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# BIO-ASSEMBLIES ONTO CONDUCTING POLYMER SUPPORT: IMPLEMENTATION OF DNA-CHIPS

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*The electrical deposition (electropolymerization) of polypyrrole, PPy, grafted by different ODN probes is the key to construct oligonucleotides, ODN, arrays on silicon devices bearing a matrix of gold microelectrodes. The straightforward functionalization of PPy allows finalizing a more general grafting based on affinity interactions between a biotinylated polypyrrole support and the biological probe and results in a surface renewable DNA sensor. The electrical monitoring of the thickness of the PPy deposit at the nanometer range allows the real time monitoring of the biological interactions using Surface Plasmon Resonance imaging. Recently, the electroconductivity of the PPy was exploited to trigger the electrochemiluminescence of a DNA sequence labelled with a luminol derivative, ABEI.*

*Keywords*: affinity grafting; biosensors; biotin; DNA chips; electrochemical polymerisation; functionalized polypyrrole



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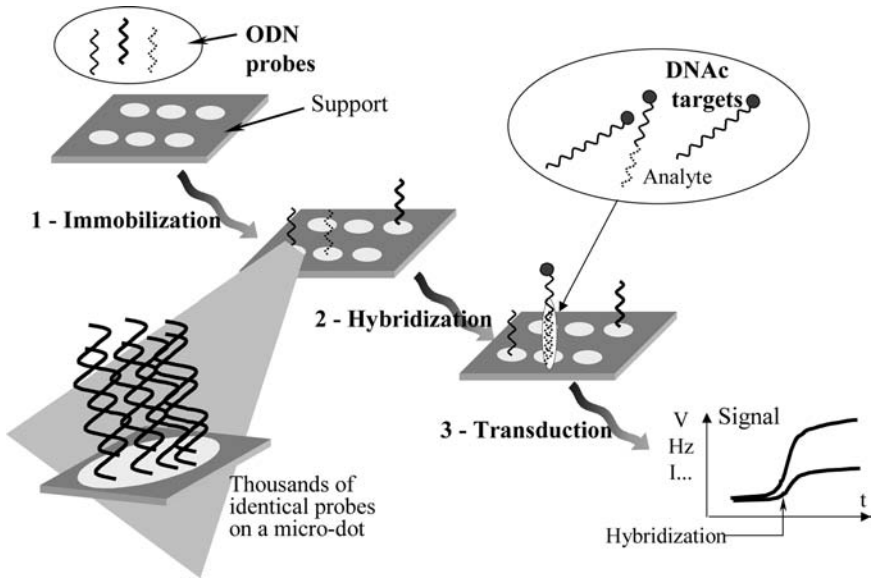
## 1. INTRODUCTION

In recent years, there has been considerable interest in the development of DNA sensor owing to their great application in the areas of medical diagnostics, food manufacturing and environmental monitoring. The basis of operation for a DNA sensor is the complementary coupling between the specific DNA sequences within a target and the specific DNA sequences (ODN probes) immobilized on a solid support. The combinatorial chemistry involved in the recognition of an unknown sequences among other [1] needs thousand of reactions carried out in parallel. The requirement of fast analysis gives rise to the concept of miniaturized and multiparametric sensor or biochips [2]. Three main steps (Sch. 1) may be considered for a biochips analysis:




**SCHEME 1** Representation of the main steps for the implementation of a DNA chips. First step is the specific immobilization of various single stranded DNA sequences, ODN probes, on a structured surface (array of gold microelectrodes, 50 μm × 50 μm each). Second step is the recognition (hybridization) between complementary sequences, (dotted lines). The chips is dipped in a solution containing various DNA targets, DNAc. The third step is the transduction of the hybridization even in a tractable signal. Triggering the label (redox, fluorescent, . . .) brings by the DNAc and usually introduced during the PCR, allows to record an electrical or fluorescent signal.

1. The immobilization of the ODN probes onto specific areas of a structured support The anchoring of DNA probe to the support could be achieved by adsorption, direct covalent attachment on gold or glass slide, or onto a polymer film. The delivery of pico- to nano-litters at specific location of the presynthesized ODN probe may be achieved by spotting or by means of jets.
2. The hybridization between the immobilized ODN probes and the corresponding complementary sequence along a single stranded DNA target extracted from living cells, virus,...
3. The transduction of the biological recognition event in a tractable signal. A label (or its precursor) is introduced during the PCR experiment. The spots were the hybridization takes place exhibit the properties of the label. In the case of a fluorescent label, the location and the intensity of fluorescence emitted after selective excitation by a laser allows characterizing the recognition.



