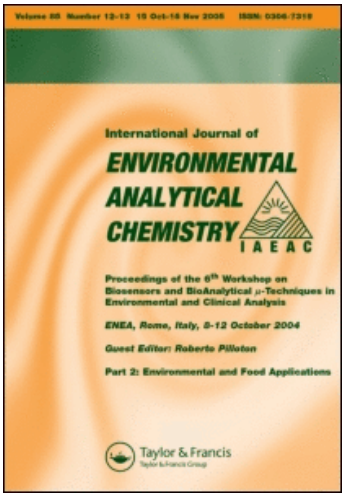

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Development of a Novel Dry Process to Functionalize Membranes for the Covalent Attachment of Antibodies Used in Immunochemical - Based Environmental Strip Tests

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DEVELOPMENT OF A NOVEL DRY PROCESS TO FUNCTIONALIZE MEMBRANES FOR THE COVALENT ATTACHMENT OF ANTIBODIES USED IN IMMUNOCHEMICAL – BASED ENVIRONMENTAL STRIP TESTS

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The controlled attachment of antibodies is a prime requirement for developing membrane-based immunoassays used in field tests. A novel dry process was developed to functionalize a nitrocellulose membrane by introducing amino groups on the surface, allowing an oriented covalent linkage of antibodies through their oxidized carbohydrate moieties. A non-equilibrium low pressure plasma of NH_3 and NH_3/H_2 mixtures was used to incorporate an average of 2.4 amine functions per nm^2 of porous surface. A mechanism of the functionalization process was proposed using on-line emission spectroscopy and mass spectrometry analysis to monitor the reaction process. The modification of the physical properties and the chemical composition of the treated material was also investigated by X-ray Spectrophotometric (XPS) analysis and scanning electron microscopy. The immobilization of radiolabelled antibodies resulted in an average binding capacity of 60 $\mu g/cm^2$. Their activity was retained as shown by an enzyme linked immunosorbent assay. This fast and reproducible process was demonstrated to be efficient in developing functionalized membranes suitable for environmental strip tests.

Keywords: Immobilization; antibodies; enzyme immunoassay; affinity membranes; environmental screening test; herbicide; phenylurea; isoproturon; plasma functionalization

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INTRODUCTION

Membrane based environmental enzyme immunoassays are always designed in the direct format where the antibodies are immobilized onto the solid support. This format has been reported to allow a better sensitivity of the resulting test[1,2]. A controlled covalent attachment of antibodies is usually preferred to random adsorption so as to achieve a better homogeneity in antibody coating. Furthermore, an oriented immobilization would allow the maximum availability of the antibody recognition sites. Procedures involving protein A or second IgG as orienting agents are considered costly and require cross-linking to allow an irreversible immobilization. Binding the antibodies via the carbohydrate moieties eliminates the risk of random orientation which is likely to occur when the multiple lysine groups present on the surface of the antibody are coupled to the aldehyde groups generally available on functionalized supports. The theoretical basis of binding oligosaccharide moieties of immunoglobulins to solid supports was reviewed by O'shannessy et al[3] who demonstrated that goat antibodies retained biological activity after binding to a hydrazide containing solid support.

Plasma processes have been widely employed for polymer surface treatment and modification resulting in cross-linking, surface cleaning, adhesion enhancement, wettability and functionalization. These processes are preferred to wet chemical treatments involving organic solvents or strong acid and bases. Plasma technology was recently used in relation to immunochemistry to generate thin plasma polymerized films over quartz crystals, incorporating amino groups for further antibody immobilization in an immunosensor application[4]. However, these functions were not used for a direct binding of antibodies exploiting the carbohydrate moieties but required different bifunctional and cross-linking agents for an oriented immobilization. Our interest was to incorporate amine functions on nitrocellulose membranes, initially used for antibody adsorption, to allow a covalent binding of oxidized antibodies. NH_3 plasma treatment was shown to be the most suitable for such a purpose since it was more reactive than N_2 plasma[5]. The modification of cellulose derivatives under plasma conditions was extensively studied. Substituted cellulose such as ethyl cellulose and hydroxyethyl cellulose showed enhanced reactivity compared to cellulose itself[6]. It was found that the concentration of the plasma-induced free radicals investigated by on line Electron Spin Resonance (ESR) was greater for the substituted material[6]. Nitrocellulose was chosen as a starting material for derivatization because of its polar structure and its ability to retain proteins[7].

Either amine or hydrazide containing membrane supports would be suitable for site directed immobilization of antibodies employed in environmental strip tests. This paper proposes a novel dry process to functionalize nitrocellulose mem-

branes by introducing amino groups on the surface using plasma chemistry. Different diagnostic tools were proposed to control this process in order to optimize its efficiency and reproducibility. The resulting supports were evaluated for their capacity to covalently bind antibodies via the oxidized oligosaccharide moieties and for their potential use in environmental immunochemical based methods.

