THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

Ammonia Plasma Treated Polyethylene Films for Adsorption or Covalent Immobilization of Trypsin: Quantitative Correlation between X-ray Photoelectron Spectroscopy Data and Enzyme Activity

Mahsa Ghasemi,[*,†,‡,§,∥] Michel J. G. Minier,[*,§,∥] Michaël Tatoulian,[†,‡] Mohamed M. Chehimi,[⊥] and Farzaneh Arefi-Khonsari[†,‡]

[†]Chimie ParisTech, Laboratoire de Génie des Procédés Plasma et Traitements de Surface (LGPPTS), EA 3492, 75005 Paris, France

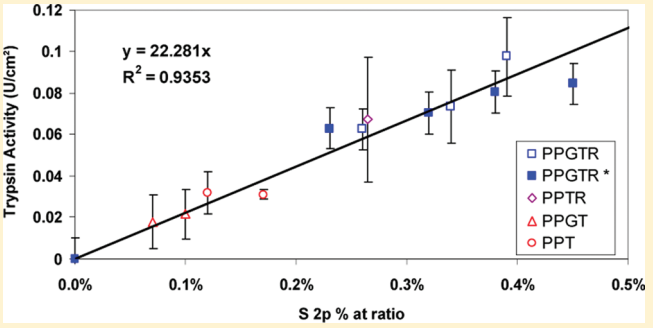
[‡]UPMC Univ Paris 06, 75005 Paris, France

[§]Chimie ParisTech, Laboratoire Charles Friedel (LCF), 75005 Paris, France

[∥]CNRS, UMR 7223, 75005 Paris, France

[⊥]Interfaces, Traitements, Organisation & Dynamique des Systèmes (ITODYS Lab), University Paris Diderot & CNRS, 15 rue Jean-Antoine de Baïf, 75013 Paris, France

ABSTRACT: The ammonia plasma process was used for generating reactive groups, particularly primary amine functions on the surface of polyethylene (PE) films, to immobilize the enzyme trypsin. The attachment of the enzyme was achieved by directly applying an aqueous solution of trypsin to the plasma-activated surface or by using glutaraldehyde as a chemical linker. In both cases, the utilization of sodium cyanoborohydride efficiently stabilized the immobilization. The surfaces were analyzed by X-ray photoelectron spectroscopy (XPS) and enzymatic activity measurements. Active trypsin was successfully immobilized on the surface with a mean activity of 0.09 ± 0.02 U/cm^2. The study of the stability of the immobilized enzyme during repetitive assays showed that some activity could be maintained during several months. An original quantitative correlation between the immobilized enzyme activity and the XPS signal intensity of the S 2p electrons present in the sulfur-containing amino acid residues was evidenced.



INTRODUCTION

The surface functionalization of various materials by bioactive compounds has seen a rapid development in the last few decades and has found applications in multiple areas such as biomedical, bioanalysis, asymmetric synthesis, bioprocess engineering, antibacterial treatment, and food packaging.[1−10] Typically, the required specifications for the application of such materials are: (i) chemical inertness of the support in the conditions used for the application, (ii) maximization of the biological activity after grafting, and (iii) stability of the functionalized surfaces over time and during repeated utilization. In addition, there is an increasing interest for surface treatment processes which minimize the use of solvents or hazardous chemicals, in agreement with the principles of green chemistry.[11]

Due to their chemical inertness and current commercial availability, polymers such as polyethylene (PE), polytetrafluoroethylene, or polystyrene are suitable platforms for the immobilization of biomolecules. Generally, such polymers require an initial preparation step which consists of drastic physical or wet chemical treatments, to generate hydrophilic and reactive functional groups on the surface.[12] Among the main methods reported, the use of plasma processing is rapidly increasing since it is a dry process which allows the functionalization of the outermost surface layer of the material with a great variety of chemical functions.[13−19] Different plasma techniques are currently used such as plasma polymerization of organic precursors[20−23] or by using nonpolymerizable plasmas such as nitrogen or ammonia which lead to the grafting of amine groups on the surface.[24−27] More recently, plasma immersion ion implantation showed its potential for protein immobilization.[28,29]

Many studies dealing with enzyme immobilization after plasma processing of surfaces have been published, the enzymes being generally tethered to the supporting substrate via a chemical linker. However, only few studies report the measurement of the immobilized enzyme activity: for example, with plasma processing using nonpolymerizable gases, the following works referring to α-chymotrypsin,[30] papain,[31] horseradish peroxidase,[32,33] oxalate oxidase,[34] glucose oxidase,[26] lysozyme,[35] and trypsin[27] can be mentioned, while plasma polymerization was used for the following enzymes (glucose oxidase,[36,37] glucose isomerase,[20,21]

Received: May 3, 2011
Revised: July 13, 2011
Published: July 19, 2011

dx.doi.org/10.1021/jp204097a | J. Phys. Chem. B 2011, 115, 10228–10238

invertase,[7] and trypsin)[38] and was combined with photografting for the immobilization of trypsin,[14] lipase,[39] and glucose oxidase.[40] In addition, to further minimize the number of wet chemical steps, a recent approach has been reported, consisting of the direct covalent attachment of enzymes, (horseradish peroxidase, catalase, and a cellulase mixture), on plasma-treated surfaces, without the addition of a chemical linker.[23,29]

The choice of the bond to be used for the immobilization of enzymes on a surface depends on the physicochemical characteristics of the protein and of the material and also on the focused application. Direct adsorption on the supporting substrate appears to be the simplest method but in some cases can lead to extensive denaturation of the enzyme[41] if the polar and hydrophobic interactions are not controlled. In contrast, cellulases have been shown to retain their activity level when adsorbed on—or directly covalently linked to—hydrophilic surfaces.[29,42] The utilization of chemical linkers and spacer-arms, ensuring a covalent attachment, a greater mobility of the enzyme, and a better accessibility to the active site,[30] is generally preferred when stability and long-term utilization are desired. However, to the best of our knowledge, there is no report on the comparison of the initial activity and stability of an enzyme directly adsorbed on or covalently attached via a chemical spacer-arm to an NH_3 plasma-processed surface over repeated assays and storage periods.

Besides enzymatic activity measurements, various spectroscopic methods are frequently used to qualitatively monitor the modification of the functionalized surfaces. X-ray photoelectron spectroscopy (XPS) is a reliable method which provides information about the chemical species comprising the successive layers arising from each treatment step, including the bound enzyme. However, very few studies[38,43] make use of sulfur XPS data to characterize the presence of proteins and still less give a quantitative correlation between the S 2p peak and the surface protein coverage.[44] To the best of our knowledge, no one has correlated XPS data with immobilized enzymatic activity.

Several reports have recently focused on the immobilization of trypsin to suppress cell adhesion in cerebral shunts[45] or to design microbioreactors for the digestion of proteins before further peptide mapping.[3,46−49] Due to the specific proteolytic properties of trypsin, the identification of the initial protein becomes possible through the analysis of peptide fragments, for example, by coupling chromatography and mass spectrometry.[50,51]

This paper reports on the immobilization of trypsin on an ammonia plasma treated polyethylene film, by direct adsorption and through covalent attachment with glutaraldehyde. The comparison of the two methods in terms of initial enzymatic activity and its behavior over time and successive assays has been achieved. A standardized washing method and a meticulous enzymatic assay protocol ensured that only immobilized activity was measured.

