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Direct and rapid electrochemical immunosensing system based on a conducting polymer

B. Piro[a], Q.D. Zhang[a], S. Reisberg[a], V. Noel[a], L.A. Dang[c], H.T. Duc[b], M.C. Pham[a,*]

[a] Laboratoire Interfaces-Traitements-Organisation et Dynamique des Systèmes (ITODYS), associé au CNRS-UMR 7086, Université Paris Diderot – Paris 7, 15 rue Jean-Antoine de Baïf, 75205 Paris Cedex 13, France
[b] Université Paris 11, IFR 89, U-542 Inserm, Groupe Hospitalier Paul Brousse – 94800 Villejuif, France
[c] Institute of Chemistry, VAST, 18 av. Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam

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ABSTRACT

A system device using multifunctional conjugated copolymer poly(5-hydroxy-1,4-naphthoquinone-co-hydroxy-2-thioacetic acid-1,4-naphthoquinone) acting both as immobilizing and transducing element for reagentless immunosensor has been constructed. Its functionality was evaluated in an antigen–antibody interaction model using ovalbumin–anti-ovalbumin. It was shown that the system specifically detects via electrochemical signal the antigen–antibody immune interaction in a reagentless context. Comparison to the conventional ELISA technique relevant to performance and sensitivity was presented.

1. Introduction

Development of biosensors in general and immune sensors in particular has evoked interest since there is a need in obtaining alternative technique that could be complementary and/or replace conventional ones such as ELISA [1]. Indeed, the latter one, although useful, remained time consuming. Our immune sensor system detected the specific interaction between defined antigen and specific antibody in a reagentless context through electrochemical process subsequently to immune complex formation. Transduction of signal by immunosensor in general was achieved by methodologies that relied on absorbancy or chemiluminescence in optical systems. Eventually in other methodology, the reactant could be conjugated to magnetic beads thus allowing to sort out the specifically recognized counterpart reactant (i.e. antigen or antibody). These methodologies actually have had large applications, but however remained complex and generally needed intermediary step such as amplification systems or label-conjugated secondary antibody system. Beside these techniques, there are label-free ones such as those using plasmon resonance [2,3], mass spectroscopy [4], gravimetry [5], near-field microscopies [6], field-effect transistors [7,8] as well as electrochemistry [9–12].

In this report, we are interested to label-free electrochemical immunosensor that made use a multifunctional conjugated copolymer poly(5-hydroxy-1,4-naphthoquinone-co-(5-hydroxy-2-thioacetic acid-1,4-naphthoquinone), poly(HNQ-co-HNQA) as the immobilizing and the transducing element. We recently developed a new class of direct DNA electrochemical biosensors using this concept [13–16]. The quinone group is used for its redox properties, and the carboxylic group as a precursor for antigen linkage via peptide bond.

It is expected that the presence of Ab/Ag complex in the vicinity of the polymer/solution interface strongly influences the switching rate of the conducting polymer. The switch includes (i) charge (ion) diffusion from the solution to the polymer/solution interface, (ii) interfacial ion penetration, (iii) mixed charge transport inside the film and (iv) electron transfer at the metal/polymer interface. For such multistep reactions, the overall rate is determined by the slowest step. The evolution of the current intensity recorded during quinone redox switch is correlated with the rate-limiting step. The main transformation between Ag and Ag/Ab complex formation occurs at the polymer/solution interface. Hence the rates of steps (i) and (ii) are the most affected by complex formation.

OVA/anti-OVA system was studied as a model. Antigen OVA, of small molecular weight (45 kD), was grafted as probe to detect OVA/anti-OVA complex formation, anti-OVA being much more heavy (350 kD). A current decrease was detected after complex formation by this immunosensor.

* Corresponding author. Tel.: +33 1 57277223.
E-mail address: mcpham@univ-paris-diderot.fr (M.C. Pham).