EXPERIMENTAL

Apparatus

The bell jar plasma reactor was designed in the Laboratory of Plasma Processes (Ecole Nationale Supérieure de Chimie de Paris) and was used in several polymer treatment applications[8]. It has an assymetrical configuration of electrodes : a hollow electrode made of stainless steel and a grounded cylinder around which the polymer to be treated can be rolled (22 cm × 22 cm^2). This reactor is equipped with different on-line diagnostic tools as displayed in Figure 1. The pressure was measured with MKS 127A capacitance manometers and controlled by MKS 252 exhaust-throttle-valve controllers. The vacuum apparatus included a TPH 170 (Balzers) turbomolecular pump and a 2012 AC chemical pump. The electrical charactecteristics of the plasma discharge were monitored by a 9400 Lecroy digital oscilloscope at a sampling frequency of 100 MHz. Mass spectrometric analyses were performed with a Balzers QMG 420 quadrupole mass spectrometer. Optical emission spectroscopy monitoring was achieved using a Jobin-Yvon spectrometer. Radiolabelled compounds were monitored using an LKB Wallac 1211 Rack Beta device. A spectrophotometer UVICON 860 was used to monitor optical densities.

Materials

Commercially available UHP gases were used without further purification (purity of $NH_3 \geq 99.6\%$; $H_2 \geq 99.7\%$). Ovalbumin (OVA), Horseradish peroxidase (HRP) (isoenzyme c, type VI, product n° P 8375), Tween 20, 2,2'-azino-di[3-ethyl-benzothiazoline-6 sulfonic acid (ABTS), 4-Chloro-Naphthol were purchased from Sigma. The virgin nitrocellulose membranes were supplied by Schleicher & Schuell (product number 439 194). LC grade water was prepared by purifying demineralized water in a Milli-Q (MQ) filtration system (Millipore, Bedford, MA). Anti-isoproturon antibodies were obtained according to a protocol previously described[9]. The IgG fraction was isolated by affinity chro-

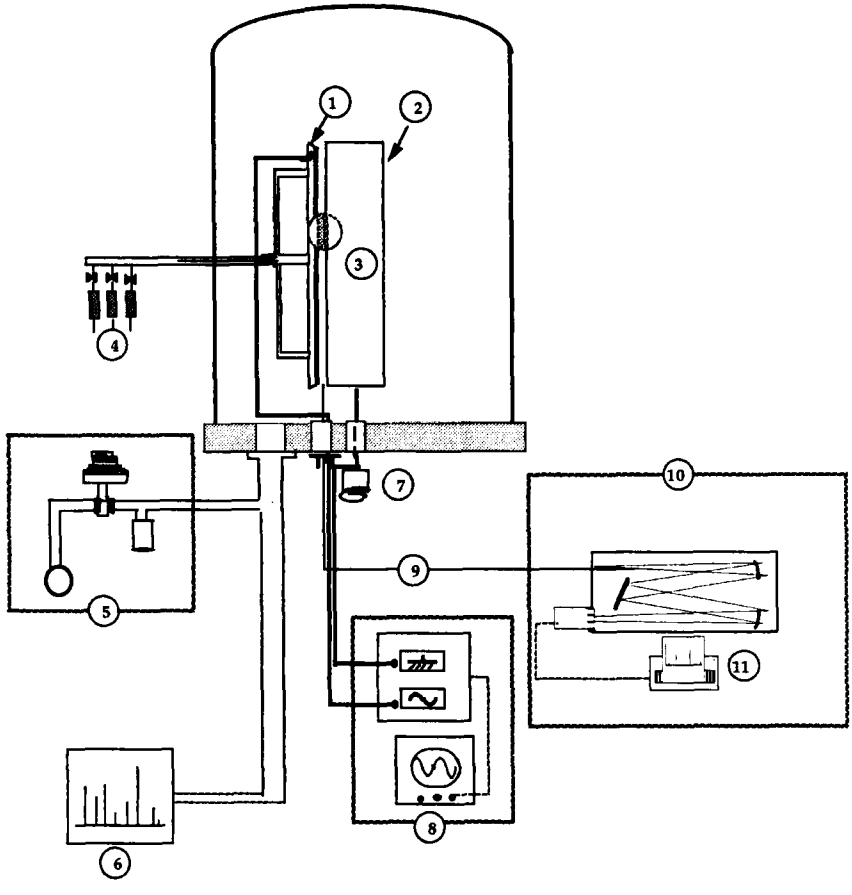


FIGURE 1 Bell jar plasma reactor used for nitrocellulose functionalization and on-line diagnostic tools. **1** Hollow electrode **2** Cylinder **3** Polymer film 22 × 22 cm^2 **4** Gas flow regulation, MKS 252 Mass Flowmeters + Gas inlet **5** Process Control :Pumping Unit, Pressure Measurements and Control, Chemical Pump **6** On-Line analysis using Mass Spectrometry **7** Motor **8** Excitation source and Power Control **9** Optical Fiber Transmission : $\lambda > 200$ nm **10** Monochromator THR 1000 Jobin-Yvon **11** Printer