XPS analysis was performed to characterize each step of surface treatment, and an original quantitative correlation between immobilized activity and XPS data of sulfur was demonstrated.

MATERIALS AND METHODS

1. Materials. Low density (0.92 $g \cdot cm^{-3}$) polyethylene film (ref ET 311201) was obtained from Goodfellow (Lille, France) and used as received. Commercially available NH_3 gas from Air Liquide (Paris La Défense, France) was used for plasma treatment (purity ≥99.96%). Glutaraldehyde 25% solution grade II (ref G6257), *N*-α-Benzoyl-L-Arginine Ethyl Ester (BAEE) (ref B4500), sodium cyanoborohydride 95% (ref 156159), guanidine (amino methanamidine) (ref 177253), trypsin from bovine pancreas (ref T1426), and Tween 20 polyoxyethylene-sorbitan monolaurate (ref P1379) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

2. Plasma Reactor. A bell jar type reactor with an asymmetric blade-type electrode configuration was used for the plasma treatment. This technique is described in more detail elsewhere.[52] One electrode is a hollow stainless steel blade which serves also for the introduction of the gases. It faced a grounded stainless steel cylinder (second electrode), onto which the polymer film (22 × 22 cm^2) to be treated was placed. The gases were introduced through mass-flow controllers (MKS), and the pressure was monitored with a capacitive gauge. The main chamber was evacuated by a TPH 170 (Balzers) turbo molecular pumping system, and a base pressure of 10^{-3} Pa was obtained. The operating pressure was maintained at 150−200 Pa by a 2012 AC rotary pump. Optimized conditions for ammonia, defined in preliminary studies, were p = 1.5 mbar, Q_{NH3} = 175 cm^3/min (sccm), P = 40 W, and t = 2 s (plasma duration time). Further experiments done on treated PE surfaces were performed on disk samples (2.4 cm diameter, 4.52 cm^2) which were punched out from the 22 × 22 cm^2 treated films.

3. Surface Characterization. Contact angle measurements. Static contact angle measurements using water (sessile drop) were performed with the help of an image processing system. The reported values correspond to the average of three measurements, performed on different parts of the samples.

XPS. X-ray photoelectron spectra were recorded using a Thermo VG Scientific ESCALAB 250 system fitted with a microfocused, monochromatic Al Kα X-ray source (1486.6 eV) and a magnetic lens which increases the electron acceptance angle and hence the sensitivity. An X-ray beam of 650 μm size was used (15 kV × 200 W). The pass energy was set at 150 and 40 eV for the survey and the narrow regions, respectively. An electron flood gun was used, under a 2 × 10^{-8} mBar partial pressure of argon, for static charge compensation. These conditions resulted in a negative but uniform static charge.

Advantage software version 2.2 was used for digital acquisition and data processing. Spectra were calibrated against the main C−C/C−H C 1s component set at 285 eV. The surface compositions, in atomic ratios, were determined by considering the integrated areas of the C 1s, S 2p, O 1s, and N 1s core-level peaks and the respective manufacturer's sensitivity factors.

4. Glutaraldehyde Activation of the Plasma Aminated Surface. The activation by glutaraldehyde (GA) was done by the incubation of the plasma-treated PE disks (PP) in a solution of 5% glutaraldehyde in ethanol/water (4:1 v/v) for 3 h at room temperature.[53] The surfaces were washed successively in absolute ethanol, water, and finally ethanol.

5. Immobilization of Trypsin onto Plasma-Treated PE Disks Activated with Glutaraldehyde. A solution of bovine pancreas trypsin (1 g/L in 0.1 M phosphate buffer pH 7.6) was prepared. Plasma-treated PE disks activated by GA (noted PPG) were placed in separate glass reactors and soaked overnight in 4 mL of the solution of trypsin at 4 °C under shaking, leading to the PPGT samples (Plasma-treated Polymer activated by Glutaraldehyde and put in contact with Trypsin). Generally, the reduction of the imine bonds was performed by reacting 0.1 M sodium cyanoborohydride ($NaCNBH_3$) on PPGT for 35 min to form stable secondary amines (disks designated as PPGTR, i.e., PPGT Reduced). Then the disks were washed according to the

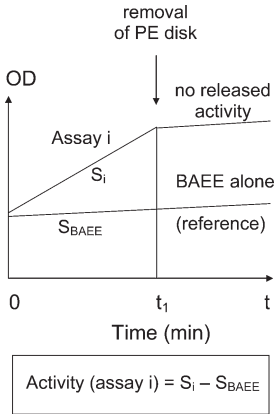


Figure 1. Measurement of immobilized trypsin activity. S_i and S_{BAEE} are, respectively, the slope of variation (OD per min) measured with the sample i and with BAEE solution alone as reference.

following five-step protocol: (i) rinsing in phosphate buffer (0.1 M; pH 7.6) for 30 min, (ii) second rinsing in new phosphate buffer for 30 min, (iii) washing solution for 10 min, (iv) rinsing in phosphate buffer for 10 min, and (v) last rinsing in new phosphate buffer for 10 min. For the third step, different washing solutions were used: 0.1 M phosphate buffer, Tween 20 (0.1 mL of Tween 20 in 100 mL of 0.1 M phosphate buffer), and a 2 M aqueous solution of guanidine−HCl. Agitation was maintained throughout the rinsing process by a reciprocal shaker. PE disks were finally dried by air.

6. Measurement of Trypsin Activity. The activity of the trypsin immobilized on the PE disks (4.52 cm^2 area) was determined by kinetic measurement of *N*-α-Benzoyl-L-Arginine Ethyl Ester (BAEE) hydrolysis. A solution of 0.25 mM BAEE was prepared in 67 mM phosphate buffer at pH 7.6. At time t_0, 3 mL of this solution was added to screw cap glass tubes, each containing a PE disk. Incubation was carried out for 2−3 h (noted time t_1 in Figure 1) at room temperature, a good contact between the PE surface and the BAEE solution being ensured by continuous agitation on a reciprocal shaker. During the incubation, the hydrolysis of the ester (formation of *N*-α-Benzoyl-L-Arginine) was monitored by measuring the absorbance of the BAEE solution at 253 nm on a spectrophotometer Uvikon 860 (Kontron Instruments). At each sampling time, 1 mL of BAEE solution was taken from each tube and replaced after the rapid measurement of its absorbance on the spectrophotometer. The disks were then removed from the glass tubes (at time t_1), and the absorbance of the remaining BAEE solution was monitored during a second phase of the assay (approximately 2 h). The absence of significant BAEE hydrolysis during this second phase of the experiment was used to prove that the enzymatic activity measured during the first phase ($[t_0, t_1]$) was actually due to immobilized trypsin and that no significant enzyme release was evidenced during the enzymatic reaction in the BAEE solution.

