Immobilization of Biomolecules on Electrodes Modified by Electrografted Films

S. Ameur,[†,‡] C. Bureau,[§] J. Charlier,[†] and S. Palacin*[,†]

Chemistry of Surfaces and Interfaces, CEA-Saclay Bât. 466, DSM-DRECAM-SPCSI, F-91191 Gif sur Yvette Cedex, France, Alchimer S.A., 15 rue du Buisson aux Fraises, Z.I. de la Bonde, F-91300 Orsay, France, and Unité de recherche de Physique des Semiconducteurs et Capteurs, IPEST, La Marsa 2070, Tunis, Tunisia

Received: April 23, 2004; In Final Form: June 9, 2004

This work shows that electrografted polymethacrylonitrile films can be used for direct immobilization of proteins through spontaneous reaction between the nitrile groups from the film and the amino groups from the amino acids of the protein, in alcoholic basic medium. The formation of the resulting imino-ethers groups is followed by XPS measurements and assessed by high precision calculations of core electron binding energies. The final protein film is stable toward aqueous rinsing and can be used for protein−substrate recognition.

Introduction

Bioengineering, biocompatibility, and nanobiosciences are interdisciplinary scientific research domains in great expansion. The adhesion of proteins to solid matrixes is particularly important in all those domains because of the fundamental role that proteins play in every contact between an organic or inorganic surface and a biological environment.[1] Many microsystems, including chemical- and bio-sensors, require the sensing layer to be strongly linked to a solid support.[2−13] Some of them even require the solid support to be conducting because the detection relies on an electrochemical process.[14−18] Grafting biomolecules on a surface generally requires a preliminary modification of the electrode, to create suitable surface functional groups.

The electroinitiated polymerization of vinylic monomers on metallic electrodes has proven useful for building functionalized surfaces in which a polymer film is chemically grafted on the metal.[19,20] The electrografting procedure has some advantages, including the following: the resulting organic film is extremely thin (<100 nm) and adherent; the film is mechanically and chemically stable; and many organic species can be polymerized by this process. Electrografting is thus a very powerful technique for immobilizing the sensing molecules on selected conducting substrates by creating a primer layer where the chemically or biologically active molecules can be easily linked via chemical couplings.

Besides the functions often used to link antibodies on a surface (such as amine and alcohol groups, which require the use of coupling reageants), the cyano-function held our attention. Indeed, it was found in the literature[21] that cyano groups can directly react with amino functions in an alcoholic basic medium to form imino-ether derivates (Pinner reaction) which are useful intermediates in preparative chemistry.

Solid matrixes functionalized by electropolymerized cyano N-substituted polypyrrole have already been used to immobilize antibodies.[22−29] By this means, antibodies were immobilized via electrostatic interactions between the cyano groups of the conductive polymer and their hydroxyl groups by using an activation protocol. Moreover, antibodies fixed onto a cyano-silanized silica substrate have been found to resist acid washing. The efficiency of the immobilization method was revealed by different experimental techniques (gravimetric and permeation measurements,[22] impedance spectroscopy[25,26]) but no direct evidence for electrostatic interactions was in fact available.

TABLE 1: Computed Core−Electron Binding Energies (CEBEs) for the (CH_3CN; CH_3OH) System, Using the UGTS Method.

	O1s	N1s	Δ(O1s − N1s) (eV)
CH_3CN/CH_3OH	533.5	400.33	133.17
CH_3CN- - -$HOCH_3$	532.43	400.71	131.72
$CH_3C(-OCH_3)=NH$	533.75	398.94	134.81

The aim of this paper is to adapt that procedure to polymethacrylonitrile (pMAN) films electrografted on metal surfaces, so that the modified electrode plays the role of primer adhesion layer and offers functional groups directly available for the anchoring of biological molecules. We first developed a procedure allowing the direct covalent fixation of the protein on the substrate. Then, we applied it to a biological system. We have studied the direct fixation of immunoglobulin G antibody (anti-rabbit IgG) to electrografted pMAN modified films and checked its biological activity by reaction with immunoglobulin G antigen (rabbit IgG). This system was chosen for its simplicity and its specificity and is already largely used as a simple biochemical model.[30]