matography using Avidchrom® gel manufactured by Unisyn Technologies (Hopkinton, MA, USA). Antibody and enzyme solutions were concentrated using Amicon Centriplus® 30 (exclusion size of 30 kD). Dialysis tubing (10 mm in diameter with 12000–14000 molecular weight cut off) was purchased from Spectnun Medical Industries Inc. (Los Angeles, CA, USA). Fast desalting columns (5 mL bed) were obtained from Pierce (Rockford, IL, USA) (product

n° 1852640). Radioactive materials such as $NaBT_4$ and ^{14}C HCHO (0,05 mCi, 53 mCi/mmol) were purchased from Amersham. Other chemicals were from Prolabo, Merck, or Fluka.

Buffers

Phosphate-Buffered Saline (PBS, adjusted to pH 7.4) contained 20 mmol of NaH_2PO_4 and 140 mmol of NaCl per liter of deionized water. PBS-OVA was obtained by adding Ovalbumin (OVA) in PBS at the concentration of 1 mg/mL. Washing buffer (PBS-T) consisted of 0.1 % Tween 20 (v/v) in PBS. Tris Buffered Saline (TBS, pH 7.5) contained 50 mmol of Tris, 150 mmol of NaCl per liter of deionized water. The substrate used for the antibody activity retention testing on the modified membranes consisted of 5 mg of 4 chloro-naphthol dissolved in 30 mL of MeOH/TBS (10/90 v/v) and 60 µL of 30 % H_2O_2.

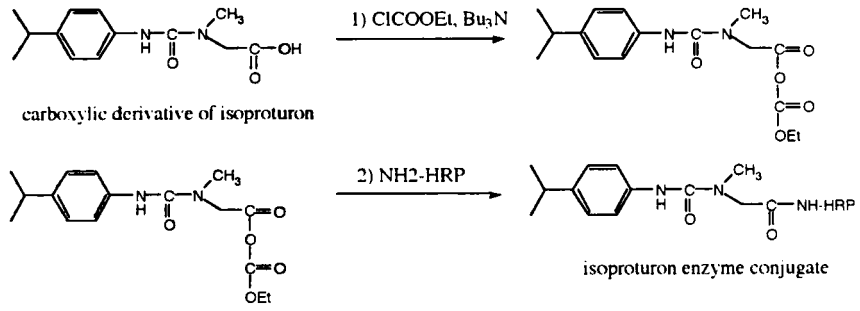


FIGURE 2 Reaction scheme of conjugation of horseradish peroxidase with isoproturon carboxylic derivative

Reactor operating and on-line analyses

The controlled vacuum system allowed an operating pressure of 100 Pa. The industrial 800 W excitation source had a frequency of 70 kHz and delivered a power of 8 W (I=50 mA ; U=1250 V). A total gas flow rate of 100 cm^3/min (pure NH_3 or NH_3/H_2 mixtures) was maintained during the experiments. Different NH_3 and NH_3/H_2 mixtures were investigated and different treatment periods were attempted varying from 2 to 10 seconds of exposition to the plasma discharge. The NH_3/H_2 mixture option was attempted as it was previously reported to enhance the total amount of grafted amine groups[10]. Plasma emission was collected by an optical fiber inserted into the discharge for emission spectroscopy analysis. Effluents were pumped through a capillary tube from the intere-

lectrode space, up to a leak valve installed on line with the mass spectrometer set at 70 eV. The pressure in the ionization chamber was held at 3.10^{-4} Pa by varying the leak rate through the sampling valve.