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2. Experimental

2.1. Chemicals, biological products

N′-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and phosphate buffer saline (PBS, 0.137 M NaCl; 0.0027 M KCl; 0.0081 M Na_2HPO_4; 0.00147 M KH_2PO_4, pH 7.4) were provided by *Sigma*. Aqueous solutions were made with ultrapure (18 MΩ) water. 5-Hydroxy-1,4-naphthoquinone (HNQ), 1-naphthol (1-NAP) and lithium perchlorate were purchased from *Fluka*. HNQA was synthesized in the laboratory [13]. Acetonitrile (ACN, HPLC grade) was supplied by *Aldrich*. All other reagents used were of analytical grade. OVA (egg albumin, 2× crystallized) was purchased from Calbiochem, La Jolla, CA, USA. KLH (keyhole limpet hemocyanin) was from Sigma (Sigma–Aldrich, L'Isle d'Abeau Chesnes, Saint Quentin Fallavier, France). Secondary Goat F(ab′)2 anti-Mouse Ig conjugated to horseradish peroxidase was purchased from Tebu (Le Perray-en-Yvelines, France). Immune sera against OVA and KLH were obtained by immunizing mice of C57BL/6 strain (H-2b haplotype) with relevant protein antigens. 2 i.p. injections (a priming followed by a boost) were done with 300 μg of the antigen in 0.2 mL PBS per mouse. Sera were harvested 15 days after the boost and pooled. Normal sera, usually from blood collected before immunization was used as control. Specificity of the immune sera was verified against the specific and irrelevant antigen (i.e. OVA vs KLH) by ELISA.

2.2. Electrochemical methods

For electrochemical experiments, a conventional one-compartment, three-electrode cell was used with an Autolab PGSTAT. The working electrodes were glassy carbon (GC) disks (*Aldrich*) of 0.07 cm^2 area, or GC plates (2 cm^2) for ELISA assays. The auxiliary electrode was a platinum grid and the reference electrode a commercial saturated calomel electrode (SCE, *MetrOhm*).

The electrochemical synthesis of the polymer films was carried out by electrooxidation of a mixture of 5×10^{-2} M HNQ + 5×10^{-2} M HNQA + 2×10^{-3} M 1-naphthol + 0.1 M $LiClO_4$ in acetonitrile on GC or gold electrodes, under dried argon atmosphere, by 50 potential scans at 50 mV s^{-1} in the domain 0.4–1.05 V vs SCE. Using this method, the quinone and carboxylic functions remain preserved as previously shown by FT-IRRAS. This electrosynthesis as well as characterizations of the resulting polymer film have been thoroughly described in previous works [13,17].

The presence of the antibody–antigen complex was detected using square wave voltammetry (SWV), an electrochemical method that allows to suppress the capacitive component of the overall recorded current. The following parameters were used: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz. The electrolytic solution was PBS, bubbled with argon for 20 min before and during SWV measurements. The SWV scans were repeated until complete stabilization of the electrochemical signal (i.e. no difference observed between two successive responses). All electrochemical experiments were conducted at room temperature (22 °C).

2.3. Antigen protein grafting and immune reaction conditions

For OVA grafting, 10.5 mg of NHS and 9.7 mg of EDC were introduced in 3 mL ultra-pure water. Then OVA was added (at various concentrations between 0.2 μg mL^{-1} and 10 μg mL^{-1}). The electrode onto which a poly(HNQ-co-HNQA) film has been deposited was then put into this solution during 20 h under stirring at 37 °C. After that, the electrode was rinsed in PBS during 2 h under stirring, at 37 °C.

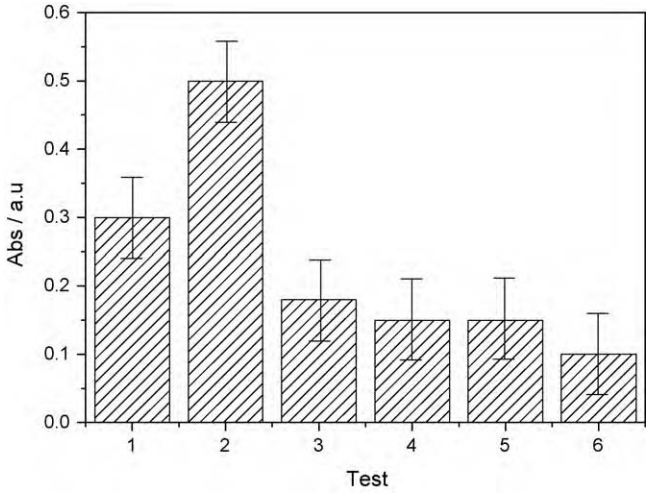


Fig. 1. Absorbances obtained for spectrophotometric ELISA assays corresponding to: 1 – OVA concentration of 3 μg mL^{-1}; 2 – OVA concentration of 10 μg mL^{-1}; 3 – without OVA; 4 – without secondary antibody HRP-Ab′; 5 – without anti-OVA (normal serum); 6 – without HRP-Ab′ nor anti-OVA (serum normal). All other conditions are detailed in Section 2.4. Reaction time: 15 min. Serum diluted 500 times (1/500).

