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Polypyrrole based DNA hybridization assays: study of label free detection processes versus fluorescence on microchips

Thierry Livache [a,*], Emmanuel Maillart [b], Nathalie Lassalle [c], Pascal Mailley [a], Bruno Corso [a], Philippe Guedon [c], André Roget [a], Yves Levy [b]

[a] *Département de Recherche sur la Matière Condensée, UMR 5819 (CEA, CNRS, Université J. Fourier), CEA-Grenoble, 17 Rue des Martyrs, 38054 Grenoble Cedex 09, France*
[b] *Laboratoire Charles Fabry de l'Institut d'Optique Théorique et Appliquée, UMR8501, Bat.503, BP147, 91403 Orsay Cedex, France*
[c] *Genoptics SA, Institut d'Optique Théorique et Appliquée, Bat.503, BP147, 91403 Orsay Cedex, France*

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Abstract

In this paper, we present different ways to detect DNA hybridization on a solid support. The grafting chemistry is based on the electro-controled copolymerization of a pyrrole-modified oligonucleotide and pyrrole. This process allows an easy functionalization of conducting materials. Three kind of devices were studied: silicon chips bearing an array of addressable 50 or 4 μm microelectrodes, quartz crystal microbalance (QCM) and a non patterned gold/glass slide bearing 500 μm spots. Each device is compatible with a specific detection process: a classical indirect fluorescence detection for the microchips, a microgravimetric measurement for the QCM and a surface plasmon resonance imaging process (SPRi) for the gold slides. Both QCM and SPRi are a label-free real time detection process whereas the fluorescence methodology gives end-point data but only the fluorescence and the SPRi give multiparametric results. Although the hybridization experiments show that the detection limit for an oligonucleotide is better for the fluorescence (1–10 pM) than that found for SPRi (10 nM) and QCM (250 nM), the information content of real time measurement techniques such as SPRi is of interest for many biological studies.

Keywords: DNA array; Polypyrrole; Silicon chip; Microelectrodes; Surface plasmon resonance imaging; Quartz crystal microbalance

1. Introduction

The identification of analytes in biological samples though their nucleic acid sequences has been receiving increasing attention in recent years [1]. Basically, most of the systems rely on hybridization between immobilized single-stranded DNA probes and the nucleic acid target. Major applications deals with diagnostic of infectious or genetic diseases, point mutations screening (cancer follow up) and forensic investigation or, in an other field, it can be applied to the public or private basic researches. In this way, two kinds of DNA assays can be designed; the first one is a

* Corresponding author. Tel.: +33-4-38-785229; fax: +33-4-38-785145.
E-mail address: tlivache@cea.fr (T. Livache).

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highly parallel miniaturized DNA sensor (DNA chip) allowing a high number of analytes (DNA sequences) to be tested in one experiment [2,3]. The second kind of assays is a low cost, easy to use in clinical chemistry laboratories (ELISA-like DNA assays) or, especially for the environment field, the devices should be compact and portable (DNA sensors). For all these purposes, the first point is to immobilize the ssDNA or the oligonucleotide (ODN) probes on the solid support and many procedures have been described [4] (review). Among them, the fixation of DNA on electrodes is of special interest because it allows to cross the fields of DNA assays and electrochemistry and then to create a link between the electronic microtechnologies and the biology. For this task, it is indeed necessary to construct the DNA active layer over a conducting materials (electrode). The electrochemical step can then be helpful for the construction of the sensor (electrochemical synthesis) or/and can be involved in the detection process (biosensing) [5]. For this task, the use of conducting polymers, especially the polypyrrole, is an attractive way to link the biomolecules and the electrode [6]. These polymers have been widely used in the field of biosensors [7] or DNA assays [8,9]. Such an interest ensues from two intrinsic properties of these materials (i) their ability to be a molecular transducer between the biological event and the electrode and (ii) especially for the polypyrrole (ppy), its ability to be electrogenerated at low potential in water at neutral pH in biocompatible solution on a conducting layer (gold for microelectrode or surface plasmon resonance substate, platinum for quartz microbalance...). For DNA testing, the former property is involved in all the electrochemical detection of DNA hybridizations [10] and the latter is needed when the DNA grafting occurs during the polymer synthesis; this can lead to the electrochemical directed construction of DNA arrays which implies a real miniaturization. In order to reach this goal, different polypyrrole DNA functionalization schemes have been investigated. These polymers can be functionalized with ODN after [11] or during their polymerization. Concerning this latter methodology, Wang [9] described a straightforward electrostatic entrapment of ODN into the polypyrrole matrix. In order to obtain a regioselective and covalent grafting of the ODN, we have developed a process based on an electro-directed addressing of the synthesis of a copolymer ODN-pyrrole/pyrrole allowing the construction of ODN arrays on microelectrode arrays [8]. Briefly, the electrochemical oxidization of pyrrole gives, in one step, a solid polypyrrole film laid on the surface of the electrode. In a same way, the copolymerization of pyrrole and ODN tethered to a pyrrole group leads to a film bearing covalently linked ODN. The synthesis of polypyrrole is limited to the surface of the electrode, so that, the size of the organic support is the same as that of the electrode. In a first approach, this copolymerization process has been used to address ODN on an array of microelectrodes layed on a silicon chip [12,13]. Then, active microelectronic chips bearing 128 multiplexed microelectrodes (Micam DNA chips) coupled with a fluorescent detection process have been recently used for clinical studies [14] (K-ras gene point mutation detection). In order to show the spatial resolution of this technology, the copolymerization was also performed in this work, on an array of 4 μm ultramicroelectrodes. For both microdevices, the DNA hybridization detection is carried out by an end-point fluorescence detection. Although this method is quite sensitive, it needs a two step protocol and quantification is not easy. Thus, we have tried to construct real time label free DNA sensors; in this way we have designed two kinds of assays based on the same polypyrrole chemistry. The first one is based on a Quartz Crystal Microbalance frequency measurement allowing a simple and convenient way to detect DNA hybridization [15]. The second detection way deals with a direct Surface Plasmon Resonance Imaging (SPRi) detection that allows the simultaneous monitoring of many hybridizations occurring on an array of DNA probes [16,17].