Experimental Section

The electrolytic medium for the electroinduced deposition of pMAN films was made of 40 vol % of dehydrated and redistilled methacrylonitrile (MAN) and 60 vol % of dimethylformamide (DMF) (i.e. 5 mol·dm^{-3} in DMF). Tetraethylammonium perchlorate (TEAP, 5×10^{-2} mol·dm^{-3}) was the supporting electrolyte. The electrolysis was performed in a one-compartment Teflon cell. The electrolysis was carried out with a standard three-electrode arrangement. The counter electrode was a 10 cm^2 platinum foil, and the reference electrode was based on the Ag^+/Ag couple (10^{-2} mol·dm^{-3} $AgClO_4$ dissolved in DMF). The electrochemical experiments were performed with an EG&G PAR model 273 potentiostat. The electroinitiated

[†] DSM-DRECAM-SPCSI.
[‡] Unité de recherche de Physique des Semiconducteurs et Capteurs.
[§] Alchimer S.A.

10.1021/jp0482262 CCC: $27.50
Published on Web 07/30/2004

TABLE 2: Binding Energies of the 1s Core Levels of the O and N Atoms after Basic Alcoholic Treatment in Different Media

		N1s (eV)		
dipping medium	O1s (eV)	majority component	minority component	Δ(O1s − N1s)[a]
electrografted pMAN (reference)		400.33		
pMAN in pure EtOH		400.28		
pMAN in EtOH/DMF, pH 9.4, bicarbonate[b]	533.84	400.33	398.78	135.06
pMAN in EtOH/DMF−H_2O (80%; 10%; 10%) (pH 9, NaOH)	533.28	400.25	398.89	134.39
pMAN in EtOH/DMF−H_2O (80%; 10%; 10%) (pH 7, NaOH)	533.85	400.37	399.12	134.73

[a] Only the minority N1s component value was taken into account for this calculation [b] Bicarbonate solution was prepared by mixing Na_2CO_3 (0.1 M) and $NaHCO_3$ (0.1 M) and adjusting the pH to 9.4.

