measured following the injection of different sucrose concentrations ranging from 0.05 % to 5 % [6]. Setting two different incident angles, investigations were done for two ranges of wavelength excitation conditions (740 and 810 nm as baselines in water respectively). For each assay, recorded signals indicated a linear relationship between wavelength shifts vs. sucrose concentration (data not shown). As expected from theoretical calculations [7], near-IR shifting resulted in a 13 % more sensitive signal.

In association with near-IR settings and differently from previous studies in the literature, signal normalization was here processed from the spectrum obtained with ethanol solution as its refractive index is sufficiently different from water one's to shift outside the detection window. That protocol offers the advantage to limit spectral distortions coming from differences of transmittance and reflectance between TM vs. TE polarizer modes ; as a result, normalized spectra offer a shape with higher symmetry for the dip (data not shown). Consequently, a 13.5 % increase of sensitivity was observed through calibration curves.

Following those experimental conditions and signal process, sensor optical sensitivity $\delta\lambda/\delta n$ was estimated to 6321 nm/RIU considering 810 nm as dip baseline.

Adopting a home-made improved version of "centroïd" signal processing published by Chinowsky *et al.* [8], we were able to obtain a dip prediction below the spectrometer resolution (0.15 nm). Signal noise standard deviation of $7.5 \cdot 10^{-3}$ nm was measured. Thus, refractive index resolution was estimated at 3.56×10^{-6} RIU.

B. Sensor functionalization

A synthetic 25-mer DNA 5'-thiolated sequence – Amin25-SH – (Table 1) coding for the 28S ribosomal subunit of *A. minutum* was immobilized onto a gold substrate via thiol chemisorption chemistry. After injection of 1 μ M Amin25-SH diluted in running buffer – HEPES 0.05 M NaCl 0.5 M –, a total wavelength shift $\Delta\lambda$ of 4.8 nm was observed. From equation (1), assuming that $\delta n/\delta C$ for nucleic acids (note C as concentration in pg/mm^3) is $1.9 \times 10^{-10} \text{ mm}^3/\text{pg}$ [9] and that penetration depth of the plasmon wave L_{ZC} is 110 nm for the range of wavelengths scanned, it is possible to determine a DNA probe density Γ of $4.40 \text{ pg}/\text{mm}^2$ at the sensor surface.

$$\Gamma = \frac{\Delta\lambda \cdot L_{ZC}}{\left(\frac{\delta\lambda}{\delta n}\right) \cdot \left(\frac{\delta n}{\delta C}\right)}. \quad (1)$$

That calculated surface density of $56.30 \text{ fmol}/\text{mm}^2$ (3.39×10^{10} molecules/ mm^2) is in good agreement with other published results for a 0.5 M NaCl containing running buffer [10, 11].

C. Models for ssDNA PCR hybridization

Hybridization reaction was explored with synthetic oligonucleotide models. As described in Table 1, we chose DNA complementary sequences of different lengths – 25, 50 and 100 bases – and overlapping the 3' end of the final ssDNA PCR product from *A. minutum*.

TABLE I. DNA SEQUENCES

Sequence Name	Sequence description
Amin25-SH	5'-AAGCCCTTACACATCAGTGCTGGCA-3'
Amin25c	5'- TGCCAGCACTGATGTGTAAGGGCTT -3'
Amin50c	5'-CAAGACGGGTCAAGCAGAAACATTTT GCCAGCACTGATGTGTAAGGGCTT -3'
Amin100c	5'-GTTTGCCACCCACGAACCTTGCACATCTGTTAGACTCCTTGGTCCGTGTTTCAAGACGGGTC AAGCAGAAACATTTTGCAGCACTGATGTGTAAGGGCTT -3'
Negative control	5'-CCTTGGTCCGTGTTTCAAGA-3'

a. Bolded bases correspond to the complementary sequence vs. Amin25-SH sequence

DNA hybridization kinetics were observed for those three sequences as shown in Fig. 2. Else, 1 μ M of non reactive DNA strand was also injected as negative control (Table 1). As expected, hybridization kinetics follow a first order Langmuir model. For all DNA complementary strands, no difference was observed during dissociation kinetic (k_D). Variation of kinetic behaviour was thus attributed to association phase (k_A). In fact, the longest the DNA sequence was, the lowest the k_A appears. That observation is in phase with a random approach of the target oligonucleotide to the probe; the probability of good matching decreases while DNA length increases.