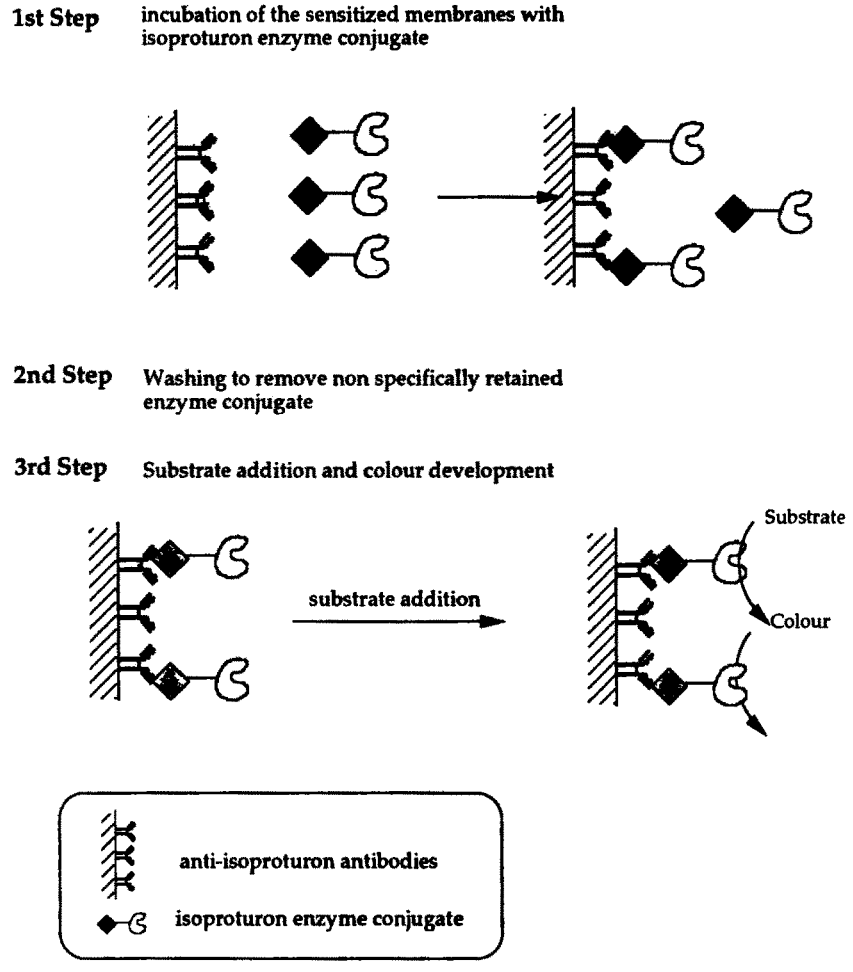


FIGURE 3 Principle of the test of activity of the covalently attached antibodies on the functionalized supports

Surface analyses

Surface morphologies of the nitrocellulose membranes were analyzed by scanning electron microscopy (SEM-JOEL T330 ; electron beam accelerating voltage

15 kV, electron gun-target distance : 40 mm ; vacuum : 10^{-6} mm Hg), from gold coated substrates. XPS analyses were performed at Laboratoire ITODYS (University of Paris VII) using a MgKα source (VG Scientific ESCALAB MK1) operating at a power of 200 W. The spectra were calibrated with respect to the Cls (C-C, C-H) signals set at 285.0 eV. Data was acquired using a Cybernetix Acquisition Data System.

Surface density measurments

^{14}C labelled formaldehyde ($H^{14}CHO$) has been used for a direct determination of the amine function density on the modified nitrocellulose membranes. This reagent has been diluted in a borate buffer ($Na_2B_4O_7$ 0.2M, PH 9) giving a specific radioactivity of 3.37 μCi/mmol. Different pieces of the plasma functionalized membrane rolls to be evaluated, having an accurately measured surface approaching 1 cm^2, were put into a multiwell cell culture plate allowing a simultaneous treatment of the different samples. Each membrane piece was pre-incubated in the borate buffer prior to the reaction with the $H^{14}CHO$ solution (0.5 mL / well). A reducing agent consisting of $NaBH_3CN$ (0.5M in 0.1N NaOH) was added at the level of 100 μL / well after 2 hours of reaction. The multi-well plate was gently agitated at a controlled temperature of 40°C for 2 hours. The membranes were then thoroughly washed with PBS-T until no significant residual radioactivity was detected in the washing solutions. The scintillation counting of the different samples was performed taking into account the response of a known quantity of the radioactive reagent (1 μL) deposited on a similar piece of nitrocellulose membrane. All the samples were monitored in triplicate and non-treated membrane pieces were also evaluated following the same procedure so as to check for the non-specific retention of the reagent.