In this paper, we will compare different detection approaches i.e. direct or undirect, end point or real time, monoparametric or multiparametric in terms of sensitivity, quality of the data obtained using for the first time the same grafting chemistry for all the dedicated substrates.

2. Materials and methods

2.1. Preparation of pyrrolylated oligonucleotides

Oligonucleotide probes bearing a pyrrole group (ODNpyr) were synthesized according to a previously reported procedure by direct oligonucleotide synthesis using pyrrole-phosphoramidite building blocks in the course of the ODNs synthesis [8]. Purification of these products was performed by HPLC on RP18E with a gradient of acetonitrile (10–20%) in 25 mM triethylammonium acetate pH 7.0.

The sequence and the name of the ODN used came from the K-ras gene study [14]: *CPpyr* $^{5'}$pyrrole-T_{10}GCC-TTG-ACG-ATA-CAGC; *M5pyr* $^{5'}$pyrrole-T_{10}TGG-AGC-TGC-TGG-CGT and their biotinylated complementary strands: *CPc* $^{5'}$biotin-GCT-GTA-TCG-TCA-AGG-C and *M5c* $^{5'}$biotin-ACG-CCA-GCA-GCT-CCA.

2.2. ODN-polypyrrole electrosyntheses on the different substrates

2.2.1. Copolymerizations on silicon chips

All the chips used were made by microelectronic technologies on a silicon support (CEA/Leti, France). The active chips contained 128 (50×50 μm^2) gold microelectrodes and nine outlet pad connections [13]. Each electrode can be individually addressed. The packaging included a reaction chamber and the external of the inlets/outlets to a PC computer. The chip was interfaced to a computer monitoring the multiplexing process using a dedicated software developed from LabWindows (National Instruments).

The ultramicroelectrode array was constructed on a passive silicon chip containing 25×25 μm^2 gold electrodes arranged in a 4×12 matrix. The surface of the chip was overlaid with a 1 μm thick layer of silicon dioxide, which was opened over the electrodes. Four 4 μm windows were then opened in the silicon oxide layer over each gold electrode.

For both chips, electrochemical reactions were carried out in a 0.5 ml Teflon cell including a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference (Tacussel). Working electrodes located on the chip were connected to an external potentiostat. The electrochemical system was connected to an EG&G Princeton Applied Research model 283 potentiostat and to a Probe pipetor (Packard Instrument). The copolymerization was carried out in 0.5 ml of a polymerization solution containing 20 mM pyrrole (Tokyo Kasei), 0.1 M $LiClO_4$ (Fluka) and 1 μM of the ODN bearing a pyrrole group. The films were synthesized on the micro-working electrode by a 1V/ECS electrochemical pulse for 0.5 s. Following syntheses, the cell was rinsed with water by the automate, another electrode was then selected and switched on and the following copolymerization was carried out. After synthesis, the chip was rinsed with water and stored at 4 °C.