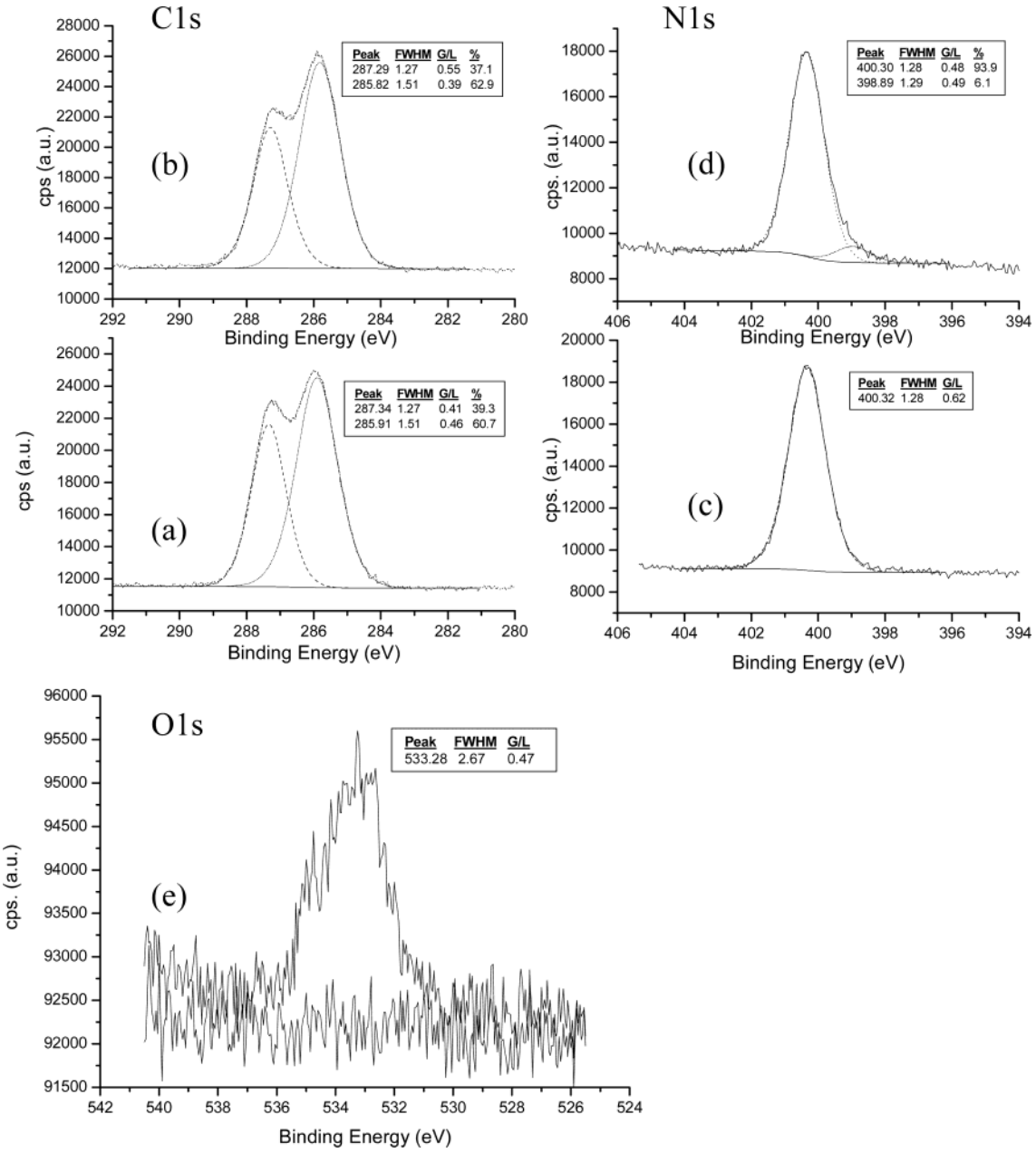

Figure 1. XPS spectra of the core levels C1s, N1s, and O1s of an electrografted pMAN film before (a, c) and after (b, d, e) treatment in a basic mixture (EtOH/DMF/H_2O, pH 9 adjusted with NaOH) (10%/10%/80%) (v/v).

polymerization technique consists of applying cyclic linear voltammetry to the solution. The grafting, on metallic substrates, takes place only beyond a threshold polarization potential (−2.4 V vs the Ag^+/Ag reference electrode for a pure gold electrode).

Before reacting with biomolecules, electrografted pMAN films were pretreated by total immersion in a EtOH/DMF/H_2O mixture (80%, 10%, 10%) (v/v) at different pH values (see Table 2) for 15 h under constant mechanical stirring and then dried under nitrogen flow before analysis.

Avidin and biotin-oligonucleotides (ODN, Biotin-5′-GCTTGCTGAAGTTCG-3′) were obtained from Sigma-Aldrich and used without further purification. The electrografted pMAN film samples were incubated at low temperature (4 °C) for 15 h in 30 mL of deionized water solution containing 2 mg/L of