Biosensors based on electronic conducting polymers [3] (ECPs) appear particularly well suited for these requirements of modern biological analysis. Indeed the ECPs were attractive materials in order to elaborate a sensitive layer at the surface of an electrode. This is due to their key properties, i.e. (i) The synthesis of films onto electrode surfaces by electropolymerization of monomers in solution. The thickness of the deposit is directly related to the amount of electricity passed during the electropolymerization reaction, with a stoechiometry of about 2.3 electrons by polymerized monomer unit. This easy electrodeposition on an electrode surface opens the route to miniaturization since the deposit cover exactly the surface of the metallic dot; (ii) a versatile functionalization by biological moieties either by grafting or doping, enabling one to perform selective recognition.

Many biosubstances such as enzymes [4] or DNA sequences [5] or peptides [6] have been immobilized on a ECPs matrix, which is mainly a polypyrrole, PPy, film. Indeed the conditions of the electrodeposition of pyrrole monomers (low potential, buffered aqueous solution) were compatible with the stability of the biological entities.

Thanks to the biocompatibility of the polypyrrole, we develop a one-step electro-addressing and immobilization of ODN onto gold micro-electrodes. The grafting of ODN probes is achieved during the electrosynthesis of a copolymer obtained by the electropolymerization of pyrrole units (Pyr) and pyrrole monomer bearing ODN (Pyr-ODN) [7] (Fig. 1). This process gives in a single step a copolymer deposited at the electrode constituted by ODN grafted irreversibly on a polypyrrole chain and presenting at the solution interface ODN probes available for recognition with complementary strands (DNAc).

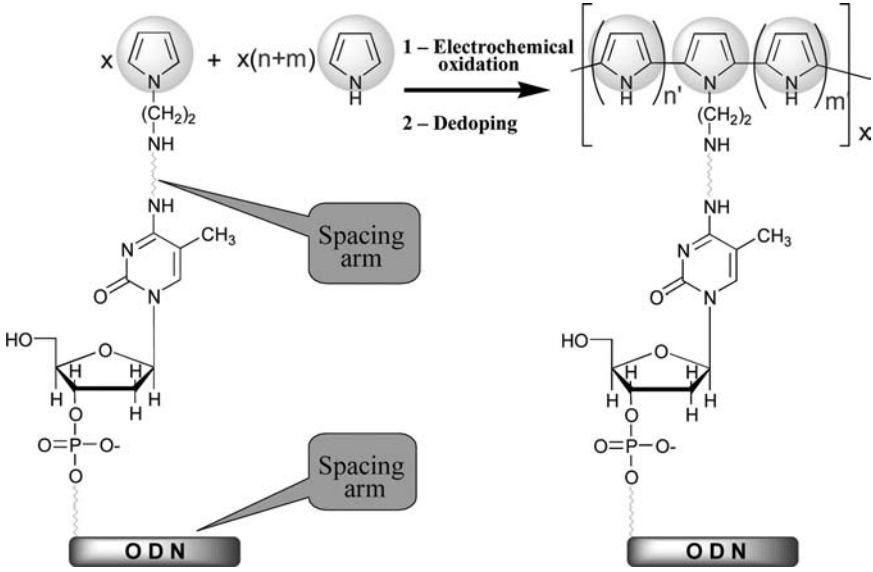
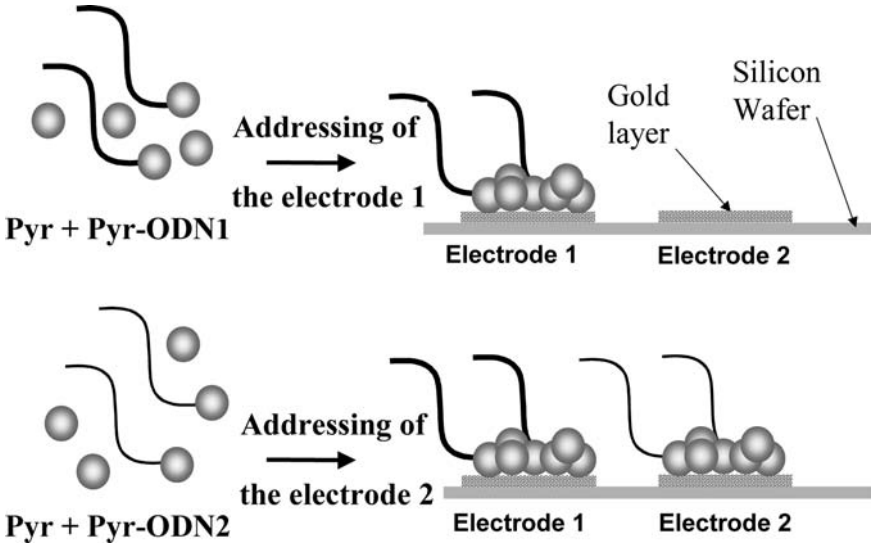



**FIGURE 1** Synthetic strategy for the immobilization of ODN probes based on the electro-copolymerization of pyrrole monomers and pyrrole-ODN monomers. Concentrations of pyrrole and pyrrole-ODN are $5 \times 10^{-7}$ M and $10^{-2}$ M respectively (m + n = 20,000), in a aqueous phosphate buffer. Electrocopolymerization is carried out either at a constant current of 1.2 mA cm$^{-2}$ or by cyclic voltammetry between −0.3 V and 0.85 vs. SCE at a sweep rate of 50 mV s$^{-1}$. The ratio in the film is m' + n' = 60,000 (2.7 pmole cm$^{-2}$) [7].