2.2.2. Polymerization on quartz crystal

The electrochemical syntheses and characterizations were realized with a computer controlled PAR 273 potentiostat (EG&G Princeton Applied Research). All the electrochemical measurements were performed in a 300 μl Teflon three electrode cell including a 4 mm diameter platinum disk as a counter electrode and a SCE as reference (Tacussel). Working electrode is a platinum film coated on an AT cut quartz crystal ($F_0 = 8.8$ MHz, 8 mm wide square shape, thickness ≈ 0.1 mm) obtained from EG&G Princeton Applied Research [15].

Electrochemical synthesis of pyrrole/pyrrole-ODN copolymer films was performed on one side of the quartz electrode by cycling the potential (three cycles) between −0.3 and 0.85 V versus SCE at 20 mV s^{-1}. The biolayer synthesis was carried out in 300 μl of polymerization solution containing 2 μM of pyrrole-ODN. Following electrosynthesis the film was stabilized in PBS (Sigma) for 10 min.

2.2.3. Copolymerizations on SPRi slides

The glass slides were covered by a 50 nm gold thin films by vacuum-evaporation onto 3×3 cm^2 glass substrates ($n = 1.717$ at $\lambda = 633$ nm).

The electrochemical copolymerization was carried out by the "electrospot" method [16] on the gold layer through the use of a 200 μl pipette tip as the electrochemical cell. Electrical contact was established inside the tip by inserting a platinum wire used as a counter electrode. The tip was filled with 10 μl of polymerization solution containing

40 μM of pyrrole-ODN and was then applied to a precise location on the gold layer used as the working electrode. This electrochemical system was connected to an EG&G Princeton Applied Research model 283 potentiostat and to an 8300 Schlumberger X/Y recorder. The polypyrrole film was synthesized by electrocopolymerization on the gold layer (working electrode) by a 2 V electrochemical pulse for 0.25 s (the voltage is defined with respect to the platinum counter electrode). Following the electrosynthesis, the tip was emptied and rinsed with water. The successive polymerizations were carried out by the same process with the different ODNs to be copolymerized on spatially defined areas of the gold slide. When all the ODN spots were synthesized, the slide was disconnected, rinsed with water and stored at 4 °C.

2.3. Hybridization and detection of nucleic acids on the different substrates

2.3.1. Fluorescence detection

The hybridization of the ODN grafted on the different substrates was carried out with biotinylated complementary (or non complementary) probes according to the procedure described for the DNA chips [12]. Briefly, the hybridization was carried out at 45 °C for 15 min in a 20 μl volume (or 200 μl for the Quartz) containing 50 nM (unless specified) of biotinylated oligonucleotide diluted in a hybridization buffer: PBS containing 0.5 M NaCl, 100 μg ml^{-1} of herring sperm DNA (Sigma) and 10 mM EDTA. Following a washing in PBS/0.5 M NaCl/0.5% tween (washing buffer) at room temperature, the substrate (silicon chips, SPR slides or quatrz crystal) was incubated for 10 min in a solution of 5% streptavidin-R-phycoerythrin (Molecular Probes) diluted in the washing buffer. The fluorescence was recorded for 0.5 or 1 s with an epifluorescence microscope (BX 60, Olympus) equipped with a Peltier cooled CCD camera (Hamamatsu) and image analysis software (IMAGEPRO PLUS, Media Cybernetics). Regeneration of the substrate was carried out by a 10 s denaturation step in 0.1 M NaOH followed by a water rinsing.

2.3.2. Quartz crystal microbalance hybridization monitoring

ODN probe hybridization was carried out at 25 °C in the same cell than that used for the polymer synthesis in 200 μl of PBS containing 500 nM of the ODN target (complementary or non complementary strands). The quartz crystal frequency variation was continuously recorded as a function of time during the overall experiment [15]. Following each experiment, the pyrrole/pyrrole-ODN copolymer films were washed with distilled water, dipped for 10 s in 0.1 M NaOH to regenerate the support and washed again with water, more than ten hybridization/regeneration cycles can be carried out.