For immune reaction between antigen and antibody, anti-OVA immune serum was added at various dilutions corresponding to 0.2, 0.6, 2, 3 and 6 μg mL^{-1} protein in water. The electrode was left to react during 1 h under stirring at 37 °C, and then thoroughly washed in PBS under stirring at 37 °C.

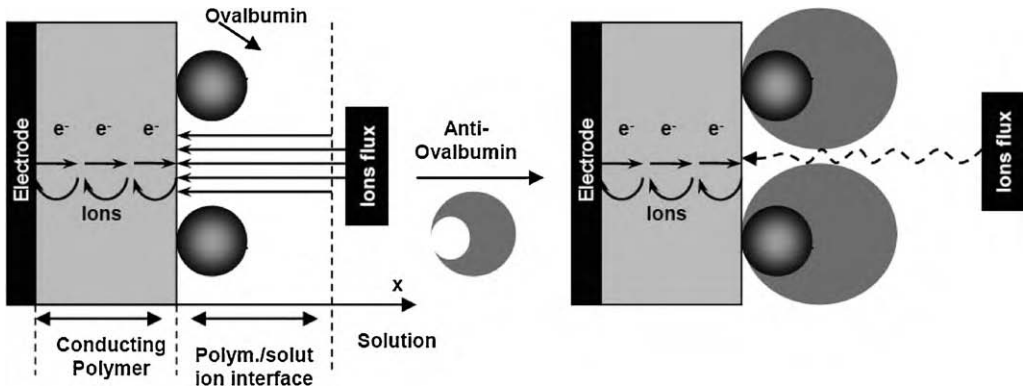
2.4. Immune reaction assessed by spectrophotometry

Immune reaction assays based on spectrophotometry recording consisted in 5 steps. In the first step, OVA was grafted on poly(HNQ-co-HNQA) film as described above. Then, anti-OVA immune sera was added (diluted 1:50, 1:500 or 1:5000), and left to react during 1 h at 37 °C. The electrode was then washed five times with PBS-0.1% Tween and horseradish peroxidase-conjugated secondary antibody (HRP-Ab) is added (at the dilution of 1:1000 as indicated by the purchased) for 1 h at 37 °C. After five washings with PBS-0.1%Tween, the electrode is ready for spectrophotometric assays. For this, HRP is used for the catalytic oxidation of a chromogen (o-phenylenediamine, OPD) by H_2O_2, giving 2,3-diaminophenazine (2,3-DAP) having λ_{max} = 450 nm. The electrode is left to react at 25 °C into 1 mL citrate buffer (5×10^{-2} M, pH 4.8) containing 0.014% H_2O_2 and OPD (0.5 mg mL^{-1}) during 15–60 min. After that, 100 μL of the reaction medium were kept aside and in which 50 μL HCl were added to stop the enzymatic reaction. 2,3-DAP was measured by absorbency at 450 nm. Absorption is therefore related to the amount of secondary antibody and, by extension, to that of anti-OVA complexed on the electrode surface.

3. Results and discussion

3.1. OVA grafting and verification by photometric assays of immune complex formation with specific antibody

The procedure for the spectrophotometric experiments consisted in 5 steps, as described in the experimental section. Results obtained in using this technique were expressed in absorbance and were not informative in terms of absolute quantity of complexed HRP-Ab, anti-OVA Ab or grafted OVA. Results corresponding to 2 different OVA concentrations, i.e. 3 and 10 μg mL^{-1}, are presented as bars 1 and 2 in Fig. 1. Values obtained in other controls (indicated as blanks) were presented that allowed to demonstrate the specificity of the immune reaction, hence the immune com-