As previously described by us [8], DNA chips have been elaborated by this method [9] (Sch. 2) from a miniaturized multiparametric device consisting in several different ODN microelectrodes (50 μm × 50 μm); each microelectrode is covered by a ODN functionalized polypyrrole copolymer electrosynthetized from a pyr/pyr-ODN monomer ratio of 1/20000. This strategy was validated by the genotyping of C virus [10].

We focuss in this paper on our recent developments of this immobilization strategy based on the binding properties of a functionalised polypyrrole. Three aspects are emphasized:

– A versatile grafting based on affinity interactions between a biotinylated polypyrrole support and the biological probe allows building up a surface renewable DNA sensor.
– The electrical monitoring of the PPy films thickness at the nanometer range allows using Surface Plasmon Resonance Imaging (SPRi) detection for the real time monitoring of the biological interactions.




**SCHEME 2** Principle of the ODN immobilization by electrodirected copolymerisation of pyrrole, Pyr, and pyrrole grafted by an ODN sequence Pyr-ODN. Pyr-ODN1 is first copolymerised on the electrode 1; following by washing of the cell, copolymerisation of the Pyr-ODN2 is carried out by activation of the electrode 2, and so on.

– The electroconductivity of the PPy was exploited to trigger the electrochemiluminescence of a luminol derivative, ABEI.

## 2. MATERIALS AND METHODS

The biotinylated pyrrole monomer was synthetized as reported previously [11]. Electrochemical syntheses of polymer films were performed by electrolysis at a controlled potential of 0.75 V vs. SCE from a $H_2O + 0.7$ M $LiClO_4$ solution containing 50 mM of monomers. The polymerization charge was 20 mC $cm^{-2}$. The gravimetric and enzymatic measurements have been performed according to a process previously described [11,12]. The elaboration of DNA sensor has been realised by immobilisation of biotinylated specific nucleotide on an avidin layer previously anchored onto a biotinylated polypyrrole film using an experimental mode previously reported [11].

A quartz crystal microbalance (QCM) QCA 917 from Seiko EG&G Princeton Applied Research and a 9 MHz AT-cut quartz crystal resonator (EG&G) mounted in a home-made Teflon cell [12] has been used for gravimetric measurements. The electrochemical equipment consisted of a PAR 273 unit from EG&G Princeton Applied research connected to a computer with a homemade software.

The preparation of oligonucleotides, the SPR Instrumentation, the SPR chips preparation by electrospotting, the hybridization and denaturation steps and the fluorescence quality control of the ODN spots have been already published [13].

The ECL measurement was achieved from a polypyrrole copolymer electrosynthetized at 0.75 V vs $Ag^+ 10^{-2}$ M/Ag passing a charge of 12 mC $cm^{-2}$ on a gold electrode ($S = 7 \times 10^{-2}$ $cm^2$) from a solution containing monomers of pyrrole (40 mM) and of N-functionalized pyrrole bearing active ester group (60 mM). The immobilization of ABEI was performed by dipping of a modified gold electrode in 20 mM of ABEI in a DMF solution during 60 minutes. After washing with a buffer solution of the copolymer/ABEI assembly the luminescence measurement were carried out in borate buffer solution (pH = 10) containing 500 μM of $H_2O_2$.

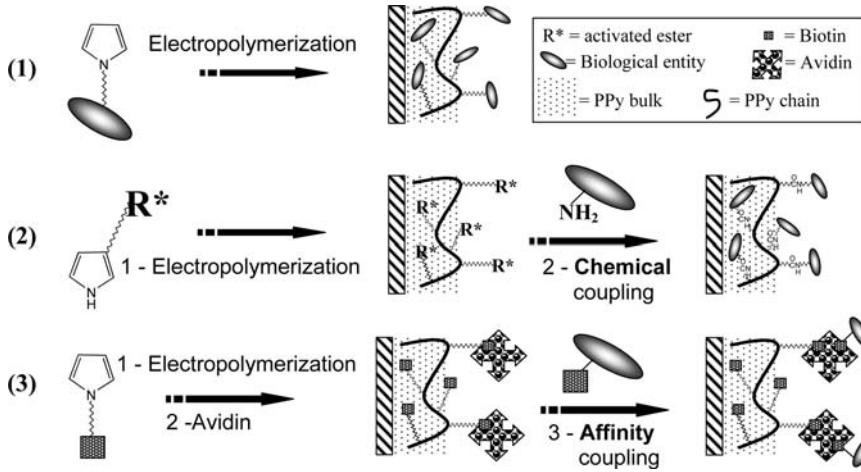
## 3. RESULTS AND DISCUSSION

### 3.1. Affinity Coupling onto a Biotinylated Polypyrrole Film

The appropriate covalent grafting of a pyrrole monomer by a biological moiety allows its one-step immobilization onto an electrode surface via the electrocopolymerization process (Fig. 1 and Sch. 3, reaction 1), but without further modification of the grafted probes. The extension of the electrocopolymerization process to other biological moieties than ssDNA sequences, such as enzymes [4], antibodies [14], or proteins needs to adjust a new grafting chemistry for each new biological family. A two-step method (so-called post-functionalization) based on the functionalization of pyrrole monomer by activated ester [5] is more versatile and allows to bind any biomolecule with amino pending group to the activated polypyrrole layer (Sch. 3, reaction 2).