Antibody immobilization and activity retention

The covalent attachment of antibodies onto the modified membranes was checked using tritiated IgGs. The IgG fraction of anti-isoproturon antibodies developed in earlier studies for analytical purposes[9], was isolated using a previously reported procedure[11] with the Avidchrom® gel. These antibodies were tritiated following a procedure described by Wilder et al.[12]. The obtained antibodies were first thoroughly dialyzed against a solution of NaCl (9 g/L) until no residual radioactivity was found in the external solution. The protein concentration of the resulting solution was adjusted to at least 4 mg/mL before oxidizing the oligosaccharide moieties using a solution of $NaIO_4$ (8mM in water) added at

the level of 50 µL per mL of antibody solution. The oxidized antibodies were then purified on a fast desalting column using a borate buffer (0.2M, pH 8.5) as an exchanging medium. The membranes to be evaluated for antibody covalent attachment were pre-incubated in the borate buffer prior to the reaction with oxidized antibodies, following the same procedure as that described for surface density determination and using the same kind of multiwell plates, allowing a systematic and simultaneous treatment of different samples. The reaction was allowed to proceed for 4 hours, at 25°C and under gentle agitation. $NaBH_3CN$ was added (0.3 mg/well) 2 hours after the beginning of the antibody reaction. Serial washing of the different wells was then performed with PBS-T and 1.5 mL of 8M urea was added to each piece of membrane in the multiwell plate. A further 12 hour incubation at room temperature was carried out before another washing cycle with PBS-T, until no residual radioactivity persisted in the washing solutions. The membranes were then put in a scintillation liquid before β emission counting.

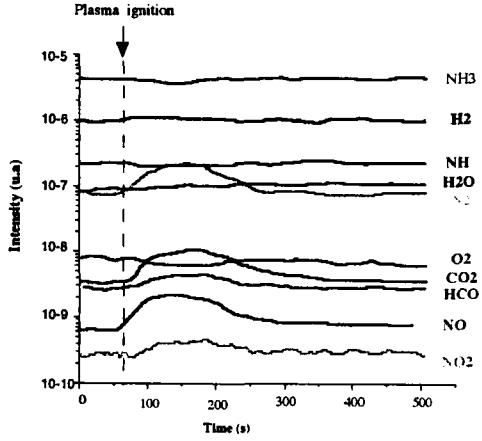


FIGURE 4 Evolution of neutral products in the plasma effluent products as a function of the treatment time before and after the plasma ignition. ($P=10^2$ Pa ; P_W = 10 W ; Q = 100 cm^3/min)

A second immobilization procedure was carried out to determine the antibody activity after covalent binding to the modified membranes. The starting solutions of non-radiolabelled oxidized antibodies were set at the concentration of 0.5 mg/mL in PBS-OVA and the immobilization occured following the same steps of reduction (100 µg of $NaBH_3CN$) and washing using pre-incubated pieces of functionalized membranes in a basic borate buffer. No desorption step with 8M urea was applied in this case and the sensitized membranes were kept at 4°C for a further use.

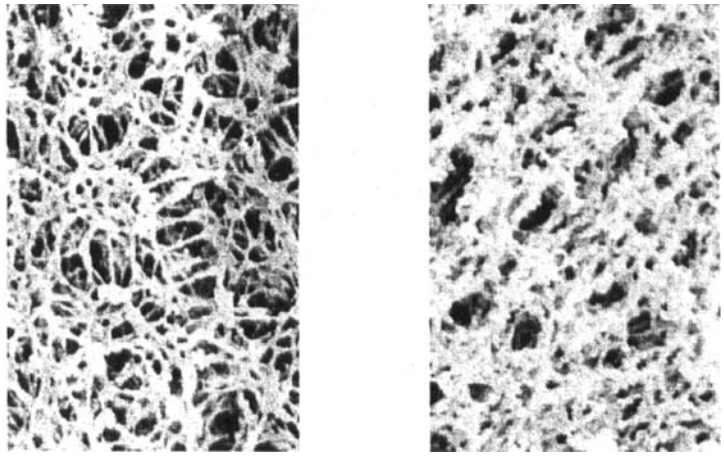
The activity of the immobilized antibodies (raised against isoproturon, a major phenylurea herbicide) was tested using an enzyme analogue of this compound. This analogue was obtained by reacting a previously reported carboxylic derivative of isoproturon[9] with horseradish peroxidase according to a thoroughly described procedure[13] and following the reaction scheme presented in Figure 2. This analogue was tested for the enzyme activity after the conjugation procedure using ABTS as a substrate and exhibited an activity of 22 UI (1UI : μmoles of ABTS transformed per minute by 1 mg of enzyme). The test for the persistance of activity of the covalently linked antibody is illustrated in Figure 3. The enzyme conjugate of isoproturon was set at the concentration of 10μg/mL and was added to the sensitized membranes (membranes incorporating covalently linked antibodies) at the level of 0.5 mL per piece of membrane. After a 30 min incubation at 4 °C and washing with PBS-T, the substrate solution (4-Chloro-Naphthol) and H_2O_2 were added. The colour development was stopped after 15 min, and the membranes were finally washed with distilled water. Non-sensitized functionalized membranes (blanks : membranes without immobilized antibodies) incubated in PBS-OVA were put in presence of the isoproturon enzyme conjugate to check for the absence of non-specific retention of the conjugate on the membrane and to exlude any possibility of false positives.