Scheme 1. Schematic representation of the ion flux, before and after immune complex formation.

plex formation. Bar 3, without grafted OVA; bar 4, no secondary antibody HRP-Ab was added; bar 5, no anti-OVA was added; bar 6, neither HRP-Ab nor anti-OVA were added. All these controls gave absorbency values at 455 nm under those of bars 1 and 2. These results thus, lend support to the specific binding of anti-OVA antibody to the grafted OVA antigen. It also appears that the ELISA method is not very specific, as shown by the ratio of only 1:3 between bar 5 (no anti-OVA, absorbency of 0.15), and bar 2 (anti-OVA, absorbency of 0.5). It will be shown below that the electrochemical approach is more selective.

3.2. Electrochemical approach

3.2.1. OVA grafting

OVA was grafted onto poly(HNQ-co-HNQA)-coated GC electrodes as described in the experimental section. The OVA immobilization was of course a determinant step in the electrochemical biosensor process. OVA was a bulky macromolecule with a molecular weight of 45 kDa and could therefore generate a strong steric hindrance at the electrode-solution interface, when immobilized. Its dimensions were known as 70 Å × 45 Å × 50 Å [18] and in terms of occupied electrode surface could be, for one molecule $(70 \times 45 \times 50)^{2/3} = 2900$ Å^2, meaning that the maximum surface concentration of OVA (considering a monolayer) was ca. 54 pmol cm^{-2}. Such a close-packed structure had to be avoided because it would lead to a complete surface coverage, i.e. complete surface blockage for ion transport into the polymer film. Of course the ideal situation would be certainly to obtain a complete blockage only after anti-OVA binding. The antibody molecule (ranging from 150 kD for IgG to 500 kD, for IgM), was bigger and more voluminous than that of the OVA probe, the average surface occupied by one anti-OVA antibody molecule could be estimated as ca. 10^4 Å^2. Therefore, the surface of anti-OVA antibody molecules available to form a uniform monolayer would be ca. 15 pmol cm^{-2}. On the basis of this estimation a complete surface blockage generated by anti-OVA antibodies could be deduced. For this reason, OVA antigen was grafted at low surface density necessary and sufficient allowing subsequently an efficient complete blockage by OVA/anti-OVA antibody complex layer. This approach was summarized in Scheme 1.

In other words, an optimal antigen concentration working surface must be found for grafted antigen (i.e. OVA, in the present work) that would lead to a minimum blockage while a maximum blockage would be obtained with the formation of antigen-specific antibody complexes. To determine the optimal conditions, several OVA concentrations (5, 15, 50, 75 and 150 nM, i.e. 0.2, 0.6, 2, 3 and 6 μg mL^{-1}) were used for grafting. Results obtained before and after OVA grafting (with 0.2 and 6 μg mL^{-1} of OVA) were presented in Figs. 2 and 3. A representation of SWV was shown in Fig. 2 while

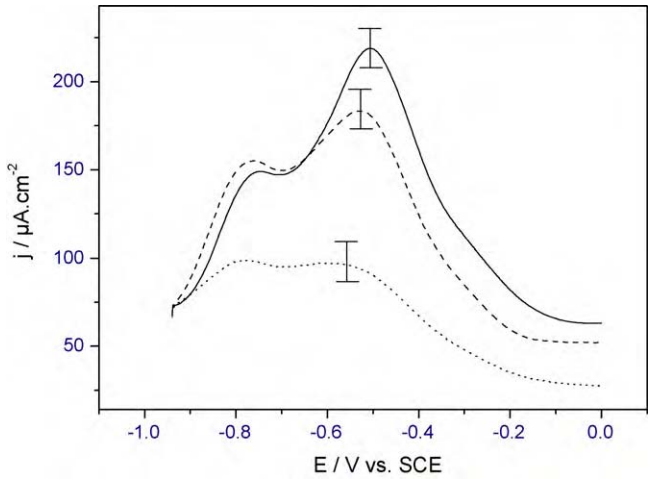


Fig. 2. Square wave voltammograms of the poly(HNQ-co-HNQA)-modified electrodes, before OVA grafting (straight line), after OVA grafting with a concentration of 0.2 μg mL^{-1} (dashed line) and after OVA grafting with a concentration of 6 μg mL^{-1} (dotted line).