Recently, an alternative method has been developed based on the biotin/avidin affinity system where the biomolecules were anchored to a biotinylated polypyrrole, PPy-B. These new biomaterials incorporating biological grafting units, so-called biotin, have been designed for the fabrication of complex bioactive surfaces in view of their use for biosensors [15,16]. These sensors are based on PPy-B and the immobilization of active biomolecules was obtained due to the high affinity between biotin and avidin entities (Sch. 3, reaction 3). This approach presents various advantages:

1. Incorporating biomolecules only at the surface of the polymer film, as a matter of fact, the bulkiness of avidin prevents its diffusion inside the polypyrrole film.
2. Avoiding chemical reagents and conditions used during the coupling reaction that may damage the PPy support and more fragile biological species.




**SCHEME 3** The three main routes for the covalent immobilization of biological entities via polypyrrole support. (1): One step functionalization from the grafted pyrrole monomer [8]; (2): Post-functionalization based on an activated ester derivatized pyrrole monomer [5]; (3): post-functionalization based on affinity coupling [15].

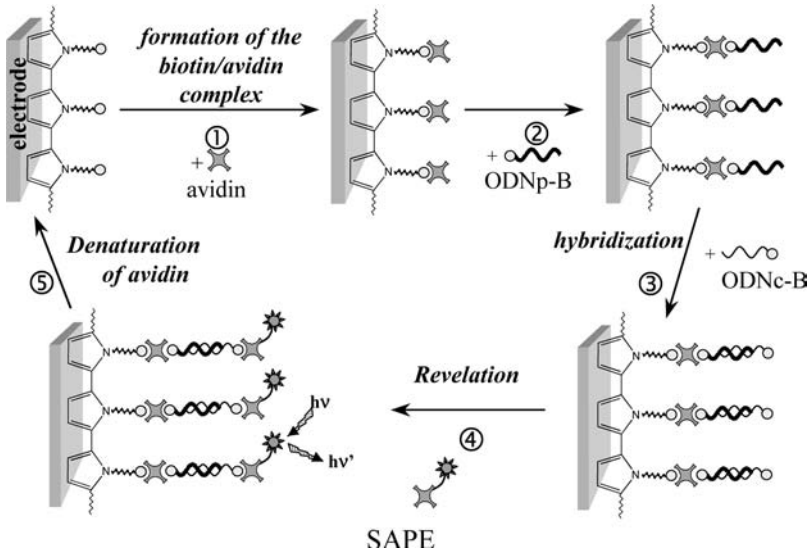
3. This approach is also a versatile process for the immobilization of biological species since a large number of biosensors could be synthesized with the wide variety of commercially available biotin conjugates.

After the validation of such an assembled system using a fluorescent-labeled avidin [15], biotinylated glucose oxidaze, B-GOx, was used as a reference enzymatic system to evaluate the efficiency of this "sandwich-affinity" immobilization [12]. The determination of the amount of immobilized B-GOx, as well as its enzymatic activity was performed by gravimetric measurements using a Quartz Crystal Microbalance (QCM) and enzymatic tests in the same condition to correlate the data. This study validated the QCM approach to evaluate bio-assembly onto polypyrrole, (in particular in our experimental conditions, viscosity can be ruled out) and resulted in the discrimination between the three paths of anchoring of B-GOx, i.e.: 1. direct adsorption of B-Gox onto the PPy-B film, 2. affinity coupling with the avidin adsorbed onto the PPy-B film and 3. the affinity coupling with the avidin coupled with the grafted biotin. The direct adsorption process was negligible, however, about 25% of the equivalent bi-layer of avidin was non-specifically adsorbed.

These results were taken into account to avoid any non-specific binding when this anchoring by affinity was extended to the immobilization of

biotinylated single stranded DNA probe [17]. Hybridization with the complementary DNA target has been performed. After that, the biotinylated surface may be regenerated by the denaturation of avidin by an aqueous solution of sodium dodecylsulfate 0.1 M at 100°C, allowing another immobilization/hybridization cycle with a new DNA probe. The entire process was followed by QCM and after each cycle, a fluorescent detection was made (Sch. 4).