2.3.3. SPRi hybridizations monitoring

The optical setup was described elsewhere [16]. Briefly, the hybridizations produce changes in the refractive indexes near the gold surface which results in the changes of the reflectivity which was recorded by a 8-bit CCD camera as gray-level contrasts. During hybridization experiments, images were recorded at fixed intervals of time (4 s). All the images captured were analyzed on a computer with IGOR PRO software (Wavemetrics). The hybridizations were carried out in a 10 μl Teflon hybridization cell connected to a peristaltic pump (Gilson) in PBS at room temperature. The flow rate of running solutions within the cell was 10 μl min^{-1}. Following the oligonucleotide injection, the sensor surface was rinsed with PBS to remove unbound molecules and then regenerated with 20 mM NaOH for 1 min and washed again with water.

3. Results and discussion

In order to compare both the sensitivity and the information content of the different detection approaches, we have chosen herein a simple oligonucleotide hybridization model. The specificity of DNA hybridization on polypyrrole microchip supports and their biological applications on clinical samples have been presented elsewhere [12,14].

3.1. ODN polypyrrole synthesis

The principle of our electrosynthesis process is summarized on the Fig. 1. The pyrrole copolymerization reaction allows a one-step preparation of polypyrrole-ODN films on the surface of an electrode. The addressing of the pyrrolylated-ODN is achieved by successive copolymerizations on the selected microelectrodes belonging to a microdevice bearing an array of individually addressable microelectrodes or by the displacement of a miniaturized electrochemical cell over the substrate. The electrochemical polypyrrole synthesis processes have been optimized for each support: the SPR based approach must be carried out on a thin layer of polymer (0.25 s electrochemical pulse $\sim$10 nm thick film) whereas the fluorescence detection can be carried out on thicker films (0.5 s electrochemical pulse $\sim$25nm thick film); concerning the QCM detection process, in order to obtain an homogeneous biological layer, it is necessary to cover the electrode with a thick polypyrrole film (cyclic electrochemical process, $\sim$200 nm thick film).

The detection of the hybridizations occurring on the substrates can be carried out either by fluorescence, microgravimetry or SPRi.

3.2. Electrochemical setup

The substrates are chosen to be compatible with the detection process used; i.e. addressed microelectrodes with fluorescence, quartz crystal with microgravimetry and homogeneous gold layer with SPRi. Then, the electrochemical setup must fit to these different substrates. The quartz and the silicon chip can be dipped in a classical three electrode electrochemical cell whereas a mobile