The enzymatic activity immobilized on the surface of a PE disk was calculated from the difference between the rate of optical density increase ($d(OD)/dt$) of the test sample during incubation time $[t_1 - t_0]$ and that of the BAEE solution alone (as reference), which could undergo a slight variation with time. According to Sigma,[54] one enzymatic activity unit (S&T unit) is the quantity of enzyme which produces a $\Delta(OD)_{253\ nm}$ of 0.001 per minute with a BAEE substrate at pH 7.6 and at room temperature (25 °C) in a reaction volume of 3.2 mL. We applied the formula below to take into account the different reaction volume (3 mL) used in our experiments

$$\text{Activity} = \frac{3}{3.2} \times 10^3 \times \frac{d(\text{OD})}{dt}$$

7. Storage Stability and Reusability of Immobilized Enzyme. The stability of trypsin immobilized on PE disks was studied by periodically repeating measurements of its activity after various storage times, up to more than 6 months. After each enzymatic activity assay, the PE disks were kept in a solution of 0.1 M phosphate buffer pH 7.6 and stored at 4 °C.

RESULTS AND DISCUSSION

The immobilization of trypsin on plasma-functionalized PE disks has been performed by using glutaraldehyde as a homobifunctional cross-linking agent and as a spacer-arm between the aminated PE support and the enzyme (lysyl residues). Generally, a reduction step by sodium cyanoborohydride was achieved, to transform hydrolyzable imine linkages into stable secondary amines (PPGTR samples). Some experiments with the direct adsorption of trypsin on the plasma-treated PE disk were also performed (PPT samples) possibly followed by sodium cyanoborohydride reduction (PPTR samples) (cf. Scheme 1).

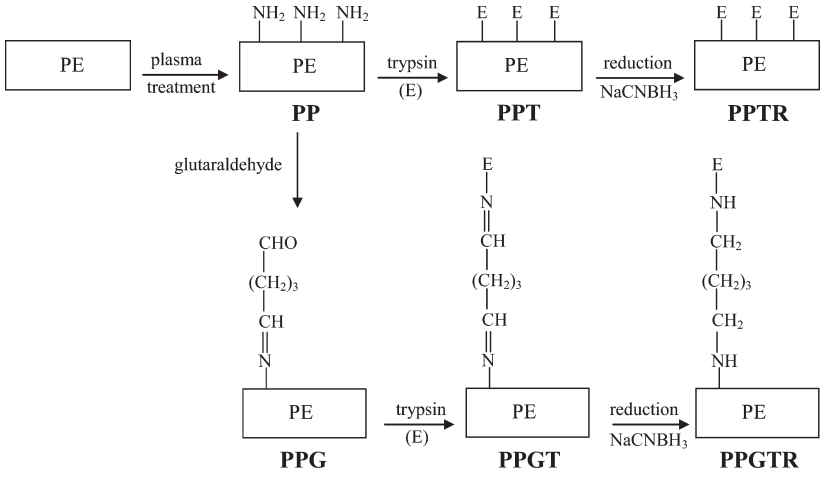
1. Physico-Chemical Characterization of Plasma-Treated PE Surfaces (PP). Ammonia plasma was selected to generate primary amine functions on the surface of low density polyethylene (PE) films. Three types of surface characterization were achieved: water contact angle, XPS analysis, and quantitative chemical derivatization.

The hydrophobic/hydrophilic character of the surface of the PE disks was evaluated via water contact angle (WCA) measurements. Results show that the WCA was 90° for nontreated PE (hydrophobic), whereas it reached 54° ± 2° for treated PE, which is in agreement with the formation of more hydrophilic oxygen and nitrogen-containing functional groups on the surface, as shown below. The knowledge of the WCA is important for evaluating the efficiency of the plasma treatment, but still more to optimize the different grafting steps. Indeed it has been shown that there exists a strong relationship between the hydration or the wettability of a surface and the aggregation or the successful immobilization of enzymes onto it.[29,42,55] Due to the hydrophilic character of trypsin, the increasing wettability of plasma-treated PE films is, a priori, favorable to its further immobilization.

The atomic composition of the modified polyethylene samples after ammonia plasma treatment (PP) was measured by X-ray photoelectron spectroscopy (XPS) and compared with that of nontreated PE samples (PE) (Table 1). The spectrum of untreated PE shows only the presence of C and O, with a very low percentage of oxygen (O/C = 0.007) due to external contamination or to the migration toward the surface of residual additives. On the contrary, in all plasma-treated samples the nitrogen uptake of the surface leads to a N/C ratio of 11−12 atom %. In all cases, surface concentration of oxygen after treatment (O/C = 0.064) is higher than for untreated PE (O/C = 0.007). The presence of oxygen on plasma-treated surfaces can be explained either by post oxidation or by the presence of residual air in the plasma reactor, which can be excited and therefore react with the surface.

XPS peak fitting (Figure 2) allows the determination of the nature and concentration of the incorporated groups. In

Scheme 1. Process of Trypsin Immobilization onto PE disks[a]



[a] E represents the enzyme trypsin.

Table 1. High-Resolution XPS Data for Successive Treatment Steps of Polyethylene Surface Samples

		C 1s			N 1s			S 2p			O 1s	N/C[b]	O/C[b]	S/C[b]
		assignment	BE[c]	%	assignment	BE[c]	%	assignment	BE[c]	%				
PE[a]	atom %		99.31%			0%			0%		0.69%	0	0.007	0
		C−C, C−H	285.0	96 ± 2										
		C-O	286.2	4 ± 2										
PP	atom %		84.34%			10.28%			0%		5.38%	0.12	0.064	0
		C−C, C−H	285.0	82.4 ± 2	C−N	399.1	41 ± 4							
		C−N, C-O	286.2	10.2 ± 2	O═C−NH2	400.1	59 ± 4							
		C═O	287.5	5.1 ± 2										
		O═C−N	288.7	2.3 ± 2										
PPG	atom %		88.84%			5.58%			0%		5.58%	0.063	0.063	0
		C−C, C−H	285.0	92 ± 2	C═N	398.9	37.5 ± 4							
		C═N	287.0	5 ± 2	O═C−NH2	400.1	62.5 ± 4							
		O═C−N, H−C═O	288.0	3 ± 2										
PPGT	atom %		76.34%			6.35%			0.07%		17.23%	0.083	0.23	0.001
		C−C, C−H, C−S	285.0	80 ± 2	C═N	398.8	12 ± 4							
		C−N, C-O	286.3	9 ± 2	O═C−NH2	400.1	88 ± 4							
		C═N	287.0	5 ± 2										
		O═C−N	288.3	6 ± 2										
PPGTR	atom %		74.23%			8.83%			0.63%		16.31%	0.12	0.22	0.0085
		C−C, C−H, C-S	285.0	64 ± 2	C−N	399.1	11 ± 4	S−S, C−S,	163.8	45 ± 2				
								C−S−R	165.2	22 ± 2				
		C−N	285.8	17 ± 2	O═C−NH2	400.1	78 ± 4	SO_x	167.8	22 ± 2				
									169.1	11 ± 2				
		C−O	286.7	9 ± 2	NH_3^+	401.1	11 ± 4							
		O═C−N	288.0	8 ± 2										
		COOH	288.8	2 ± 2										