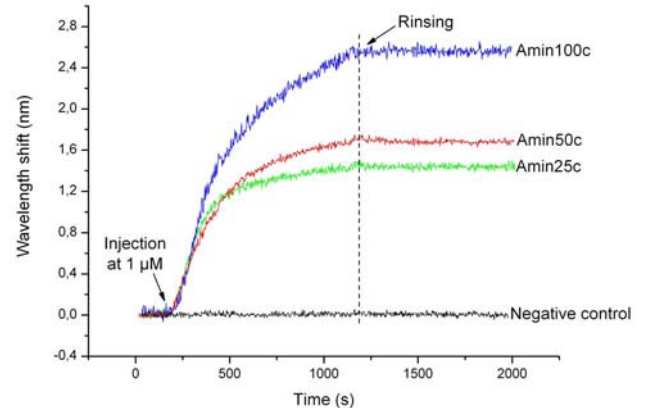


Figure 2. Hybridization kinetic signals for different lengths of DNA targets ; injections were carried at a concentration of 1 μ M diluted in running buffer.

More, considering that molecular weight stills proportional vs. surface plasmon resonance wavelength shift, steady-state hybridization signals indicated a capture efficiency of 31.2 %, 21.8 % and 11.9 % for Amin25c, Amin50c and Amin100c respectively. That observation suggests a steric hindrance of the hybridization reaction over the sensor

surface. As PCR product is a 677 bases long DNA, further optimization of immobilization chemistry will be necessary to better control probe density in order to increase target DNA capture.

Afterwards, an estimation of the biological sensor sensitivity was assayed for Amin100c. In this way, Fig. 3 represents aligned hybridization kinetic signals obtained after injection of Amin100c at different concentrations ranging from 1 μ M down to 10 nM. No dissociation was observed during the rinsing phase.

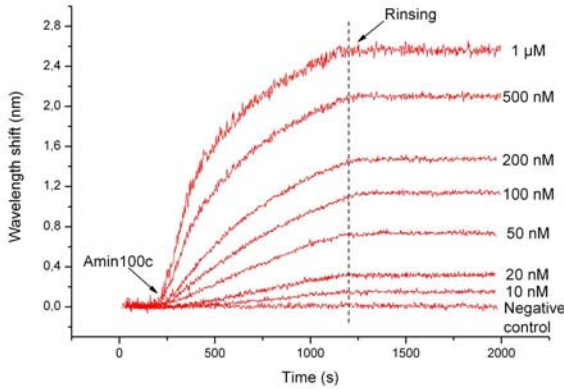


Figure 3. Hybridization kinetic signals for Amin100c at different concentrations ranging from 1 μ M to 10 nM diluted in running buffer.

From Fig. 4, signal variation adopts an exponential shape with a plateau corresponding to saturation concentrations. More, from their linear part, those results indicate an estimated limit over detection (LOD) in the range of 4.5 nM, that is in the same order of magnitude than other SPR based systems [12] and is compatible with PCR assays. Like above mentioned, a negative control assay was performed indicating no signal during the injection.

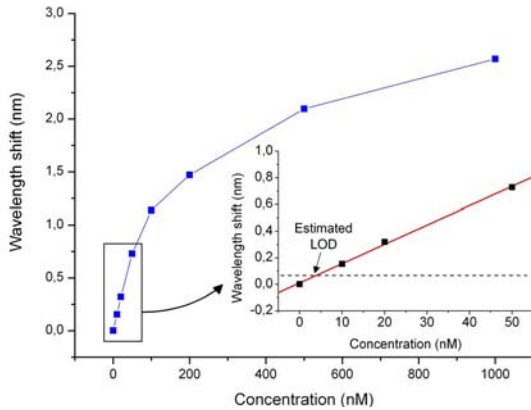


Figure 4. Sensor sensitivity for Amin100c hybridization ; insert indicates an estimated LOD based on 3σ noise signal ($2.25 \cdot 10^{-2}$ nm).

Sensor reproducibility was examined too ; biosensor showed the same signal amplitude even after more than 30 cycles (data not shown). Such a lifetime would be compatible with an autonomous *in situ* monitoring application.

D. PCR based assay for *A. minutum*

From previous results, we expected to detect *A. minutum* genome *via* a PCR based assay. For that, a culture of *A. minutum* (AM89BM) was harvested and lysed following a CTAB DNA extraction protocol. Then, a D1-D2 specific sequence coding for 28S ribosomal subunit of *A. minutum* genome was amplified by PCR. Then, dsPCR product was digested by λ -exonuclease. Finally, ssDNA was eluted in water using Qiaquick (Qiagen) and quantified spectrophotometrically. Afterwards, PCR product was diluted in running buffer just before injection.