RESULTS AND DISCUSSION

On-line characterization of the plasma discharge

Despite the complexity of the reactions involved in plasma techniques, the diagnostic tools available within the reactor provided information about the reactive species involved in the derivatization mechanism. Emission spectroscopy spectra obtained in the wavelength range 300–500 nm allowed the identification of the probable species involved in the nitriding process. An intense peak located at 336 nm corresponded to the excited NH bi-radicals which can be easily chemisorbed and react with the generated radicals on the nitrocellulose membrane surface, to give rise to amine groups. Figure 4 shows the evolution of the neutral components of the plasma effluent as a function of the treatment time before and after plasma ignition (data generated using mass spectrometric analysis). The increase in intensity observed for the ion mass m/z=28 attributed to N_2 and that of m/z=2 attributed to H_2 can be correlated with the dissociation of NH_3 observed through the decrease of the ion mass m/z=17 (NH_3^+). The peaks at m/z=44 (CO_2 and m/e=18 (H_2O) correspond to commonly observed phenomena

(such as desorption of water, oxidation and decarboxylation) when hydrocarbon based substrates react in a plasma medium. However, the signal detected at m/e=30, after the plasma ignition and assigned to NO^+ was not noticeable with other non nitrogen containing polymers such as polypropylene, exposed to the same discharge. The increase in intensity of this peak can be only related to the involvment of the nitrate groups ($-ONO_2$) of nitrocellulose in the functionalization process.



(A) *(B)*

FIGURE 5 Scanning electron microscopy morphologies of native nitrocellulose (A) and plasma functionalized membranes (B)

Membrane modification and amine function incorporation

Scanning Electron Microscopy (SEM) observations have indicated different surface morphologies for virgin and plasma treated nitrocellulose substrates. As shown in Figure 5, a higher density was obtained for the plasma treated sample. Cross-linking recombinations occuring between the radicals generated on the activated surface of the membrane appear to be responsible for this effect. The porous state seems to be fully preserved after the functionalization process which maintained an acceptable specific capacity of antibody immobilization compared to non-porous material. Elemental analysis by XPS showed the influence of the NH_3 plasma treatment on the chemical composition of the nitrocellulose mem-

brane surface. Comparative analyses of the N1S spectrum are displayed on Figure 6. The intensity of the peak corresponding to the nitrate group at 405.9 eV decreased significantly on NH_3 treated membranes while a new peak centered at 398.5 eV and assigned to amino groups, increased. The contribution of imine functions detected at 400.3 eV seamed to be very weak. This was confirmed further by the specific reactivity of the introduced functions. The deduced relative surface atomic ratios O/C and N/C are presented in Figure 7 for both non-treated and treated membranes under several conditions involving different treatment durations and NH_3/H_2 mixtures. In all cases, the contribution of the $-ONO_2$ groups decreased in favour of the NH_2 functions. The N/C ratio remained unchanged after any NH_3 plasma treatment, whereas the O/C ratio showed marked decrease after the functionalization process. These observations, added to mass spectrometry analyses suggest that the major derivatization mechanism involves the abstraction of the nitrate groups leading to active radicals prior to the incorporation of amine species induced by NH_3 dissociation in the plasma. However, other mechanisms involving hydrogen abstraction from the membrane leading to other radicals should not be neglected. These modifications under plasma conditions have been widely observed for several organic polymers.

The ability to control the density of the incorporated functions is the most important challenge while developing a functionalization procedure. This is carried out by using a probe molecule which is allowed to selectively react with the primary amine functions. Such a way of measurement is preferred to any solid surface scanning, since it checks for the potential reactivity of the introduced species. ^{14}C radiolabelled formaldehyde enables a direct measurement of the reactive amine functions on the solid support as shown by the reaction scheme in Figure 8. Using this approach, a maximum surface density of 1630 molecules/nm^2 of planar surface was determined. Taking into account the porosity of the nitrocellulose membrane which increases the specific surface by 670 fold, and assuming its slight alteration after the plasma modification process, the calculated surface density reached 2.4 molecules/nm^2. This yield of function incorporation was obtained for a NH_3/H_2 (90:10) plasma treatment during 5 seconds and was similar to what was reported by Griesser et al. who developed deposited plasma polymerized films featuring between 0.25 and 2 amine functions/nm^2 on their surface[14]. There was no obvious direct correlation between the treatment time applied and the surface density in amine functions. However, the introduction of H_2 in the discharge resulted in a higher functionalization and a better homogeneity of the treated membrane (SD for NH_2 determination < 10%). This can be explained by the involvment of $H\cdot$ radicals in either saturating the reactive species preventing a further oxidation process or reducing other nitrogen containing functions in amine groups.