[a] Nontreated polyethylene. [b] Atomic ratio. [c] Binding energy (eV).

the literature, one can find many divergences for values indicating binding energies of the same species. In the case of our samples, four peaks could be generated in the C 1s region: 285.0, 286.2, 287.5, and 288.7. The chemical assignment of these peaks is C−C/C−H (C_1), C−N/C−O (C_2), C═O (C_3), and O═C−N (C_4), respectively. After treatment, we noticed that

dx.doi.org/10.1021/jp204097a |J. Phys. Chem. B 2011, 115, 10228–10238

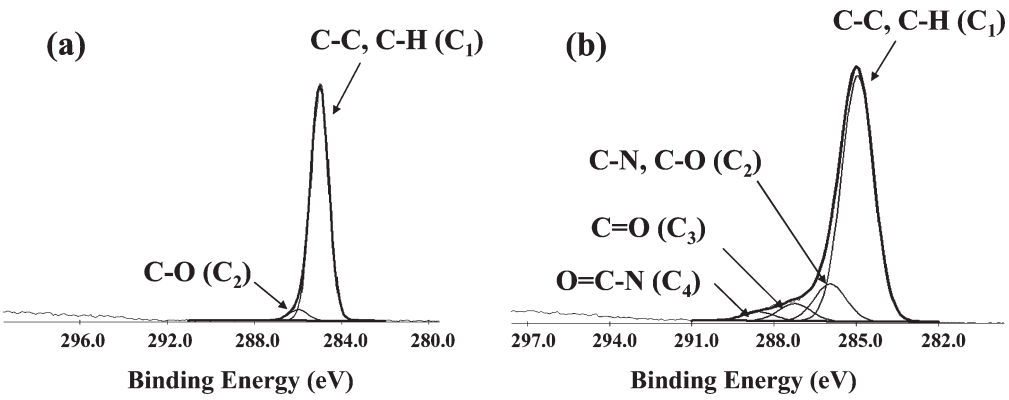


Figure 2. High-resolution XPS C 1s spectra of (a) nontreated PE and (b) PE after treatment by ammonia plasma (PP).

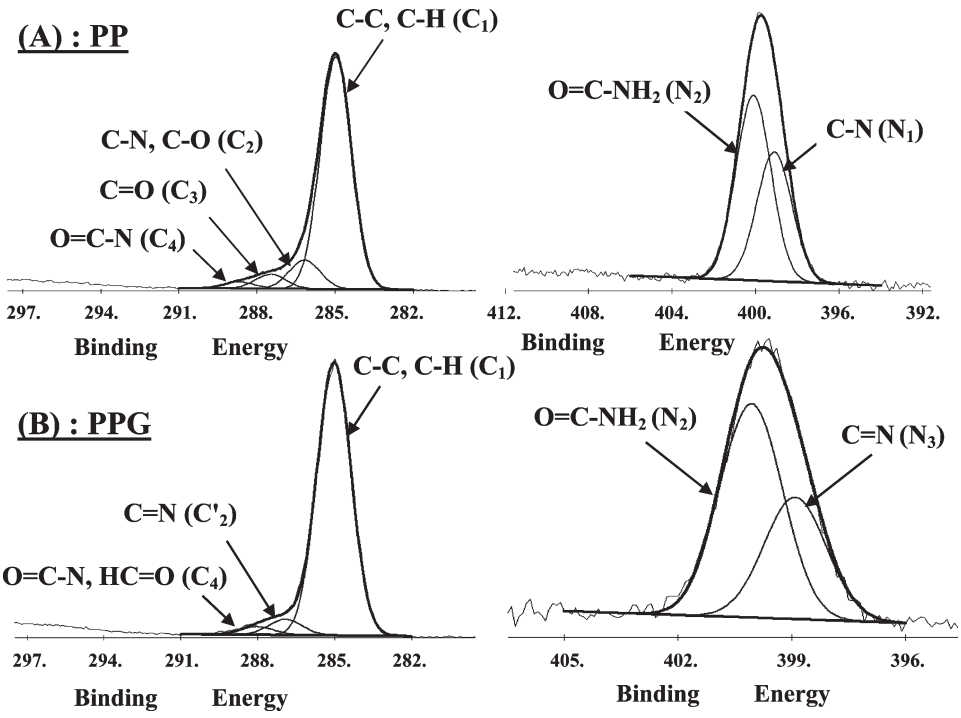


Figure 3. High-resolution XPS C 1s and N 1s spectra of (A) PE after treatment by ammonia plasma (PP) and (B) after incubation with glutaraldehyde (PPG).

the area of the C_1 component decreases, while the area of the C_2, C_3, and C_4 peaks increases. The area of the C_2 group which contains amine functions was always superior to those of C_3 and C_4.

The N 1S spectrum of PP (Figure 3) shows the presence of amine (peak N_1 at 399.1 eV) and amide functions (peak N_2 at 400.1 eV) on the surface of treated PE. One can note that the N 1s peak consists of 59% amide and 41% amine. However, the XPS analysis did not allow us to clearly separate the different classes of amines.

Finally, different chemical derivatization methods have been used for the quantitative determination of primary and secondary amines. The principal results, described elsewhere,[56] show that about 1.3 molecules per nm^2 of primary amine and 3.9 molecules per nm^2 of secondary amine were grafted on the plasma-treated PE surface.

2. Physico-Chemical Characterization of the Plasma-Activated Surface after Reaction with Glutaraldehyde. As described in Scheme 1, glutaraldehyde has been used to covalently link proteins to the aminated surface of plasma-treated disks since it readily condenses with primary amines to yield Schiff bases, providing a convenient way to conjugate two amine-containing molecules together. The five carbon atom chain length of the cross-linker may also allow an increase in the distance between the protein and the PE surface.

Figure 3 shows the C 1s and N 1s spectra before and after reaction of GA on PP. The C 1s spectra exhibit both a dominating C−C and C−H (C_1) contribution at 285.0 eV. The appearance on PPG of a new contribution at 287.0 eV (C'_2), assigned to carbon in the intramolecular imine bonds C═N, testifies to the coupling reaction of glutaraldehyde with the primary amines generated by plasma on PP. At the same time, the amine function peak (C_2) disappears on PPG due both to the reaction of primary amines and to the masking of the secondary and tertiary amines by the GA layer. A weak contribution at 288.0 eV, likely due to

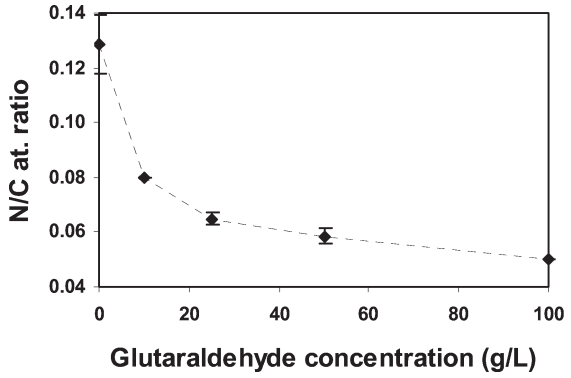


Figure 4. Glutaraldehyde grafting on PP (plasma-treated PE) disks: effect of the GA concentration on N/C atomic ratio.