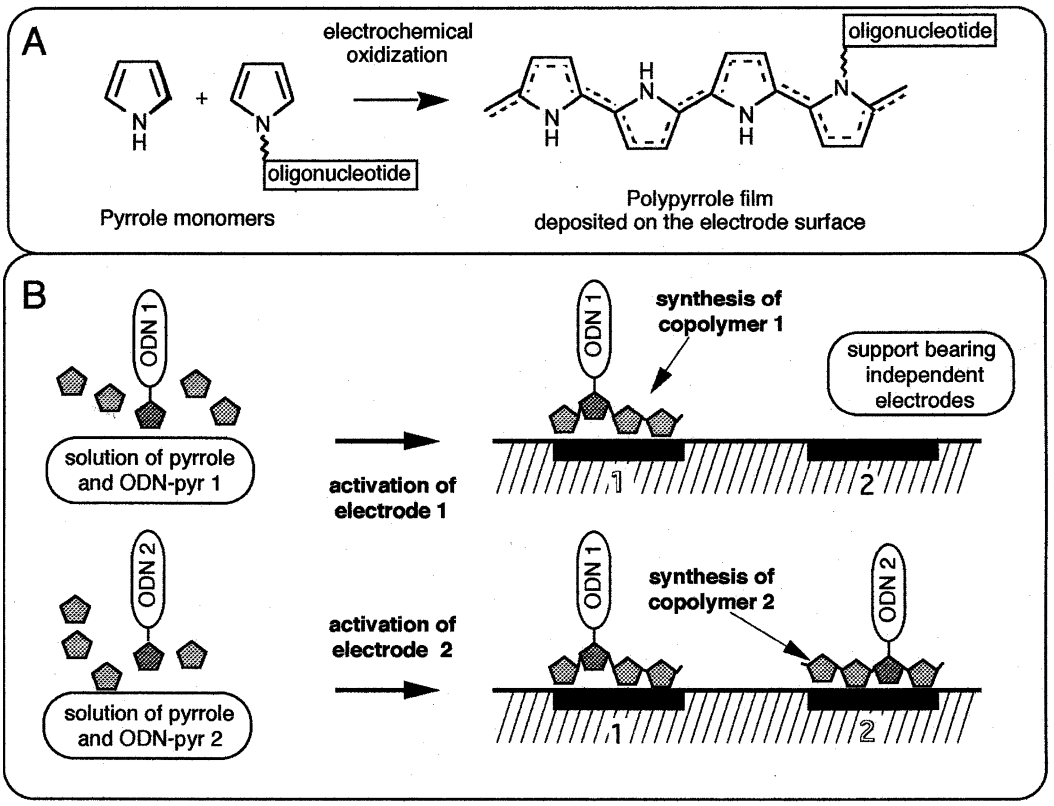

Fig. 1. (A) principle of pyrrole and pyrrole-ODN electrocopolymerization. (B) Principle of the electrochemical addressing of the ODN on addressable microelectrode array.

electrochemical cell must be used to array the polymers on the SPRi substrate (homogeneous gold layer). The basic setup of this 'electrospot'' method is outlined in Fig. 2: a pipette tip including a Platinum wire used as the counter electrode is filled up with the polymerizable solution containing the pyrrole-ODN and moved to a precise location on the gold layer used as the working electrode. Then, during the deposition of a droplet of solution, a 0.5 s electrochemical pulse allows the synthesis of the polypyrrole film. Successive copolymerizations with the different ODNs lead to a straightforward construction of a submillimetric (diameter is close to 500 μm) spot ODN array. The main differences between these different setups concern the sample volume needed for the copolymerizations; it is in the range of 500 μl for silicon chip and quartz crystal (three electrode cells) and only 10 μl for the electrospot process (two electrode cell).

3.3. Design of the biological assays

The design of the assays is illustrated on the Fig. 3. The first point deals with the number of parameters that can be analyzed in one sample. The classical Micam DNA chip includes 128 individually addressable microelectrodes that is to say 128 different ODN sequences can be theoretically grafted on the chip (Fig. 3a). The SPRi substrate (Fig. 3c) is also a multiparametric sensor (currently 25 parameters can be monitored simultaneously). Both of these two chips can include all the necessary controls i.e. positive and negative controls etc. The analysis results are given in a gray scale level; it is interesting to note that for the SPRi detection, this grey level scale is directly related to the mass of the analyte recognized/adsorbed on the surface. The quartz crystal (Fig. 3b) is a mono-sensor giving quantitative results [15]. However, for a quality control procedure, an hybridization followed by a fluorescence detection step can be carried out on all the substrates (QCM, silicon chips or SPRi slides). Among the different detection processes studied herein, two of them give real time biological interaction data without the need of a tracer (QCM and SPRi) but only the SPRi is compatible with a multiparametric detection; that is to say comparative kinetic data dealing with adsorption (or recognition) and desorption are obtained with SPRi. On the other hand, the fluorescence process used with the Micam chips is an undirect end point measurement.

3.4. Evaluation of the sensitivity of the hybridization detection

The detection limit of each process was evaluated by hybridization with various concentrations of biotinylated targets (CPc-bio) assuming that the detection limit is reached when the signal/back-

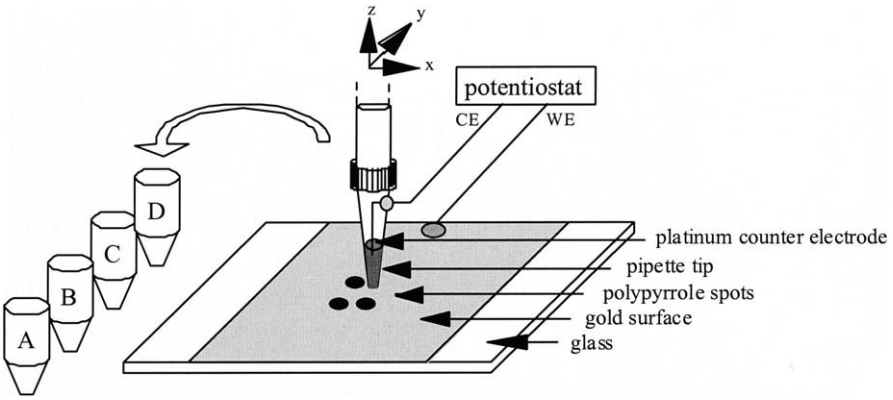

Fig. 2. General scheme of the ODN addressing on an homogeneous gold layer (electrospotting methodology): The different tubes A, B, C, D containing different pyrrole-ODN and pyrrole monomer solutions are on the left. The "electrospotting" is carried out on the gold surface via the plastic tip containing the solution to be copolymerized. The reaction occurs during a 0.5 s electrochemical pulse.