On the unmodified PPy (blank experiment, Fig. 2a) only the reaction 1 results in a small detectable frequency change ($35 \pm 5$ Hz), the following additions of the ODNp-B probe (reaction 2), ODNc-B complementary target (reaction 3) and fluorophore streptavidin-phycoerythrin, SAPE, (reaction 4) induces only negligible changes. At the opposite, deviations on the PPy-B Fig. 2b) correspond to approximatively 1.6 of avidin monolayer. Once the complex PPy-biotin/avidin/ODNp-biotin probe/ODNc-biotin target/SAPE is obtained, fluorescence intensity is measured to control the efficiency of the sensing process. The average fluorescence intensities




**SCHEME 4** Synoptic of the ODN sensor design and ODN sensing. After electropolymerization of pyrrole monomers bearing the biotin entities (O), the sensor assembly is build up at the surface by successive addition of avidin (reaction 1) and the biotinylated ODN probe (reaction 2); then the sensing even is carried out by incubation (reaction 3) with the complementary biotinylated ODN target and detected by addition (reaction 4) of the fluorophore streptavidin-phycoerythrin, SAPE, that is excited by a laser. After recording the fluorescence, the substrate is regenerated by denaturation of avidin by a solution 0.1M SDS at 100°C.

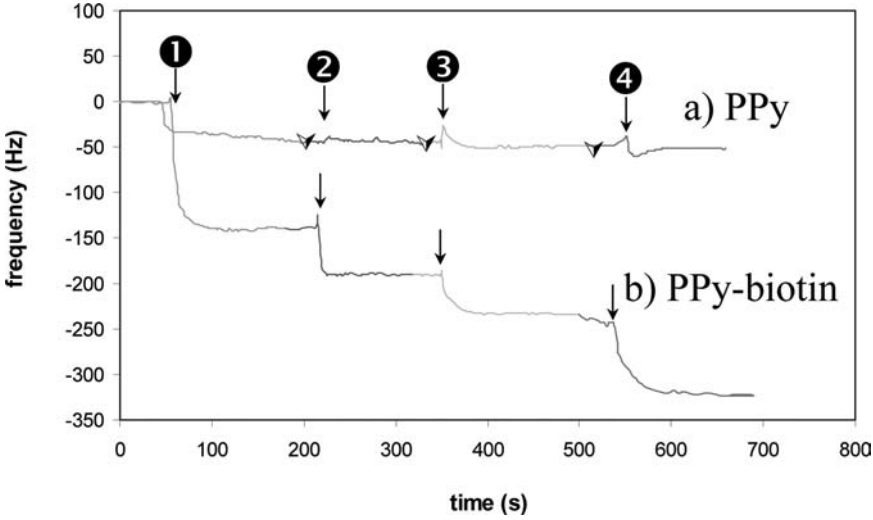



**FIGURE 2** QCM frequency change as a function of time into (a) an unbiotinylated polypyrrole film and (b) a biotinylated polypyrrole film after injection in 700 µl of phosphate buffer solution (PBS) of (1) 35 µl of avidin (final concentration = 0.125 g/l), (2) 10 µl of biotin-ODN (final concentration = 0.6 µM), (3) 10 µl of biotin-ODNc (final concentration = 0.6 µM), (4) 35 µl of SAPE (final concentration = $5.10^{-3}$ g/l). The biofilms was submitted to a washing process in PBS between each step of the assembly (↓). The both films are electrosynthesized at 50 mV/s by repetitive potential scans between −0.3 and +0.8 V vs. $Ag^{+}$ $10^{-2}$ mol $L^{-1}$/Ag of 10 mM monomer with a synthesis charge of 20 mC/$cm^2$ in 0.1 M $nBu_4PF_6/CH_3CN$ on a quartz crystal electrode (AT-cut with a basic resonance frequency of 8.8 MHz).

(FI) obtained for the biotinylated film and the unbiotinylated film are respectively, of 2100 and 200 (versus a grey level scale of 2300 levels) after the first cycle. After completion of a second cycle, the FI was 1950; thus proving the stability of the PPy support and the renewability of the anchoring points.

## 3.2. Real time Monitoring of DNA Hybridization by Surface Plasmon Resonance Performed through a Nanometer Thick Polypyrrole Film

As previously pointed out, hybridized samples are classically detected on biochips by an end-point detection process through the use of fluorescent labels. Although this method is quite sensitive, it needs a two-step protocol (pre-labeling then revelation) and quantification is not so easy. In order to

reach a real time monitoring of biological interactions, we have developed an oligonucleotide array substrate that can be fully compatible with the use of a Surface Plasmon Resonance (SPR) detection process. This latter technique combines many advantages for biosensing like real-time, label-free recognition, fast quantification and measurements of affinity constant. In order to use the polypyrrole chemistry in conjunction with a SPR detection process on a non-patterned homogenous gold layer, we have developed a new electrodirected system or "electrospotting" [13,18]. Electrospotting involves the use of a pipette tip as an electrochemical cell allowing a localized one-step synthesis of a polypyrrole film including covalently linked ODN (Fig. 3).