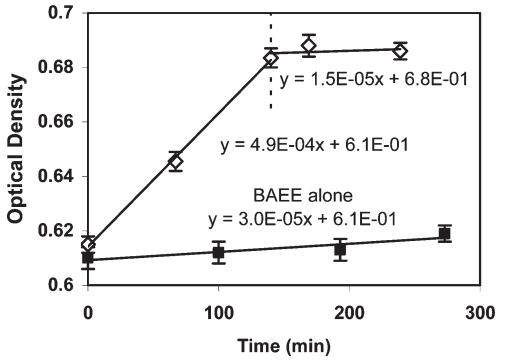


Figure 5. Kinetic measurement of immobilized trypsin activity. The dotted line represents the time (140 min) of removal of the PE disk: the second part of the experiment shows that no active enzyme release could be detected in the BAEE medium during the assay.

free aldehyde functions,[5] is also visible. These interpretations were confirmed by the observation of the N 1s spectra: binding of GA is accompanied by the disappearance of the amine component of the N 1s peak (N_1), the appearance of a peak at 398.9 eV (N_3) attributed to imine bonds, and the decrease of the N/C atomic ratio from 0.12 to 0.063 (Table 1) due to the addition of a glutaraldehyde layer.

On the PPG sample, it can be noted that both N/C and O/C ratios present the same value, which is theoretically the case in a GA molecule with one free aldehyde function and linked to the surface by the other end through an imine bond (Scheme 1). However, the lower value of N/C═O/C = 0.063 on PPG, as compared to the ratios in a single glutaraldehyde molecule fixed to the surface by an imine function (N/C═O/C = 0.2) is likely due to the presence of polyethylene carbon atoms present under the upper layer of GA and detected by XPS.

The influence of glutaraldehyde concentration on the coupling reaction with PP primary amines was also evaluated by XPS, for GA concentrations ranging from 0 to 100 g/L (0−10% v/v). As can be seen in Figure 4, the N/C atomic ratio decreases significantly with glutaraldehyde concentration up to 20−25 g/L and reaches a constant level from 50 g/L, suggesting that it corresponds to a saturating concentration for the PP surface. Therefore, a 50 g/L concentration value was chosen for the following assays of enzyme immoblization, in agreement with other results reported in the literature.[57]

The stoichiometric transformation of amine functions in imines, leading to the binding of a GA molecule, has been indirectly confirmed by the quantification of the free aldehyde functions on the PPG surface, through chemical derivatization and colorimetric measurements.[56] In this previous work, the same density of 1.3 ± 0.1 primary amine functions per nm^2 on PP, and free aldehyde functions on PPG, has been determined, by four independent analytical methods. This result is also in agreement with the saturation curve observed in Figure 4: for a sufficient concentration of the incubation GA solution, a stoichiometric primary amine + GA reaction takes place, with the saturation of all primary amine sites, without "bridges" linking two amines at the same GA molecule.

3. Immobilization of Trypsin on Polyethylene Disks. *3.1. Validation of the Rinsing Protocol.* To evaluate the presence of active enzyme covalently immobilized on, or strongly tethered to, the surface of PE disks, a standardized experimental protocol has been systematically applied to the PE disks after their incubation with trypsin solution. It consisted of: (i) a five-step rinsing process, as described in Material and Methods; (ii) the measure of enzymatic activity by monitoring the optical density of a BAEE solution in contact with the PE disk sample; and (iii) the search for possible enzyme release by continuing to take optical density measurements after the PE disk was removed from the BAEE solution.

A typical result is presented in Figure 5, showing both the presence of active enzyme immobilized on the disk and the absence of enzyme release during the experiment. It must be noted that the five-step rinsing protocol was chosen because some release of enzyme—that is a measurable trypsin activity in BAEE solution after removal of the disk—could be observed when using a shorter rinsing process, for example, only 3 steps, from step 2 to step 4.

Table 2 shows the immobilized enzyme activity of different treated PE disks. It can be noted that maximal activities, around 8×10^{-2} U/cm^2 on average, are found with PPGTR disks and that both rinsing protocols using phosphate buffer or Tween 20 as "washing solutions" for the third step give similar results. In contrast, the use of 2 M guanidine hydrochloride washing solution, which is an excellent agent for desorbing noncovalently bonded molecules, leads to an almost total loss of activity for all samples, likely due to their denaturation.[58] The very low activities of the PPGT samples confirm the necessity of reducing imine linkages to secondary amines by $NaCNBH_3$.[38] A more surprising result is the similar activity measured for the PPTR and PPGTR samples, showing that direct contact between a protein solution and a plasma-activated PE disk could lead to a strong immobilization of active enzyme, strengthened by further $NaCNBH_3$ treatment. The efficiency of this reductive reagent suggests the formation of covalent bonds between trypsin and the reactive species of the plasma-treated surface, e.g., nitrile and amines.

To compare the values of enzymatic activities measured in the present work with some results reported in the literature, we considered the data of Boulares-Pender et al.,[27] who used a nitrogen plasma for the amino functionalization of polystyrene surfaces. They immobilized trypsin on microtiter plates and used the substrate *N*-benzoyl-D,L-arginine-*p*-nitro-anilide (BAPNA) at pH 8 for the determination of its activity. The exploitation of their Figure 6 allowed us to estimate the release of *p*-nitroaniline by the trypsin-functionalized surface to be 1.56×10^{-3} OD/min (Absorbance at 405 nm), that is, a BAPNA hydrolysis rate of 0.164 μmol L^{-1} min^{-1} (calculated with a molar extinction coefficient of *p*-nitroaniline of 9500 M^{-1} cm^{-1} at 405 nm[59]).

dx.doi.org/10.1021/jp204097a |*J. Phys. Chem. B* 2011, 115, 10228–10238

Table 2. Immobilized Trypsin Activity on Various PE Samples[a]

trypsin activity for different washing solutions (third rinsing step)	0.1 M phosphate buffer	0.1% Tween20 solution	2 M guanidine hydrochloride solution
PPGTR (U/cm^2)	8.4×10^{-2}	7.9×10^{-2}	1.5×10^{-2}
	SD = 2.4×10^{-2}	SD = 1.9×10^{-2}	SD = 0.6×10^{-2}
	$n = 17$	$n = 12$	$n = 8$
PPGT (U/cm^2)	1.8×10^{-2}	2.2×10^{-2}	$0.7.10^{-2}$
	SD = 1.3×10^{-2}	SD = 1.2×10^{-2}	SD = 0.7×10^{-2}
	$n = 5$	$n = 6$	$n = 4$
PPTR (U/cm^2)	6.6×10^{-2}	$6.8.10^{-2}$	0
	SD = 0.7×10^{-2}	SD = 3.2×10^{-2}	
	$n = 3$	$n = 2$	$n = 4$
PPT (U/cm^2)	3.3×10^{-2}	3.1×10^{-2}	1.1×10^{-2}
	SD = 2.2×10^{-2}	SD = 1.7×10^{-2}	SD = 0.1×10^{-2}
	$n = 7$	$n = 5$	$n = 3$

[a] n represents the number of independent assays, and SD is the standard deviation for the n measurements.