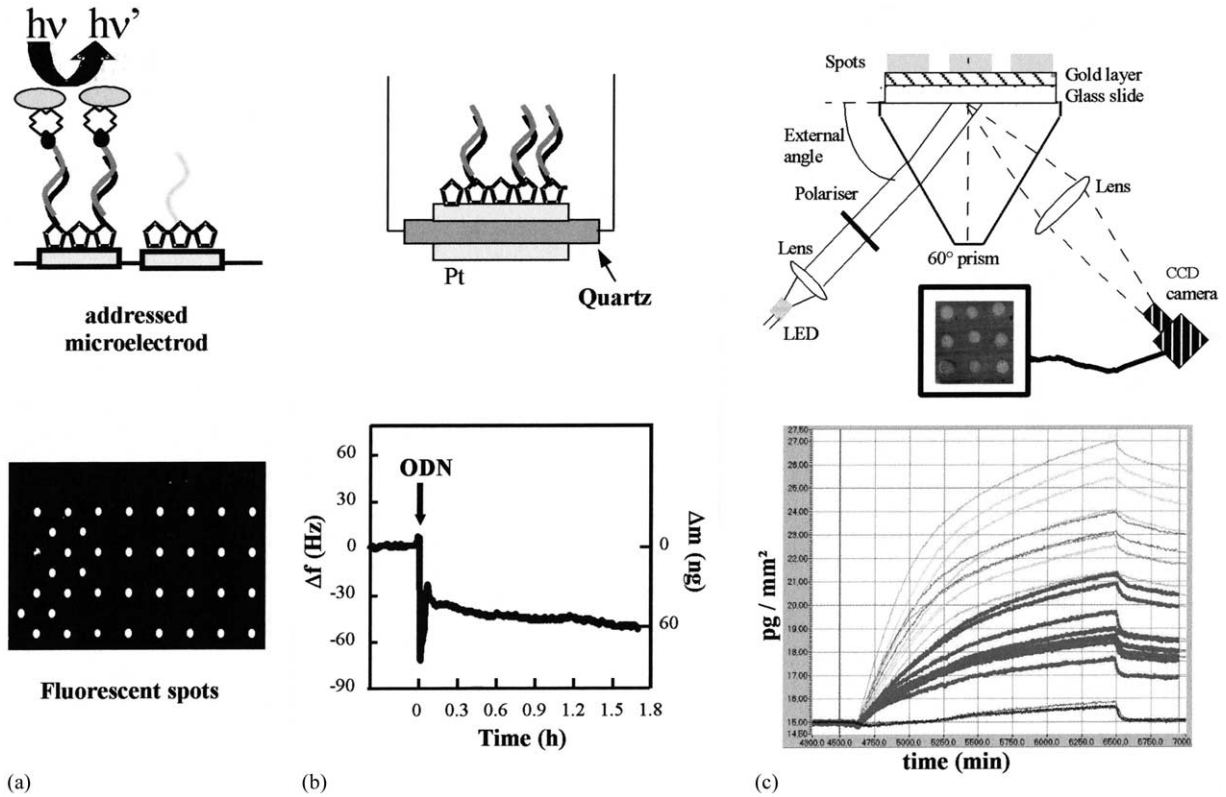

Fig. 3. Principle and example of the three detection processes used. (A) Fluorescence detection of hybridized DNA on Micam silicon chip; (B) real time QCM microgravimetric measurement of an ODN hybridization; (C) SPR imaging of a DNA hybridization on an ODN array.

ground ratio is higher than 2 (the background is the signal from a spot bearing the M5 probe). It is important to underline that the basic chemistry (polypyrrole grafting) is the same for all the substrates and that the ODN grafting density is roughly 10 pmol cm^{-2} for the quartz crystal and the SPRi substrate [16] and 1 pmol cm^{-2} for the microchip. However, the grafting density was optimized for each detection process then different ODN-pyrrole/pyrrole ratios were used during the copolymerization process; ratios of 1/20 000, 1/10 000 and 1/500 were used for the construction of the microchip array, quartz crystal and SPRi substrate, respectively. The results are summarized on the Table 1. An example of fluorescence detection is shown on the Fig. 4: the detection limit with classical Micam chip is in the range of 10 pM (Fig. 4a) of complementary ODN whereas it reaches the pM range on ultramicroelectrode (Fig. 4b). Assuming a saturating monolayer of streptavidin-R-phycoerythrin (roughly 20 nm in diameter), we can estimate that less than 20 000 molecules are visible on this 8 μm^2 electrode that is to say less than 2500 molecules μm^{-2}. According to Ekins [18], this sensitivity shift between 50 and 4 μm spots can be explained by a concentration effect on such microelectrodes, it is important to note that these experiments were carried out in static conditions because the use of flow conditions can minimize these effects [19]. In the same way, if the fluorescence detection process is carried out on bigger spots (SPRi substrates, Fig. 5a), the result shows a lower detection limit (1 nM) which falls within the same range as found elsewhere [20]. The detection limit of the QCM for the complementary ODN is 250 nM which is in the same