recorded intensities of the SWV peak current in five different concentrations (plain squares) were depicted in Fig. 3. To distinguish between covalently grafted vs simply adsorbed OVA, experiments were performed under the same experimental conditions as for the

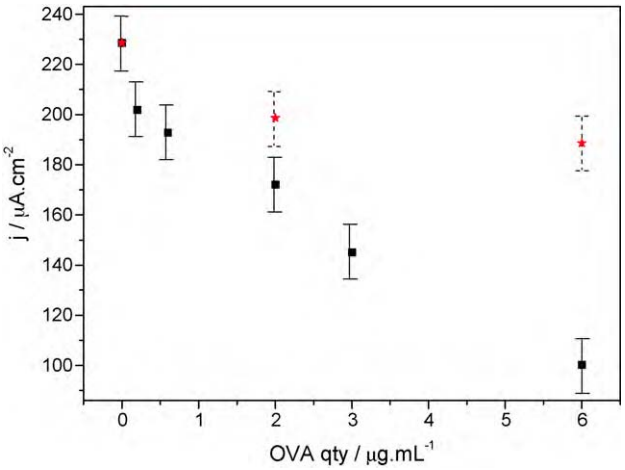


Fig. 3. Current densities obtained by SWV on poly(HNQ-co-HNQA)-modified electrodes (peak at around −500 mV), for different OVA concentrations. Plain squares: OVA grafting with coupling reagents NHS and EDC. Plain stars: OVA adsorption in the same conditions but without EDC or NHS.

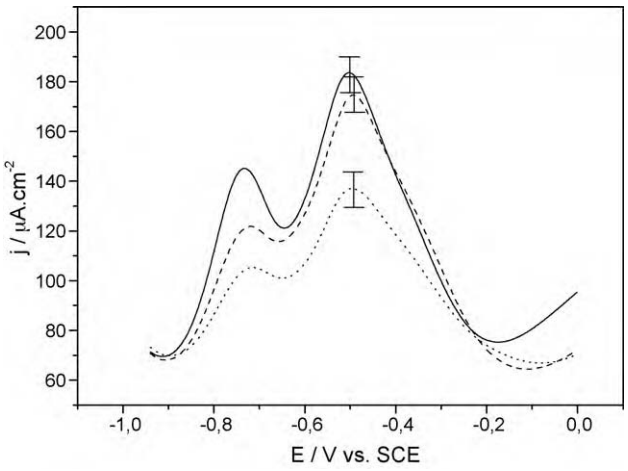


Fig. 4. SWV obtained for poly(HNQ-co-HNQA)-modified electrodes before OVA grafting (straight line), after OVA grafting (dashed line) and after anti-OVA complex formation (dotted line). Concentration of OVA for grafting: 2 μg mL^{-1}. Serum dilution: 1/50.

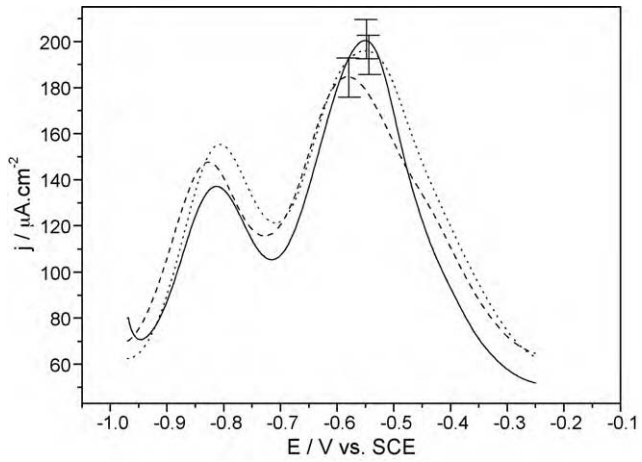


Fig. 5. SWV obtained for poly(HNQ-co-HNQA)-modified electrodes after OVA grafting (straight line, after anti-KLH addition (dotted line, serum dilution 1/50), and after addition of normal serum (dashed line, dilution 1/50). Concentration of OVA used for grafting: 2 μg mL^{-1}.

covalently grafted OVA, but in the absence of coupling agents (EDC and NHS, plain stars, Fig. 3). The two plots were significantly different demonstrating the effectiveness of covalently grafted OVA onto poly(HNQ-co-HNQA) film. OVA antigen protein that could be adsorbed onto the film was accessory and accounted approximately 15% only of the current change. In the following experiments, OVA concentration of 2 μg mL^{-1} was exclusively used, which corresponded to an effective covalent binding, while generating small surface blockage.