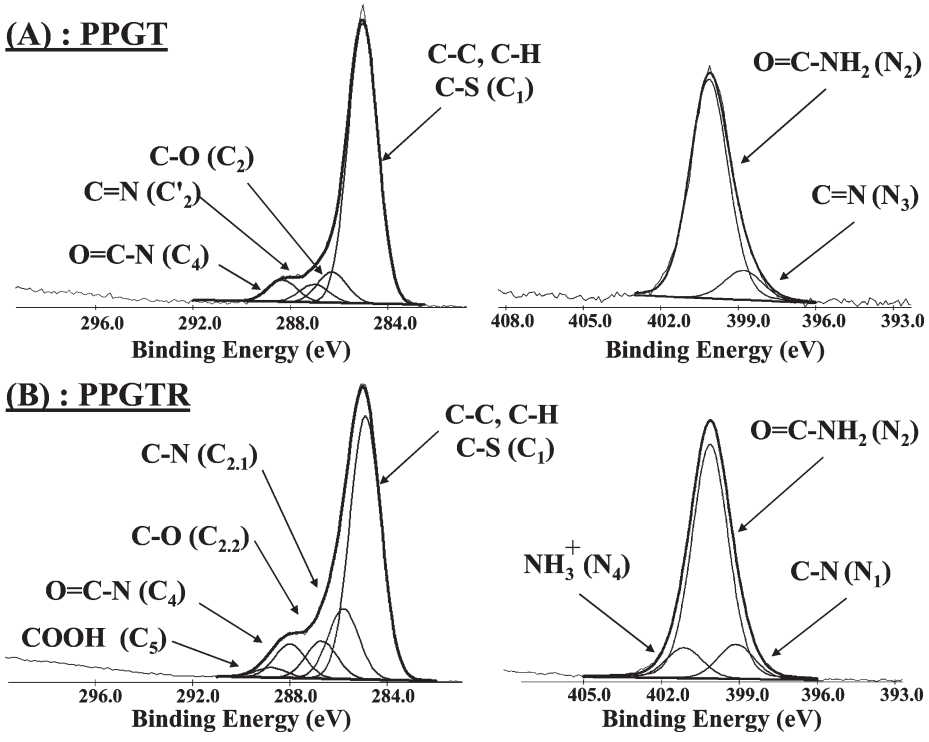


Figure 6. High-resolution C 1s and N 1s peaks: (A) PPGT and (B) PPGTR samples.

Since the reaction volume used was 0.28 mL per well, the amount of trypsin immobilized on the surface corresponds to an activity of 4.59×10^{-5} μmol D-L BAPNA released per minute that is 4.59×10^{-5} D-L BAPNA units, according to Temporini et al.[50] By using the conversion factor given by these last authors between the D-L BAPNA unit and the BAEE unit, $U_{BAEE}/U_{D\text{-}L\ BAPNA} = 1968$,[50] the immobilized enzyme activity can be estimated to be 0.09 BAEE units. Finally, to compare the immobilized enzyme activity per unit of surface, it was necessary to estimate the well surface in contact with the substrate solution. From the geometric dimensions of a NUNC Nunclon U-96 wells microtiter plates[60] microwell filled with a 280 μL substrate solution, the surface in contact was estimated to be approximately equal to 1.84 cm^2, which leads to a value of 0.05 BAEE unit per cm^2. It can be noted that this value, obtained with sophisticated hyperbranched graft polymers as spacer arms, is very comparable to our results (0.08 unit per cm^2 on average for PPGTR) obtained with glutaraldehyde grafting.

3.2. Surface Characterization by XPS. XPS analysis of the PPGTand PPGTR disks shows that the N/C ratio significantly increases after trypsin immobilization, in parallel with the high-resolution C 1s peak at 288.1 ± 0.2 eV attributed to the peptide bond and characteristic of proteins (Table 1 and Figure 6). In PPGT, the reactions between surface aldehyde groups and primary amines of trypsin form another Schiff base linkage (peak C′2 at 287.0 eV), which disappears in PPGTR samples, after being transformed into more stable secondary amines by cyanoborohydride treatment.[38] These trends are confirmed by the high-resolution N 1s spectrum.

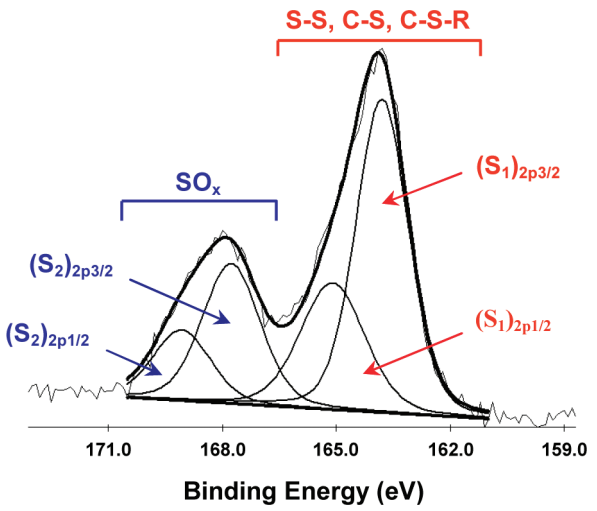


Figure 7. High-resolution S 2p XPS spectrum of the PPGTR sample.

Special attention has been focused on PPGTR samples, which present significant immobilized enzymatic activities. The high-resolution C 1s spectra were fitted into five peaks (Figure 6 B). Chemical assignments for fitted peaks were based on binding energies quoted in the literature:[43,61−64] peak C_1 at 285 eV (C−C, C−H, and C−S of cysteine and methionine residues of trypsin), $C_{2.1}$ at 285.8 eV (C−N of lysine, arginine, histidine, tryptophane residues), $C_{2.2}$ at 286.7 eV (C−O of serine, tyrosine, and threonine residues), C_4 at 288.0 eV (peptide bond), and C_5 at 288.8 eV (O═C−O carboxyl group of aspartic and glutamic acid residues), respectively.