Table 1
Summary of the different substrates used and of the main characteristics of their associated detection processes

Substrate	Feature size (diameter in μm)	Detection process	Information contents	Analysis duration (min)	Detection limit (ODN hybridization)
Ultra microelectrode array	3	Fluorescence	End point	40	1 pM
Microelectrode array	50	Fluorescence	End point	40	10 pM
Homogeneous gold layer	500	Fluorescence	End point	40	1 nM
Homogeneous gold layer	500	SPRi	Kinetic	5–20	10 nM
Quartz crystal	5000	Mass	Kinetic	5–20	250 nM

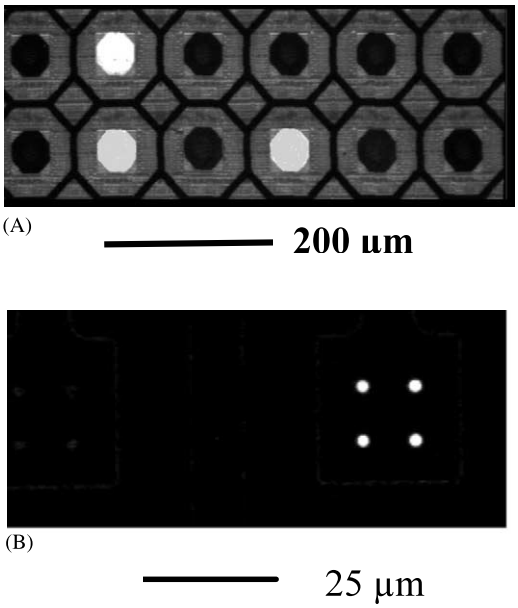

Fig. 4. DNA hybridization detection by fluorescence on a Micam silicon chip bearing (A) 128.50 μm electrodes in presence of 100 pM of CPcbio and on a silicon chip and (B) bearing an array of 4 μm ultramicroelectrodes in presence of 10 pM of Cpcbio (on the left, four M5 spots, on the right four CP spots).

order of magnitude as that found by Wang et al. [21] (220 nM for a short 15mer ODN). The quantitative QCM result can be given in nanogram. However, QCM the signal can be amplified through the addition of a high molecular weight protein such as avidin [22] or through an enzymatic process [23]. The Fig. 5 shows a comparison of the hybridization results obtained by undirect fluorescence (Fig. 5a) or SPRi (Fig. 5b) carried out on the same gold slide with various concentrations of complementary ODN. Concerning the SPRi kinetics plots (Fig. 5b) show the hybridization and the adsorption of a 17mer ODN (Cpcbio, MW 5100) on the glass/gold chip at various concentrations. The detection limit is in the range of 10 nM when long hybridization time are used; at higher concentration, the kinetics are faster. It should be noted that the SPRi detection is carried out without any labeling of the DNA target; the sensitivity of this detection process is then directly related to the molecular weight of the analyte. Using a same SPRi process based on an other grafting chemistry, Nelson et al. [24] have recently reported a detection limit of 25 and 2 nM for short RNA sequences (18mer) and long 16S RNA, respectively.