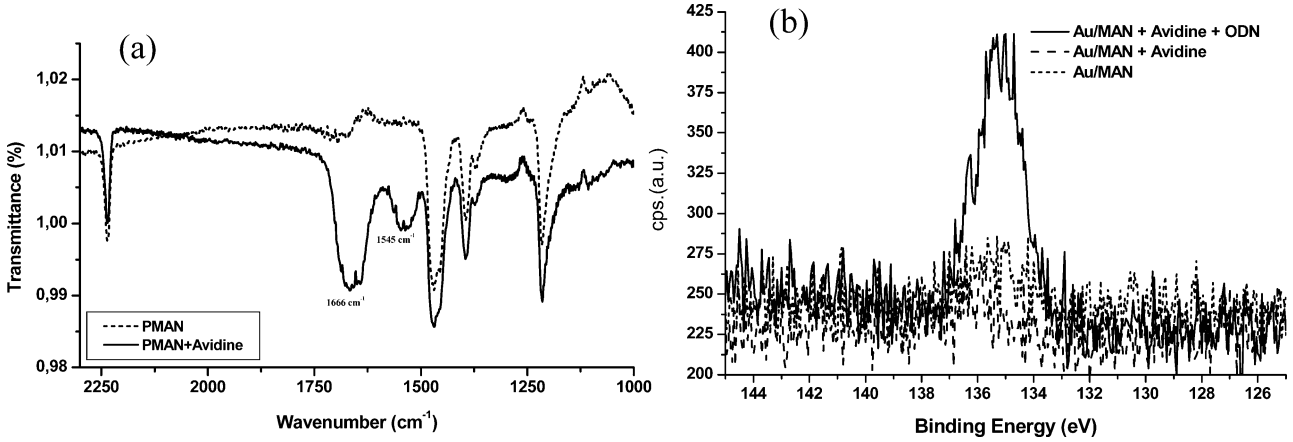

Figure 2. (a) IRAS spectra of a gold substrate coated with electrografted pMAN before (dots) and after avidin fixation (solid); (b) XPS spectra of phosphorus 2p levels recorded on the reference pMAN (dots), after avidin fixation (dash), and after avidin fixation plus biotin-ODN (solid).

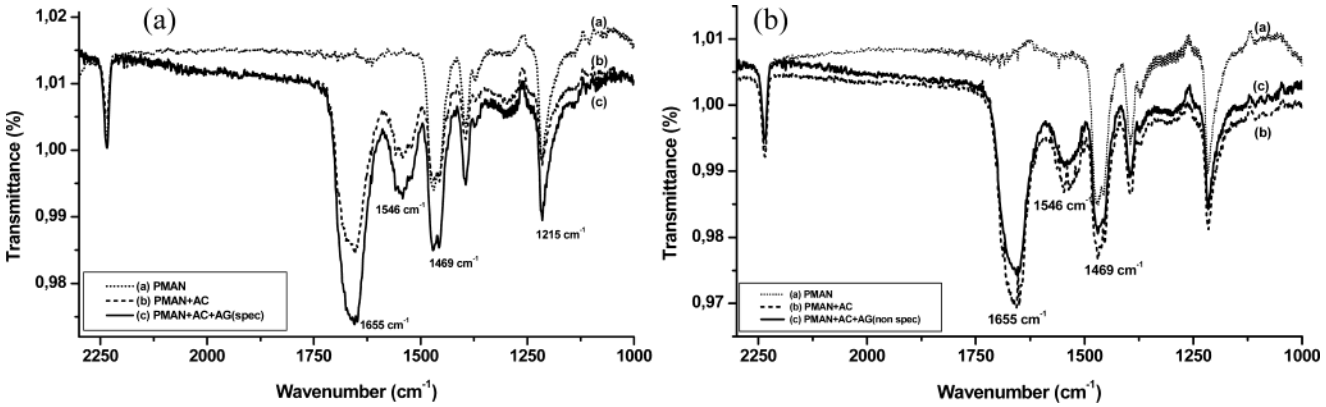

Figure 3. (a) IRAS spectra of electrografted pMAN film before (dots) and after reaction with anti-rabbit IgG (AB, dash) and then treated with a specific antigen solution *(*AC, rabbit IgG, (solid)); (b) IRAS spectra of electrografted pMAN film before (dots) and after reaction with anti-rabbit IgG (AB, dash) and then treated with a nonspecific antigen solution (AC, sheep IgG, (solid)).

avidin in a saline phosphate buffer (PBS, pH 7.2) in a hermetically closed vessel. The samples were then abundantly rinsed with water, dried under air, and immediately analyzed. Some of those samples were treated at room temperature with a solution containing 2.5×10^{-8} mol/mL of ODN (PBS, pH 7.2) for 15 h. They were rinsed and dried as above before analysis.

The fixation of anti-rabbit immunoblobulin (IgG) antibody (purchased from Sigma-Aldrich) on electrografted pMAN film was performed by dipping for 15 h into anti-rabbit IgG solution (2 mg/L) in PBS (pH 7.2) at low temperature (4°C). After that it was rinsed abundantly with deionized water and then immersed in a specific (rabbit-IgG) or nonspecific (sheep IgG) antigen solution (2 mg/L) in PBS (pH 7.2) for 15 h at room temperature. At the end of the experiment, samples were rinsed and dried as above and stored in dry nitrogen atmosphere before analysis. All solvents and chemicals used were of analytical grade. Water was purified with a Millipore Milli-Q system. All solutions were prepared just before use.

The chemical modifications of the substrates were studied by X-ray photoelectron spectroscopy (Vacuum Generator Escalab 210 spectrometer, Al Kα monochromatic) and by infrared spectroscopy in reflection−absorption mode (IRAS, Bruker IFS66).