Another indication of the presence of the immobilized trypsin was the appearance of a substantial sulfur peak in the S 2p XPS spectrum. Figure 7 shows a representative high-resolution S 2p spectrum with two peaks, S_1 and S_2, at two different bonding energies. Each one of these peaks is formed from a doublet $(S)_{2p\ 3/2}-(S)_{2p\ 1/2}$ of close energy (energy gap 1.2−1.3 eV) depending on the spin of electrons. These doublets have been fitted using a 2:1 peak area ratio (2p 3/2:2p 1/2) determined by the spin−orbit splitting effect.[65,66] The lower binding energy S_1 doublet at 163.8 and 165.2 eV was attributed to sulfur containing amino acid residues, C−S (cysteine residue), and C−S−R (methionine residue) and to intramolecular disulfide bonds S−S[43] present in the immobilized protein (Table 1). The high-energy S_2 doublet (167.8 and 169.1 eV) was attributed to oxidized sulfur species.[43,66,67] It can be noticed that the S_1 doublet only appears when the surface was previously coated with protein, while S_2 could be sometimes detected on samples which had never been in contact with the protein. This seems to be due to a contamination by sulfate ions,[68] likely brought by the rinsing with phosphate buffer. Some experiments where phosphate buffer was replaced by pure water as rinsing solution support this explanation. However, the contribution of oxidized sulfur functions belonging to the protein and appearing after its exposition to air or due to the XPS experiment itself has to be considered too.

The XPS experimental atomic ratios have been compared with the theoretical ratios of the protein. These theoretical ratios have been calculated from the sequence of the bovine trypsin studied elsewhere[69] and referenced as 1UTO in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank. The overall formula of trypsin calculated from these data leads to $C_{1012}H_nN_{279}O_{324}S_{14}$ which corresponds to the following

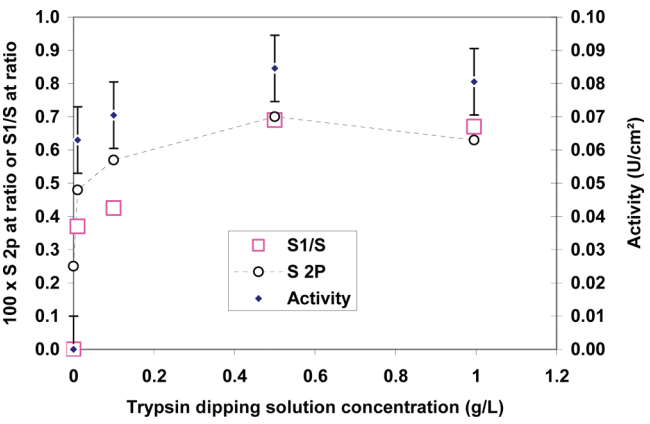


Figure 8. Effect of trypsin concentration of the dipping solution on S 2p atomic ratio, S_1/S atomic ratio, and immobilized activity.

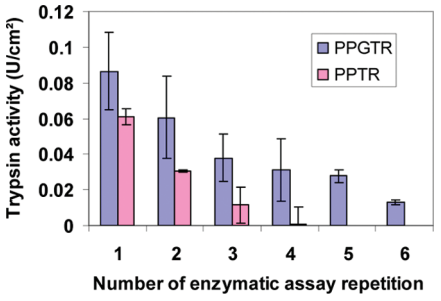


Figure 9. Comparison of immobilized trypsin activity of PPTR and PPGTR samples along storage periods and repeated enzymatic assays; for PPGTR the time of storage corresponding to the last (6th) assay was 6.5 months.

theoretical atomic ratios: O/C = 0.320 (XPS 0.22); N/C = 0.276 (XPS 0.12); S/C = 0.014 (XPS 0.009). These results show that if from PPG to PPGTR the ratios N/C, S/C, and O/C increase, following protein immobilization, they remain 56% ± 13% lower than the theoretical values, as reported elsewhere by others.[64] This can be due to an incomplete coverage of the surface[70] or to the fact that the successive layers grafted on the PE sample are not completely masked by trypsin and continue to contribute to the XPS signal.[71]

3.3. Influence of the Concentration of the Trypsin Dipping Solution on the Immobilized Enzyme Activity. The concentration of trypsin in the incubation bath which receives the PPG samples for enzyme grafting (cf. Materials and Methods) has been varied between 0.01 and 1 g/L. Figure 8 shows that the activity of PPGTR samples obtained after these different immobilization treatments slightly increases with the concentration of trypsin solution between 0.01 and 0.5 g/L and then reaches a plateau. This result justifies the choice of 1 g/L as the usual concentration of the trypsin dipping solution for the immobilization step. However, the relatively low increase of activity from 0.01 to 1 g/L suggests that, already in the least concentrated 0.01 g/L solution, the amount of trypsin present (40 μg) was much higher than the quantity strictly needed to form a complete monolayer on one face (0.9 to 1.4 μg estimated from Abbas et al., 2009).[38]

XPS analysis of the different samples was also performed: Figure 8 shows that the variation of S 2p atomic ratio follows the same evolution as the surface activity with increasing trypsin concentration, reaching a plateau close to 0.7 atom %. For the

Table 3. Comparison of XPS Results: Just Prepared versus Stored and Re-Used PPGTR Samples[a]

	C 1s			N 1s			S 2p			O ls	N/C[b]	O/C[b]	S/C[b]
	assignment	BE[c]	%	assignment	BE[c]	%	assignment	BE[c]	%				
PPGTR atom %		74.23%			8.83%			0.63%		16.31%	0.12	0.22	0.0085
	C−C, C−H, C−S	285.0	64 ± 2	C−N	399.1	11 ± 4	S−S, C−S, C−S−R	163.8	45 ± 2				
								165.2	22 ± 2				
	C−N	285.8	17 ± 2	O═C−NH2	400.1	78 ± 4	SO_x	167.8	22 ± 2				
								169.1	11 ± 2				
	C−O	286.7	9 ± 2	NH_3^+	401.1	11 ± 4							
	O═C−N	288.0	8 ± 2										
	COOH	288.8	2 ± 2										
aged [a]													
PPGTR atom %		74.56%			7.87%			0.24%		17.33%	0.11	0.23	0.003
	C−C, C−H, C−S	285.0	62 ± 2	C−N	399.1	18 ± 4	S−S, C−S, C−S−R	163.9	24 ± 2				
								165.2	12 ± 2				
	C−N	285.8	9 ± 2	O═C−NH2	400.1	70 ± 4	SO_x	167.9	43 ± 2				
								169.2	21 ± 2				
	C−O	286.6	16 ± 2	NH_3^+	401.1	12 ± 4							
	O═C−N	288.0	9 ± 2										
	COOH	288.9	4 ± 2										

[a] Aged PPGTR: 6 enzymatic assays and 6.5 months of storage in phosphate buffer. [b] Atomic ratio. [c] Binding energy (eV).

first point of the series, that is, for a trypsin concentration equal to zero, the XPS experiment leads to a S 2p value of 0.25 atom %, while no protein had been in contact with the PPG sample. The peak fitting of the XPS spectra allowed us to calculate the atomic ratio S_1/S by dividing the area of S_1 (sum of the two S_1 doublets) by the total ($S_1 + S_2$) area and to show that the sulfur detected on the sample without protein ($C = 0$) was only due to oxidized species, characterized by a high-energy S_2 peak ($S_1/S = 0$). As we noticed previously, these oxidized species seem mostly due to impurities brought by the rinsing phosphate buffer. The S_1/S ratio has also been reported in Figure 8 and follows a evolution trend similar to surface activity, pointing out its interest as a marker for active trypsin.