3.2.2. *Electrochemical evidences of immune complex OVA antigen:anti-OVA antibody formation*

With the use of SWV we could therefore demonstrate the presence of complex formation between antigen and specific antibody. Thus as expected, formation of the OVA:anti-OVA antibody complex induced a current drop (Fig. 4, dotted line), in comparison to the unmodified poly(HNQ-co-HNQA) film (Fig. 4, plain line), or to the grafted OVA alone (Fig. 4, dashed line). In the representative experiment presented above with the anti-OVA serum diluted 1/50 a current drop of 43 ± 10 μA cm^{-2} was observed.

Table 1 summarizes the current density changes recorded for different anti-OVA dilutions (1/50, 1/500 and 1/5000). Considering the standard deviation between samples, the dilution 1/500 represented the detection limit. Below this dilution, no significant change was observed. It was noteworthy that these results were obtained with whole immune serum, but not with purified antibodies.

In order to demonstrate that the signal change was due to specific recognition by anti-OVA antibody forming complex with OVA experiments were performed with an irrelevant antibody directed against KLH (Keyhole Limpet Hemocyanin), whose molecular weight was 400 kD, close to that of anti-OVA. No complex formation inducing signal drop was observed between anti-KLH and OVA as in case where anti-KLH was replaced by normal serum diluted at 1/50. See Fig. 5.

Table 1
Differential current densities (Δj) upon addition of anti-OVA for different serum dilutions. Δj corresponds to the difference between the peak current densities recorded before and after complex formation (i.e. addition of diluted serum). Each value corresponds to a new electrode.

Dilution of serum	Δj (μA cm^{-2})
1/50	43 ± 10
1/500	20 ± 10
1/5000	12 ± 10

To summarize the main results, SWV give a signal change of $43/(180-70)\times 100 = 40\%$ for OVA:anti-OVA interaction (Fig. 4), and $10/(200-70)\times 100 = 8\%$ for OVA:anti-KLH interaction (Fig. 5), giving a ratio of 8/40 = 1:5. Therefore, if we compare, in terms of selectivity, the ratio obtained by ELISA (1:3) and that obtained by SWV measurements (1:5), it seems that the electrochemical approach is slightly more selective.

4. Conclusions

The multifunctional conducting copolymer poly(5-hydroxy-1,4-naphthoquinone-co-5-hydroxy-2-thioacetic acid-1,4-naphthoquinone) was shown to act as both the immobilization and the transduction element for a reagentless electrochemical immunosensor in a model based on OVA antigen:anti-OVA antibody. Due to the chemical nature of the quinone group and its redox process, poly(HNQ-co-HNQA) film allowed to avoid non-specific adsorption of proteins on its surface (the film being neutral or negatively charged, but never positively charged). The electrochemical immunosensor was endowed with a capacity to directly detect the presence of immune complexes by recording the electrochemical signal with the use of square wave voltammetry. Furthermore spectrometry assays have confirmed the presence of the relevant immune complex on the electrode that gave rise to the signal detected by electrochemical immunosensor. No signal was observed when grafted OVA was left in contact with anti-KLH antibody or normal serum. It was shown that the electrochemical approach is slightly more selective than ELISA method.

It is proposed that the presence of OVA–anti-OVA antibody immune complex was detected via change in signal transduction due to steric hindrance. This event would lead to changes in ion diffusion and its kinetics that finally modified the redox process of the quinone group.

Work is now in progress to replace Ag probe by smaller fragments, such as oligopeptide from a dozen to 30 aminoacids, presenting a smaller size than a bulky antigen. This approach might improve the system in terms of sensitivity, but also in terms of stability (oligopeptides being more stable than the whole three-dimensional protein molecule). Finally a better control of surface morphology by using self-assembled monolayers or ultra-

thin organic films had being undertaken in work that was now in progress.

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