Results and Discussion

It is reported in the literature[31] that, by a simple treatment in basic medium, nitrile functions can be transformed into imino-ether groups according to reaction 1 and thus used for the covalent binding of proteins according to reaction 2.[21]

$$\mathrm{R{-}CN + R'\text{-}OH \rightarrow R{-}C(-OR')=NH} \quad (1)$$

$$\mathrm{R{-}C(-OR')=NH + H_2N{-}protein \rightarrow R{-}C(-N{-}protein)=NH} \quad (2)$$

We have adapted reaction 1 to electrografted pMAN by dipping the films in ethanol in different basic conditions (NaOH, bicarbonate, phosphate, Table 2). As ethanol alone does not swell the grafted pMAN, it was necessary to add dimethyl-formamide (DMF) to ensure the diffusion of the solution through the film. The thickness of the electrografted pMAN film was limited to 5−10 nm so that diffusion occurred within the whole thickness of the films. The chemical treatment did not appreciably modify the thickness as seen on the global XPS spectra recorded before and after treatment.

The chemical modifications within the grafted films were followed by XPS (Figure 1). The main features of the C1s and N1s core levels of the grafted pMAN were not drastically modified by the basic treatment, but some evolution with respect to the pMAN reference film could, however, be observed. Indeed, we detected on the N1s spectrum recorded on the treated film a new component at a lower binding energy (near 399 eV) that could be related to the formation of the imino-ether group (Figure 1d). At the same time, we observed a small decrease of the high binding energy component C1s (related to the CN function) with respect to the component at lower binding energy (Figure 1b). This suggests that some of the CN groups were

transformed to imino-ether groups so that the contribution of the alkyl carbon increases to the detriment of the nitrile groups. Last but not least, oxygen could clearly be detected within the sample after the basic treatment (Figure 1e).

To determine without ambiguity the origin of those new contributions (imino-ether groups or traces of solvent trapped in the film), we compared our experimental results with precise calculations of the binding energies of the core levels N1s and O1s. Indeed, Chong and co-workers have recently developed a procedure for computing very accurate Core−Electron Binding Energies (CEBEs) via Density Functional Theory.[32−34] With this method, which is based on a generalization of the unrestricted Transition State approach of Slater, the calculation of XPS chemical shifts can be predicted with an accuracy of about 0.2 eV[32,35,36] and has already proven to be reliable to highlight intermolecular effects such as hydrogen-bond interactions.[37,38] More details about this technique can be found elsewhere.[32−35,39−42]

We applied this technique to calculate the binding energies of the N1s and O1s core levels for the system (CH_3CN; CH_3OH): first, by considering both molecules separately, then by taking into account possible hydrogen-bond interactions (CH_3CN- - - $HOCH_3$), and finally by considering the imino-ether itself ($CH_3-C(-OR)=NH$). The results are summarized in Table 1. Those computations clearly show that the energy gap between the O1s and N1s components (Δ(O1s − N 1s)) can be used as a pertinent criterion to determine whether imino-ether groups are actually formed or not in the medium.

The various experimental results are reported in Table 2. In each case, the core level N1s contains a minority component with low binding energy (∼399 eV) in a proportion close to 1:1 compared to the oxygenated component. The experimental values of the parameter Δ(O1s − N 1s), calculated for the minority component of the N1s level, clearly indicate the formation of imino-ether type functions.

Reaction 2 was tested with avidin. This protein is chemically associated with the electrografted treated polymer in a conformation that preserves its biological activity (Figure 2a). Indeed, we used the avidin−biotin strong affinity[43−45] to immobilize biotin-linked oligonucleotides on the pMAN surface. The ODN fixation on the film was detected by XPS measurements focusing on the 2p level of phosphorus atoms coming from the phosphates groups of the ODN bases (Figure 2b).