Fig. 5 exhibits an overlay of hybridization kinetics obtained for a range from 50 nM down to 6.25 nM of PCR product injected on the DNA sensor during 10 000 s. Here, a 1.35 nm saturation signal was attained with 50 nM of PCR product ; thus, an estimation of hybridization efficiency is 1.1 % that is lower than for its 100 bases analog. Wavelength shifts behave quasi-linearly vs. time, but rather slower than as shown in Fig. 3 for Amin100c. Such a limited and slow response can be explained by steric hindrance over the biosensor surface and by the possible folding of ssDNA products. Further experiments about injection protocol will attribute the origin of those limitations.

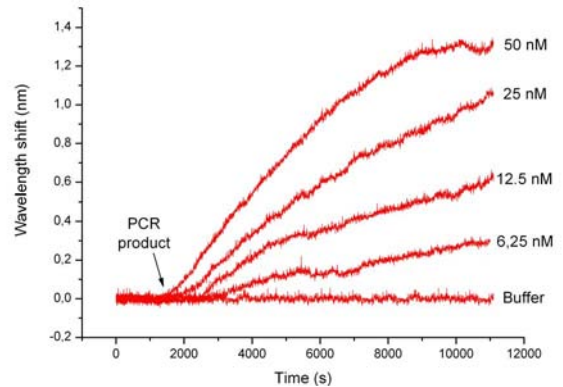


Figure 5. Hybridization signal recorded during a typical PCR assay followed by a sensor regeneration phase (0.3 M NaOH).

As indicated in Fig. 6, considering PCR product concentrations below 12.5 nM, a linearity is observed. By linear regression, an estimated LOD of 1.6 nM was determined that is consistent with Zezza *et al.* work [13].

Finally, specificity of the biosensor was assayed ; for that purpose, a sequence coding the 18S ribosomal subunit of *A. minutum* was amplified by asymmetric PCR and purified as described above. After injection, no significant signal was observed, proving thus the good specificity of the analytical technique (data not shown).

Several PCR assay were tried with the same sensor surface using NaOH 0.3 M as regeneration cocktail, proving that our system would be able to get reused for many analysis (data not shown).

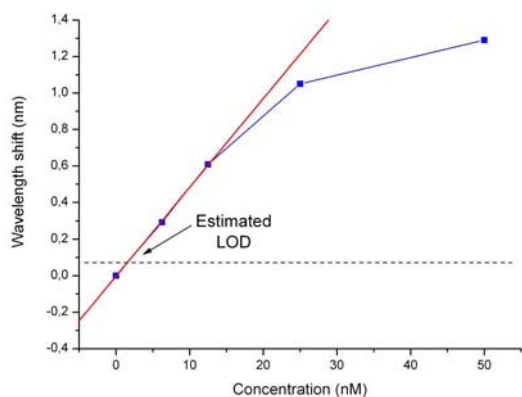


Figure 6. Sensor sensitivity for PCR product hybridization ; insert indicates an estimated LOD based on 3σ noise signal ($2.25 \cdot 10^{-2}$ nm).

V. CONCLUSIONS

This study has demonstrated that improved signal treatment for SPR sensing – i.e. upgraded centroid algorithm and ethanol spectrum normalization – , leads to a costless powerful SPR spectrometer with a resolution of 3.56×10^{-6} RIU.

In a first part, specific genome detection of *A. minutum* was assayed through targets of various lengths ; our results showed a decrease in sensor efficiency for longer synthetic oligonucleotides but stilled sensitive enough to detect less than 10 nM of 100 bases DNA. In a second part, as far as we know and for the first time, PCR detection was assayed with a SPR spectrometer. Results indicated here a possible quantification of the PCR product below 12.5 nM ; this constitutes the first step before a future quantification of *A. minutum* concentration in seawater.