4. Conclusion

This study deals with the comparison of the information contents and the sensitivity of different DNA hybridization detection approaches on different substrates bearing micro or macro DNA spots synthetized by basically the same grafting chemistry. For this study, the model chosen is a simple oligonucleotide hybridization. The results show that the fluorescence detection is intrinsically the more sensitive although the signal was obtained without optimization (indirect and single R-phycoerythrin labeling, conventional fluorescence microscope...). It probably remains the best methodology when kinetic data are not necessary and is widely used with DNA-chip technologies. The DNA hybridization quartz crystal balance monitoring is easy to carried out and can be done without any labeling but the sensitivity remains lower than that found for SPRi and remains

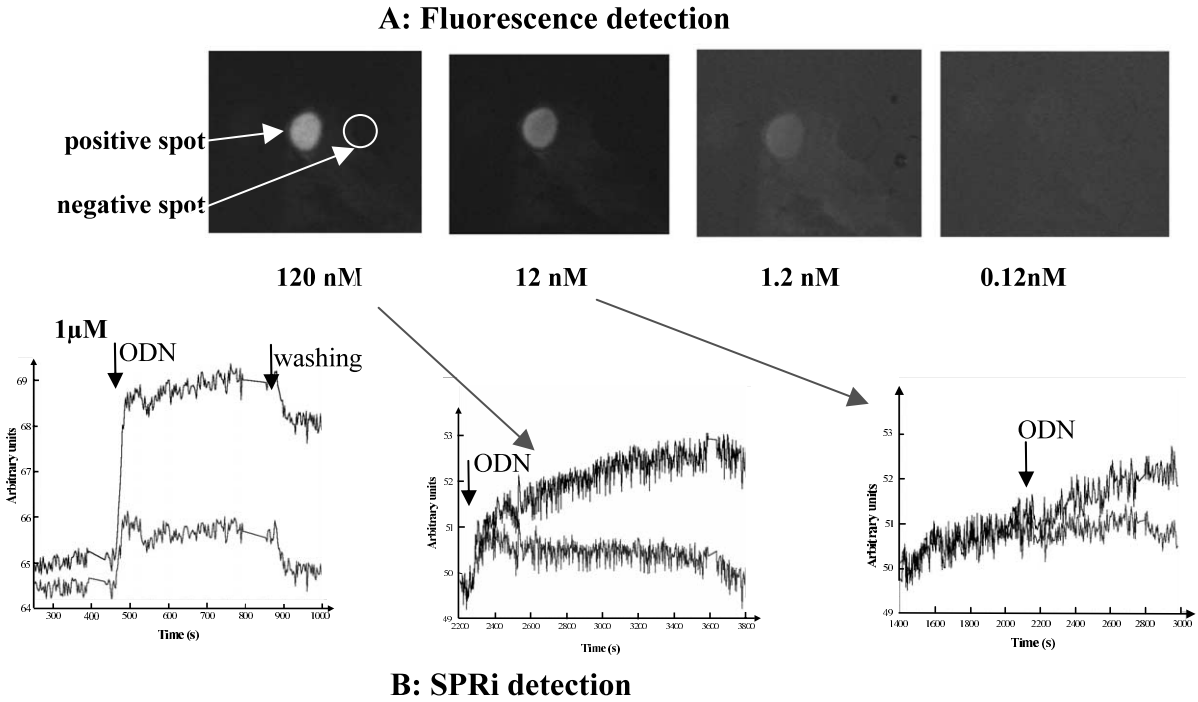

Fig. 5. Comparison of fluorescence and SPRi detections of DNA hybridization on a same substrate bearing CP (left, positive spot) and M5 (right, negative spot) probes. (A) Fluorescence images following an hybridization with 120, 2, 2 nM and 120 pM of CPcbio. (B) SPRi real time detection on the same chip during hybridization with 1 µM, 100 and 10 nM of CPcbio. The plots show the hybridizations/adsorption of CPc on the Cppyr spot (up) and CPc on the M5pyr spot (down, background).

difficult to miniaturize. Moreover, the SPRi allows a real time multiparametric detection and more than 25 parameters can be monitored in one experiment. It should be noted that a direct label free electrical detection process on conducting polymers is not easy to carried out not so sensitive; recent approaches based on the use of redox intercalating agents as tracers [25,26] are currently studied in our laboratory. However, end point fluorescence measurement and SPRi technologies are real complementary; the former is useful when a high sensitivity is needed (clinical or complex samples) but need a labeled sample or a multistep procedure (sandwich assays) whereas the latter approach (SPRi), gives very interesting comparative kinetic data if we assume a sacrifice concerning the analytical sensitivity intrinsically due to the label free approach. Thus, this methodology is probably more dedicated to biochemical studies. However, further works involving the direct SPRi detection of point mutations in clinical samples and dealing with the miniaturization of the spot size are underway. This approach is also currently extended to the multiparametric studies of protein/DNA (example of P53) or protein/protein interactions that could open the way of the development of new tools useful for the target validation and the drug discovery.

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