Thus, electrografted polymethacrylonitrile films can be used for direct immobilization of proteins through direct and spontaneous reaction between the nitrile groups from the film and the amino or thio groups from the amino acids of the protein (lysine, arginine, cysteine). The final protein film is stable toward aqueous rinsing and can be used for protein−substrate recognition, as shown in Figure 3 in the case of IgG antibody and its corresponding antigen. The analysis of these IRAS spectra (Figure 3a) reveals the bands amide I (1655 cm^{-1}) and II (1546 cm^{-1}), as well as the bands of the skeleton of protein at 1469 cm^{-1}. In addition, the activity and the specificity of the antibody were checked by reaction with a specific (rabbit IgG) and a nonspecific (sheep IgG) antigen. In the former case, we observed an increase of the bands amide I (1655 cm^{-1}) and II (1546 cm^{-1}), and the bands of the protein skeleton at 1469 cm^{-1}, proving that the quantity of proteins actually fixed on the surface increased (these bands are more or less twice more intense after the coupling with the antigen, whose size is roughly the same as that of the antibody). On the contrary, when a nonspecific antigen (sheep IgG) was used, we observed only a very weak increase in the intensity of the bands described above (probably due to nonspecific adsorptions) (Figure 3b).

Conclusions

We have shown that electrografted polymethacrylonitrile films can be used for direct immobilization of proteins through spontaneous reactions between the nitrile groups from the film and the amino groups from the amino acids of the protein. The final protein film is stable toward aqueous rinsing and can be used for protein−substrate recognition.

Supporting Information Available: XPS spectra of an electrografted pMAN film on gold substrate before and after treatment in a basic mixture. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Williams, D. F. In *Materials Science and Technology*; Williams, D. F., volume editor; VCH: Weinheim, Germany, 1992.
(2) Didenko, V. V. *Biotechniques* **2001**, *31*, 1106.
(3) Stenger, D. A.; Georger, J. H.; Dulcey, C. S.; Hickman, J. J.; Rudolph, A. S.; Nielsen, T. B.; McCort, S. M.; Calvert, J. M. *J. Am. Chem. Soc.* **1992**, *114*, 8435.
(4) Sackmann, E.; Tanaka, M. *Trends Biotechnol.* **2000**, *18*, 58.
(5) Wagner, P.; Nook, S.; Spudichi, J. A.; Volkmuth, W. D.; Chu, S.; Cicero, R. L.; Wade, C. P.; Linford, M. R.; Chidsey, C. E. D. *J. Struct. Biol.* **1997**, *119*, 189.
(6) Deutsch, J.; Motlagh, D.; Russell, B.; Desai, T. A. *J. Biomed. Mater. Res.* **2000**, *53*, 267.
(7) Hodneland, C. D.; Mrksich, M. *J. Am. Chem. Soc.* **2000**, *122*, 4235.
(8) Strother, T.; Cai, W.; Zhao, X.; Hamers, R. J.; Smith, L. M. *J. Am. Chem. Soc.* **2000**, *122*, 1205.
(9) Park, S.-J.; Taton, T. A.; Mirkin, C. A. *Science* **2002**, *295*, 503.
(10) Strother, T.; Hamers, R. J.; Smith, L. M. *Nucleic Acids Res.* **2000**, *28*, 3535.
(11) Nakanishi, K.; Muguruma, H.; Karube, I. *Anal. Chem.* **1996**, *68*, 1695.
(12) Tang, A.; Wang, C.; Stewart, R.; Kopecek, J. *Bioconj. Chem.* **2000**, *11*, 363.
(13) Collaud Coen, M.; Lehmann, R.; Gröning, P.; Bielmann, M.; Galli, C.; Schlapbach, L. *J. Colloid Interface Sc.* **2001**, *233*, 180.
(14) Jawaheer, S.; White, S. F.; Rughooputh, S. D. D. V.; Cullen, D. C. *Biosens. Bioelectron.* **2003**, *18*, 1429.
(15) He, P.-G.; Takahashi, T.; Hoshi, T.; Anzai, J.-i.; Suzuki, Y.; Osa, T. *Mater. Sci. Eng. C* **1994**, *2*, 103.
(16) Berney, H.; Roseingrave, P.; Alderman, J.; Lane, W.; Collins, J. K. *Sens. Actuators B* **1997**, *44*, 341.
(17) Ameur, S.; Maupas, H.; Martelet, C.; Jaffrezic-Renault, N.; Ben Ouada, H.; Cosnier, S.; Labbe, P. *Mater. Sci. Eng. C* **1997**, *5*, 111.
(18) Oroszlan, P.; Thommen, C.; Wehrli, M.; Duveneck, G.; Ehrat, M. *Anal. Methods Instrum.* **1993**, *1*, 43.
(19) Bureau, C.; Delhalle, J. *J. Surf. Anal.* **1999**, *6*, 159.
(20) Palacin, S. *ChemPhysChem* **2004**, in press.
(21) Inman, J. K.; Dubois, G. C.; Appella, E. Amidination. In *Methods in Enzymology*; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1989.
(22) Ouerghi, O.; Senillou, A.; Jaffrezic-Renault, N.; Martelet, C.; Ben Ouada, H.; Cosnier, S. *J. Electroanal. Chem.* **2001**, *501*, 62.
(23) Falipou, S.; Chovelon, J. M.; Martelet, C.; Margonari, J.; Cathignol, D. *Sens. Actuators A Phys.* **1999**, *74*, 81.
(24) Mazeran, P.-E.; Loubet, J.-L.; Martelet, C.; Theretz, A. *Ultramicroscopy* **1995**, *60*, 33.
(25) Maupas, H.; Saby, C.; Martelet, C.; Jaffrezic-Renault, N.; Soldaktin, A. P.; Charles, M.-H.; Delair, T.; Mandrand, B. *J. Electroanal. Chem.* **1996**, *406*, 53.
(26) Billard, V.; Martelet, C.; Binder, P.; Therasse, J. *Anal. Chim. Acta* **1991**, *249*, 367.
(27) Falipou, S.; Chovelon, J. M.; Martelet, C.; Margonari, J.; Cathignol, D. *Bioconjug. Chem.* **1999**, *10*, 346.
(28) Saby, C.; Jaffrezic-Renault, N.; Martelet, C.; Colin, B.; Charles, M.-H.; Delair, T.; Mandrand, B. *Sens. Actuators B* **1993**, *16*, 458.
(29) Gheorghiu, E.; Andreescu, D.; Oporanu, M.; Gheorghiu, M.; Cazacu, S.; Balut, C.; Ursu, A. *Songklanakarin J. Sci. Technol.* **2002**, *24* (suppl.: Membrane Sci., Technol.), 794.
(30) Dübendorfer, J.; Kunz, R. E.; Schürmann, E.; Duveneck, G. L.; Ehrat, M. *J. Biomed. Opt.* **1997**, *2*, 391.
(31) Compagnon, P. L.; Miocque, M. *Ann. Chim.* **1970**, *5*, 23.