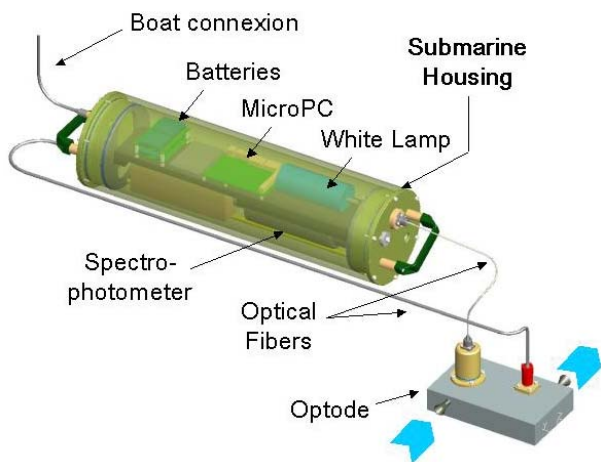


Figure 7. Schematic representation of our marine SPR spectrometer.

Finally, a marine conception of the instrument was designed for *in situ* detection of algal species. In that way, we have already built a submarine home-made SPR spectrometer (Fig. 7) for the need of future investigations.

ACKNOWLEDGMENT

We thank the region of Brittany for funding support to the PRIR-BIRMA program.

REFERENCES

- [1] M. Campas, B. Prieto-Simon, and J. L. Marty, "Biosensors to detect marine toxins: Assessing seafood safety," *Talanta*, vol. 72, no. 3, pp. 884-895, May, 2007.
- [2] S. Diercks, K. Metfies, and L. K. Medlin, "Development and adaptation of a multiprobe biosensor for the use in a semi-automated device for the detection of toxic algae," *Biosensors & Bioelectronics*, vol. 23, no. 10, pp. 1527-1533, 2008.
- [3] E. Turrell, E. Bresnan, C. Collins *et al.*, "Detection of *Pseudo-nitzschia* (Bacillariophyceae) species and amnesic shellfish toxins in Scottish coastal waters using oligonucleotide probes and the Jellet Rapid Test (TM)," *Harmful Algae*, vol. 7, no. 4, pp. 443-458, 2008.
- [4] M. Piliarik, and J. Homola, "SPR Sensor Instrumentation," in *Surface Plasmon Resonance Based Sensors*, vol. 4, O.S. Wolfbeis, Eds. Springer Ser Chem Sens Biosens, 2006, pp. 95–116.
- [5] R. R. L. Guillard, "Culture of phytoplankton for feeding marine invertebrates," *Culture of Marine Invertebrate Animals*, W. L. Smith, Chanley, M.H., ed., pp. 26–60, New York, USA: Plenum Press, 1975.
- [6] D.R. Lide, *Handbook of Chemistry and Physics 88th* CRC Press. Boca Raton, FL, 2007-2008.
- [7] K. Johansen, H. Arwin, I. Lundstrom *et al.*, "Imaging surface plasmon resonance sensor based on multiple wavelengths: Sensitivity considerations," *Review of Scientific Instruments*, vol. 71, no. 9, pp. 3530-3538, Sep, 2000.
- [8] T. M. Chinowsky, L. S. Jung, and S. S. Yee, "Optimal linear data analysis for surface plasmon resonance biosensors," *Sensors and Actuators B-Chemical*, vol. 54, no. 1-2, pp. 89-97, Jan, 1999.
- [9] E. Bouffartigues, H. Leh, M. Anger-Leroy *et al.*, "Rapid coupling of Surface Plasmon Resonance (SPR and SPRi) and ProteinChip based mass spectrometry for the identification of proteins in nucleoprotein interactions," *Nucleic Acids Res*, vol. 35, no. 6, pp. e39, 2007.
- [10] S. Peeters, T. Stakenborg, G. Reekmans *et al.*, "Impact of spacers on the hybridization efficiency of mixed self-assembled DNA/alkanethiol films," *Biosens Bioelectron*, vol. 24, no. 1, pp. 72-7, 2008.
- [11] A. W. Peterson, R. J. Heaton, and R. M. Georgiadis, "The effect of surface probe density on DNA hybridization," *Nucleic Acids Research*, vol. 29, no. 24, pp. 5163-5168, 2001.
- [12] X. D. Su, Y. J. Wu, and W. Knoll, "Comparison of surface plasmon resonance spectroscopy and quartz crystal microbalance techniques for studying DNA assembly and hybridization," *Biosensors & Bioelectronics*, vol. 21, no. 5, pp. 719-726, Nov, 2005.
- [13] F. Zezza, M. Pascale, G. Mule *et al.*, "Detection of *Fusarium culmorum* in wheat by a surface plasmon resonance-based DNA sensor," *Journal of Microbiological Methods*, vol. 66, no. 3, pp. 529-537, 2006.