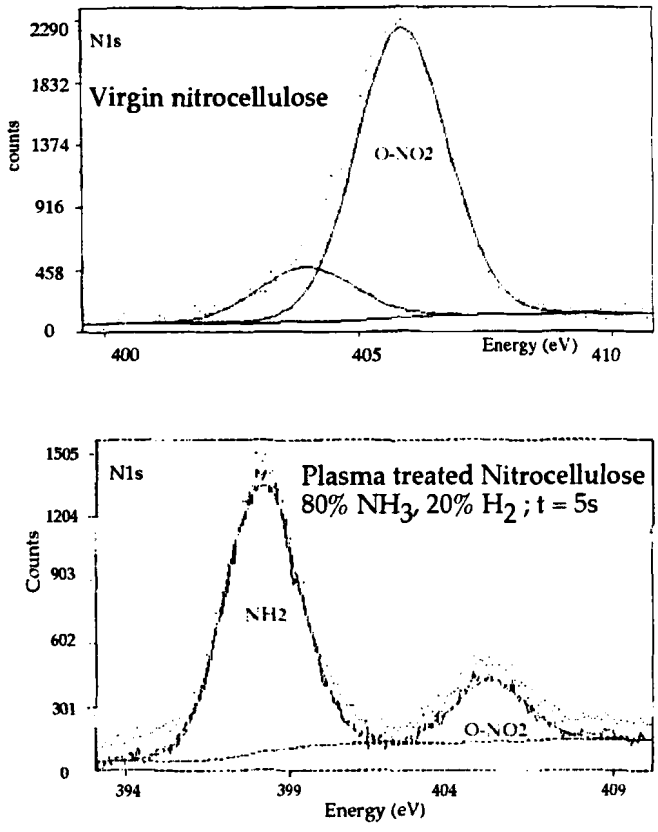


FIGURE 6 Comparative analysis of the N1S spectrum obtained by X ray photoelectron spectroscopy (XPS) for the virgin and the plasma functionalized membrane

Covalent immobilization of antibodies on the functionalized support and potential use for a membrane based enzyme immunoassay

In order to have an accurate measurement of the capacity of the functionalized membrane for covalent attachment of the oxidized IgGs, it was important to determine the non-specific adsorption properties. Different amounts of non-oxidized ^{3}H labelled IgGs were deposited on treated membranes and allowed to bind by passive adsorption. Treatment with 8M urea for 15 minutes removed an average of 94% of the deposited proteins. Extending the treatment time of the desorption agent to 12 hours did not result in further desorption from the treated membrane. Thus, a measurement of the amount of covalently bound oxidized

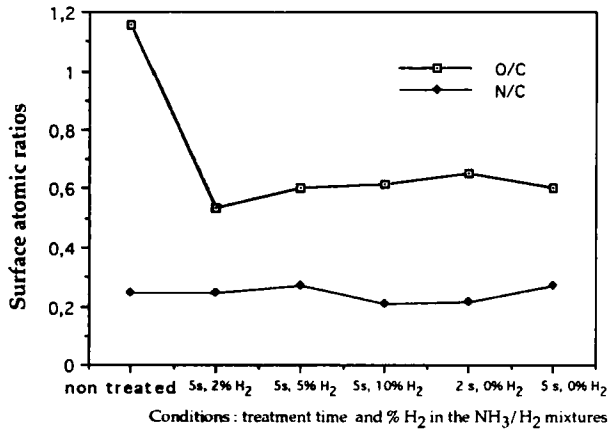


FIGURE 7 Comparative surface atomic ratios O/C and N/C for non-treated and plasma functionalized membranes under several treatment conditions

IgGs, after 8M urea treatment for 12 hours, may include an over-estimation of the linked proteins due to the adsorption contribution by no more than 6%. As shown in Figure 9-a, the immobilization capacity of oxidized antibodies on the functionalized nitrocellulose membrane averaged 60 $\mu g/cm^2$. The supports treated with gaseous mixtures including H_2, presented a better homogeneity when different batches of membranes were used for antibody immobilization. It was interesting to compare the function incorporation yield with the quantity of antibodies covalently immobilized. It appears from Figure 9-b that the latter amount does not increase proportionately with the number of available functions on the support, showing that the limiting factor for antibody immobilization is essentially due to steric hindrance related to the antibody size (150 kDa). A treatment duration as short as 2 seconds of native nitrocellulose would introduce enough reactive functions for antibody immobilization.