By successive copolymerizations, this new electrospotting process allows straightforward preparations of ODN matrices directly onto a non-structured gold substrate without the need of thiolated reagents or multi-step synthesis. Moreover, it allows an easy monitoring of both the film thickness through the charge control and the grafting density through the copolymerization ratio resulting in the synthesis of dots 500 μm in diameter thin films (11 nm) bearing a high quantity of ODN (10–20 pmole/$cm^2$). In order to reach a real time monitoring of biological interactions, the electrospotting process have been used with a Surface Plasmon Resonance Imaging (SPRi) detection (Fig. 4) [13].

For that purpose film thickness and pyrrole-ODN/pyrrole ratio have been optimized. The optimal thickness value of 11 nm lies between the sensitivity-limiting values (above 20 nm) and the values leading to inhomogeneous and consequently non-reproducible spots (below 10 nm) (Fig. 5).

Optimization of the probe density on the sensor surface to obtain the best sensitivity was obtained by varying the ratio of oligonucleotides per

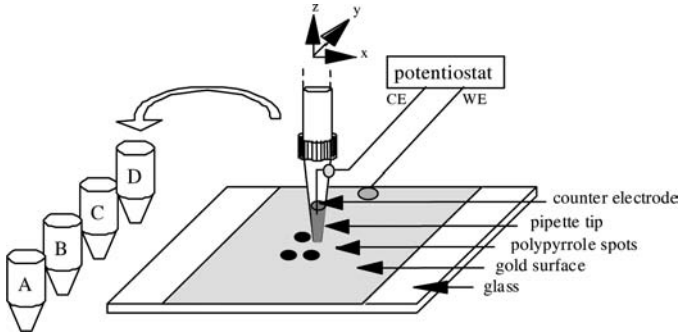



**FIGURE 3** Electrospotting setup. The different tubes A-D containing different pyrrole-ODN and pyrrole monomer solutions are on the left. The spotting is carried out on the gold surface via the plastic tip containing the solution to be electrocopolymerized [13].

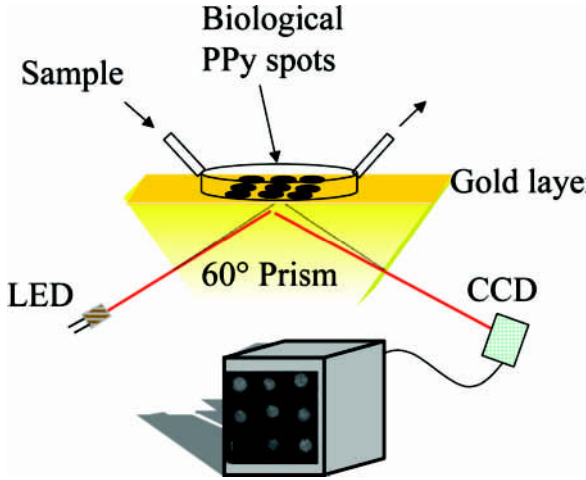



**FIGURE 4** Schematic representation of the SPR imaging device. A collimated, p-polarized LED beam illuminates the sensor surface via a coupling prism (n = 1.717). Reflected light, which contains all of the SPR response information is collected on a CCD camera.

pyrrole monomer during the electrocopolymerization (Fig. 6). The ratio ofpyrrole/pyrrole-ODN in these very thin films (10 nm) is roughly the same as the ratio from the feed solution, whereas hybridization on thick film (200 to 600 nm) leads to different results [7]. We demonstrated that a surface density of the 15-mer DNA probes on polypyrrole of $\approx 130\,\text{fmol/mm}^2$ optimizes the hybridization signal that can be detected directly (Fig. 6). This SPR imaging is currently under development for the discrimination of single mismatch [19] in Kras specific sequences.

## 3.3. Electrochemiluminescence Detection onto Polypyrrole Film

The fluorescence technique remains mainly the routine method used to detect the association between two ODN strands even if it requires the grafting of a fluorescence molecule on the ODN target. The ODN recognition event is then evaluated through the intensity of fluorescence emitted by the selectively excited fluorophore. Although this detection method possesses intrinsically a high sensitivity, a background signal could also be detected due to the illumination of the support bearing the biosensor layer. Moreover in the case of protein immobilization, their intrinsic fluorescence generates a side signal. In order to overcome these disadvantages,