(32) Chong, D. P. *Chem. Phys. Lett.* **1995**, *232*, 486.
(33) Chong, D. P. *J. Chem. Phys.* **1995**, *103*, 1842.
(34) Chong, D. P.; Hu, C. H.; Duffy, P. *Chem. Phys. Lett.* **1996**, *249*, 491.
(35) Bureau, C.; Chong, D. P. *Chem. Phys. Lett.* **1997**, *264*, 186.
(36) Bureau, C.; Chong, D. P.; Endo, K.; Delhalle, J.; Lecayon, G.; Le Moel, A. *Nucl. Instrum. Methods Phys. Res., Sect. B* **1997**, *131*, 1.
(37) Kranias, S.; Bureau, C.; Chong, D. P.; Brenner, V.; George, I.; Viel, P.; Lécayon, G. *J. Phys. Chem. B* **1997**, *101*, 10254.
(38) Bureau, C.; Chong, D. P. *J. Electron Spectrosc. Relat. Phenom.* **1998**, *88/91*, 657.
(39) Bureau, C. *Chem. Phys. Lett.* **1997**, *269*, 378.
(40) Bureau, C.; Chong, D. P.; Lécayon, G.; Delhalle, J. *J. Electron Spectrosc. Relat. Phenom.* **1997**, *83*, 227.
(41) Endo, K.; Kaneda, Y.; Okada, H.; Chong, D. P.; Duffy, P. *J. Phys. Chem.* **1996**, *100*, 19455.
(42) Endo, K.; Maeda, S.; Kaneda, Y. *Polym. J.* **1997**, *29*, 256.
(43) Lee, S.; Anzai, J.; Osa, T. *Sens. Actuators B* **1993**, *12*, 153.
(44) Anzai, J.; Hoshi, T.; Osa, T. *Chem. Lett.* **1993**, 1231.
(45) Pantano, P.; Morton, T. H.; Kuhr, W. G. *J. Am. Chem. Soc.* **1991**, *113*, 1832.