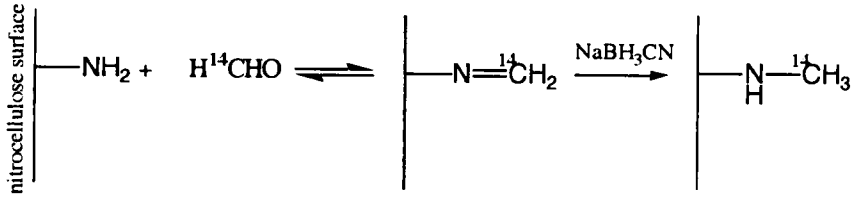


FIGURE 8 Reaction scheme allowing the determination of the surface density in amine functions using radiolabelled formaldehyde as a probe molecule

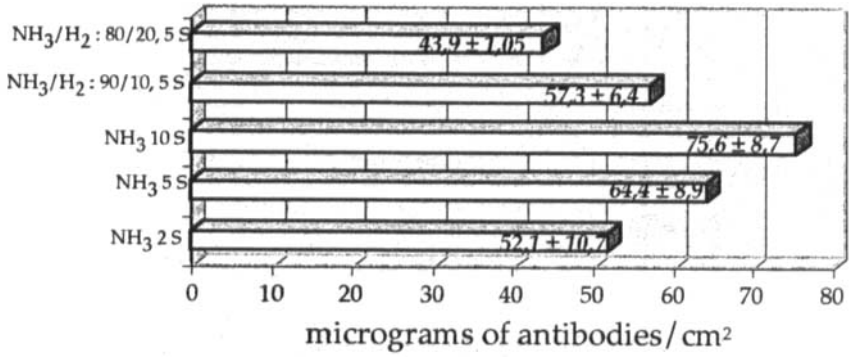


FIGURE 9-a Capacity of the functionalized membranes to covalently attach radiolabelled antibodies

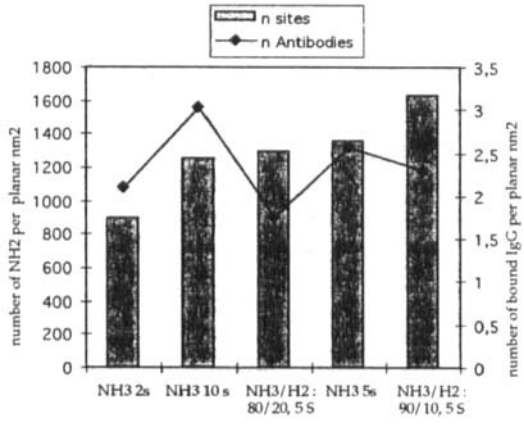


FIGURE 9-b Number of bound IgGs compared to the available amine functions after the plasma functionalization

The potential use of this novel process for antibody covalent attachment for membrane-based environmental immunoassays has been checked by studying the persistance of activity of the bound IgGs. Antibodies developed in previous studies[9] against isoproturon, a commonly used herbicide in Europe, were immobilized through their oxidized oligosacharide moieties on NH_3/H_2 (90:10) plasma functionalized membranes. The principle of the activity test was presented in Figure 3. The sensitized membranes were allowed to react with an isoproturon enzyme conjugate. As shown in Figure 10, all the tested membranes incorporating antibodies gave a positive enzymatic reaction, indicating the reten-

tion of antibody activity after the immobilization. The possibility of a false positive response, due to non-specific adsorption of the enzyme conjugate was not significant, since it was not retained on non-sensitized membranes after the washing steps. These results indicate the potential of covalently immobilizing antibodies onto plasma-activated polymer surfaces for use as supports for direct immunoassays and biosensors.

(A) (B)

FIGURE 10 Test for the persistance of the activity of the immobilized anti-isoproturon antibodies on the plasma functionalized membranes. (A) Non sensitized membranes checked for the absence of non specific retention of the isoproturon enzyme conjugate. (B) Sensitized membranes incubated with isoproturon enzyme conjugate

CONCLUSION

Plasma technology was exploited to develop a new dry functionalization process for nitrocellulose membranes. A major functionalization mechanism involving the removal of the nitrate groups prior to the incorporation of amine species generated from ammonia decomposition in the discharge, was identified. The treatment conditions led to a homogenous functionalization and covalent incorporation of antibodies. Such a facile and reproducible process is adaptable for a reliable industrial production of functionalized membranes. The potential use of these modified supports in immuno-analytical procedures has been demonstrated by the full retention of the biological activity of covalently immobilized anti-herbicide antibodies. This is to our knowledge, the first attempt to devote a plasma functionalization process for the set-up of environmental immuno-chemical techniques.

Acknowledgements

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