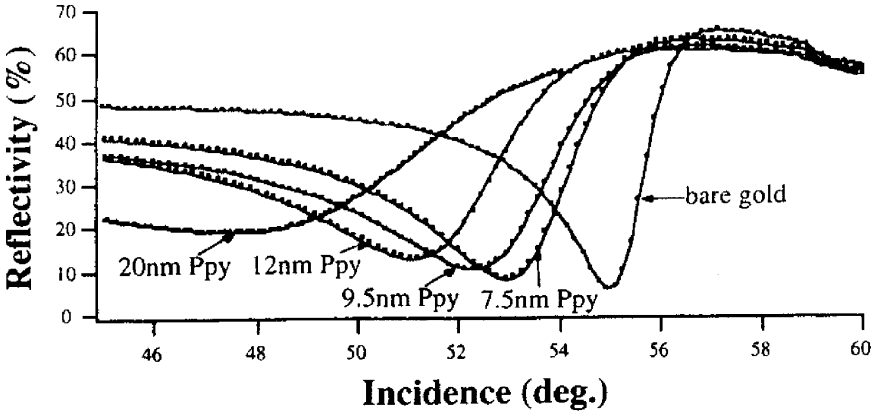



**FIGURE 5** Reflectivity curves versus incident angle performed with the regular SPR device for different polypyrrole thicknesses. Solid lines are the results of the Fresnel calculations; the points are for experimental data. Parmeters used are gold permittivity, $-11.9+j1.3$; gold thickness, $47.5 \pm 0.2$ nm; polypyrrole index $1.7 - j0.3$, at $\lambda = 633$ nm [13,18].

a detection method based on the electrogenerated chemiluminescence (ECL) could be used with a luminol derivative label N-(4-aminobutyl)-N-ethylisoluminol (ABEI) as ECL probe (Sch. 5).

ECL is widely used in analytical chemistry [20] and shows several advantages such as high sensibility and selectivity and a relatively simple

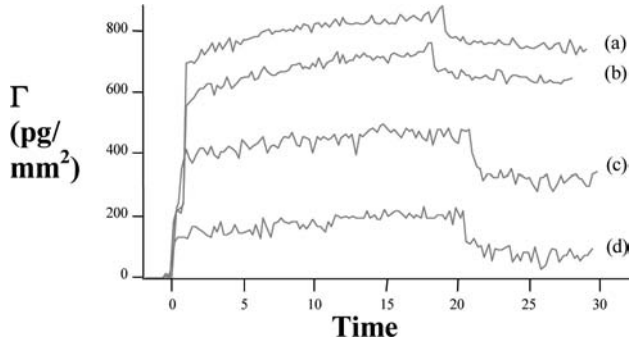
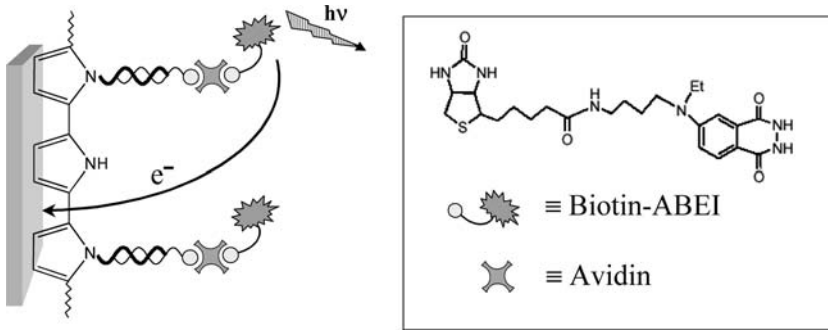



**FIGURE 6** Kinetics of hybridization reactions performed on a four-channel sensor with the SPR imaging device. Each channel bears a different oligonucleotide/pyrrole ratio on its surface. Surface coverages given respectively in regard to the volume ratio: (a) $\Gamma = 740\,\mathrm{pg/mm^2}$ (1/300), (b) $\Gamma = 660\,\mathrm{pg/mm^2}$ (1/700), (c) $\Gamma = 340\,\mathrm{pg/mm^2}$ (1/1500), (d) $\Gamma = 160\,\mathrm{pg/mm^2}$ (1/3000) [18].




**SCHEME 5** Principle of the detection of DNA hybridization by electrochemiluminescence of the ABEI label. After electrodeposition of the copolymer Pyr/Pyr-ODNp (ODNp ≡ thick wavy line), the modified electrode was (i) incubated with the complementary biotinylated ODN target (thin wavy line); (ii) dipped in an avidin solution; (iii) then dipped in a solution of biotinylated ABEI. The chemiluminescence was triggered between 0.4 − 0.8 V vs. SCE.

instrumentation. ABEI is a marker commonly used as an ECL label in homogeneous immunoassay. Recently Yang et al. [21] have shown that the hybridization of an ABEI labelled ODN probe in solution with its complementary DNA target simply adsorbed onto a polypyrrole film could be detected by the ECL reaction of ABEI.

ECL is particularly well adapted to our biochips design; it combines the advantage of the straightforward monitoring of the electrical addressing of each DNA-modified dot (micro-electrode) with the sensitivity of the luminescence. The usefulness of the ECL reaction of ABEI was evaluated by the control of various parameters such as the triggering potential, the nature of the buffer, the thickness of the underlying polypyrrole film. The electrochemical generation of the ECL reaction is controlled by the potential value applied at the electrode coated with the ODN functionalized polypyrrole film. Consequently, the potential value to activate the ECL reaction must be in a potential range where the polypyrrole matrix is or remains conducting for the time required for the ECL reaction to take place. The other corner stone to address was the electrical accessibility of the ECL label through the coPPy-ODNp/ODNc/avidin/biotin-ABEI assembly and the efficiency of the ECL reaction in biological conditions.

In order to study the efficiency of the electrical accessibility of the ECL label, we have achieved a first experiment where ABEI was directly anchored onto a polypyrrole copolymer by post-functionalization using a coupling reaction between a leaving group linked to the polypyrrole network and the amine entity of ABEI. The Figure 7 shows that an ECL response of ABEI was observed when the ECL potential was applied to the

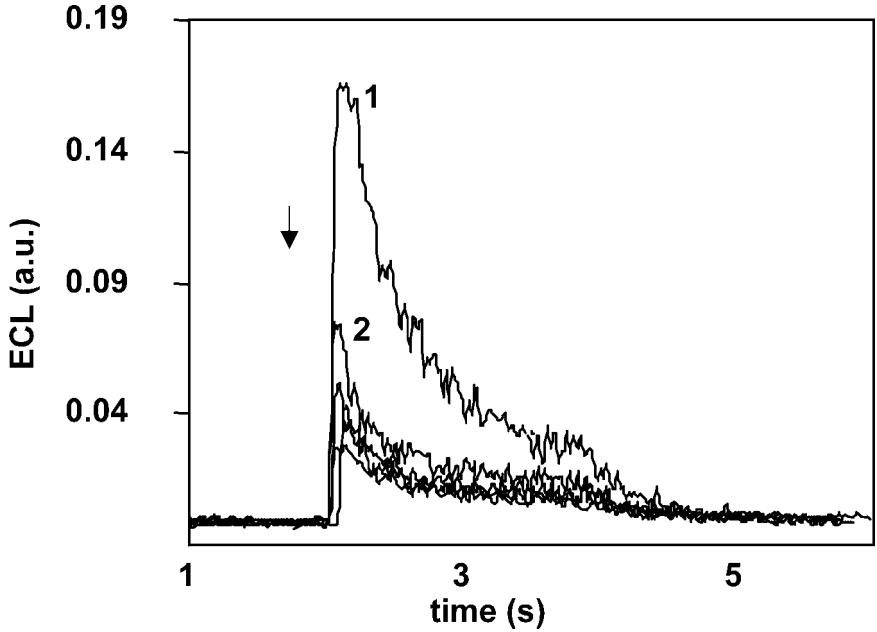



**FIGURE 7** Successive triggered ECL emissions at a constant potential of 0.6 V applied during 2 seconds of a polypyrrole copolymer/ABEI assembly on a gold electrode ($7 \times 10^{-2}$ cm$^2$) in a borate buffer solution (pH = 10) containing $H_2O_2$ 500 μM.

electrode support bearing the polypyrrole copolymer/ABEI assembly. When the potential of +0.6 V was again applied to this modified electrode, ECL intensity decreases resulting from the consumption of ABEI and perhaps the degradation of the polypyrrole conductivity. Our results demonstrated that the ECL response of ABEI is not inhibited by a polypyrrole film coated on an electrode surface. The ECL phenomenon can then be used to detect the hybridization phenomenon from a DNA sensor elaborated according to parameters used in our technology of biochip preparation i.e. an array of gold electrodes individually electrically addressable [22].

## 4. CONCLUSION

Main actual or potential applications of ECPs, e.g.: light-emitting diodes, photovoltaic devices, supercapacitors, batteries, electrochromic devices, exploit the electronic properties of these materials. However, their synthesis by electropolymerization is not a trivial reaction and allows the

straightforward preparation of multiparametric bio-sensors. As a matter of fact, the possibility to electrically address and control a polymer deposit onto any conductive surfaces (ITO on glass, metal on glass or silicon, ...) structured by the classical processes of the microelectronics technologies is a very versatile method to produce arrays of modified micro- or nano-dots. When this process is combined with the bio-functionalization of the polymers or of its precursor monomer by DNA sequences, this opens the route for the implementation of DNA chips. We have shown that the direct grafting of ODN sequence may be extended to a two-step assembly using a biotin/avidin/biotin sandwich. Considering the large number of commercially available biotin or avidin conjugates of biological moieties, this method allows to activate any conductive surface for the immobilization of a large panel of biological substances.

These results demonstrate that polypyrrole is biocompatible with a lot of biological reactions and it offers very stable binding properties. The control of the thickness of the PPy deposit at the nanometer level allows to detect biological assemblies at the surface of a SPR prism, avoiding in this way to screen the change in refractive index during the biological event. The electrical conductivity of PPy is also an important advantage in comparison with other polymeric supports used to bind ODN. It has been possible to trigger the electrochemiluminescence of a luminol derivative, ABEI, through the PPy support. For the future, the knowledge of the pathway of the electronic transfer will allow to optimize this reaction.

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