3.4. Reusability of the PE Samples. The determination of the initial enzymatic activity of the trypsin-grafted PE samples was performed about 1 h after the last step of sample preparation. After this first assay, some of the samples were kept at 4 °C in phosphate buffer 0.1 M pH 7.6 and tested again with BAEE solution, following the same experimental protocol. Generally, the second assay was performed after one or two weeks and the third one between two weeks and one month of storage. Figure 9 shows the evolution of the immobilized trypsin activity for successive enzymatic assays for the PPGTR and PPTR samples. It can be noted that both types of samples exhibit a decrease of immobilized activity with storage time and successive utilizations. This decrease is markedly less pronounced for the PPGTR samples in which enzymes are covalently linked to the surface via glutaraldehyde than for PPTR. In this last case, it has been suggested above that some covalent bonds could be involved in the attachment of the protein to the plasma-treated PE surface; however, the present experiment shows that the aging behavior of PPTR is actually less favorable than when a sufficient amount of glutaraldehyde linker reacts with all primary amine sites[56] before protein immobilization.

XPS analyses were performed on the aged PPGTR sample (6.5 months of storage in phosphate buffer 4 °C and six repeated enzymatic assays). In Table 3, the comparison of XPS data of PPGTR samples just after preparation and after aging shows that there was no fundamental change in the functional groups present on the PE surfaces, but a decrease of N/C and S/C ratios was observed for the aged sample. These results suggest that the protein was still grafted on the aged PE surface even if a part seems to have been lost. However, the peak fitting of the S 2p peak points out a drastic decrease of the S_1/S atomic ratio from 67% to 36%. This increase of protein oxidation, along with the slight increase of the O/C ratio, appears to be correlated with the measured loss of enzymatic activity of the aged sample. It must be noted that S 2p XPS data have to be achieved with great care (time of exposure, standardized experimental conditions) since X-rays have been shown to be able to induce damages on sulfur species.[67]

4. Correlation between Immobilized Trypsin Activity and XPS Results. To highlight the correlations existing between XPS measurements and enzyme activity, both types of experiments were performed on the same sample, and the results are reported in Figure 10. It must be noted that various samples, PPGTR, PPGT, PPTR, and PPT, prepared in different experimental series, that is, four different plasma treatment batches, have been tested. Three different XPS data have been reported: the S 2p atomic percentage, the S_1/S atomic ratio resulting from high-resolution S 2p spectra, and the (C_{Amide}/C) atomic ratio obtained from high-resolution C 1s spectra. In the literature, the C 1s and N 1s XPS data are the most commonly used for establishing, qualitatively, the success of the grafting of different chemical functions on surfaces. However, we have shown (Figure 10A and B) that the results obtained from S 2p spectra are closely correlated to the corresponding surface trypsin activity. The S 2p atomic percentages obtained in the above-reported experiment in varying the dipping solution trypsin concentration have also been reported on the graph. The same adjustment of −0.25% was applied to all five points, corresponding to the presumed sulfate contamination by the rinsing buffer. This

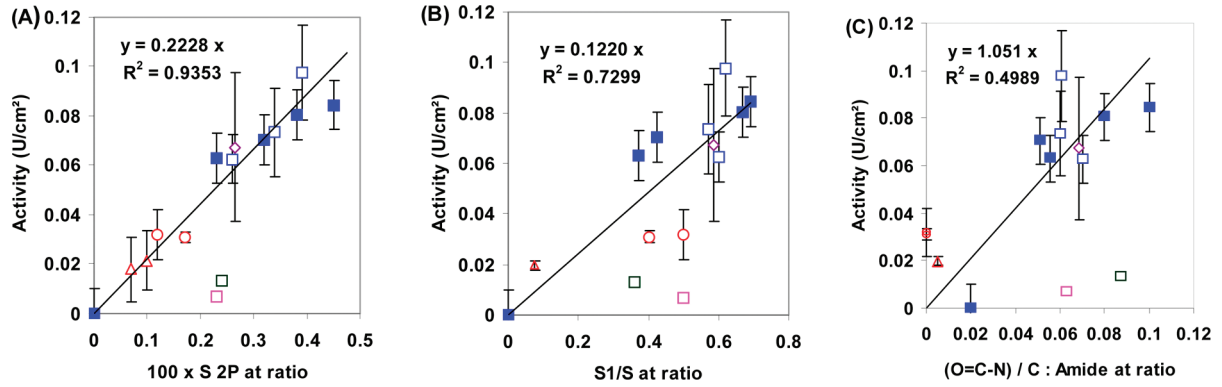


Figure 10. Quantitative correlation between immobilized trypsin activity and XPS data. (A) S 2p atomic ratio. (B) S_1/S atomic ratio. (C) C_{Amide}/C atomic ratio. Blue open box, PPGTR; blue filled box, PPGTR (experiment with variable trypsin concentration in dipping solution); purple open diamond, PPTR; red open triangle, PPGT; red open circle, PPT. The last points (pink open box, PPGTR washed with guanidine solution; black open box, aged PPGTR) are not included in the linear correlation.

resulted in a good linear correlation between the immobilized enzyme activity and the atomic ratio of sulfur S 2p atom %, with a regression coefficient $R^2 \sim 0.94$, result which, to the best of our knowledge, has never been reported. In addition, Figure 10 (B and C) shows that the correlation obtained between immobilized activity and S_1/S atomic ratio leads to a better linear fit ($R^2 \sim 0.73$) than that obtained with the (C_{Amide}/C) ratio, which is much more dispersed ($R^2 \sim 0.5$). It can be noted that, although less tightly correlated than the S 2p atomic percentage, the S_1/S ratios reported in Figure 10B correspond to direct experimental results without taking into account any corrections due to a presumed buffer contamination.

According to the nature of the protein, the surface, and the experimental immobilization conditions, the distribution of the proteins on the surfaces can take different forms, such as monolayer, bi- or multilayers, aggregates, or islands.[5,28,29,38,55] As reported above, the S 2p data from XPS analysis, which provides information on a thin molecular layer, less than 10 nm depth (corresponding to approximately the diameters of two trypsin molecules), can be linearly correlated to surface enzyme activity. This is in agreement with the common assumption that the activity lies in one or two upmost enzyme layers, the underneath enzymes showing generally no significant activity, due to steric hindrance, lack of substrate, hampered product diffusion or conformational changes.[29,72,73]

Figure 10 also gives information on the effect of (i) guanidine and (ii) repeated tests and aging on PPGTR samples. These two points, which are not included in the correlation calculation, confirm the presence of the inactive enzyme on the PE sample: guanidine is known to denaturate proteins, but the XPS results show that a part still remains linked to the support. It appears also that after 6.5 months of storage and 6 assays a significant amount of initial trypsin is still present on PPGTR but that it has lost practically all its activity.

CONCLUSION

The immobilization of active trypsin on a plasma-treated polyethylene surface has been accomplished. The best results, in terms of enzyme activity and stability, have been obtained when glutaraldehyde is used as a covalent linker between the surface and the enzyme (PPGTR samples: trypsin activity present after 6 successive assays and more than 6 months of storage). However, it was also shown that trypsin could be directly attached to a NH_3 plasma-treated polyethylene surface and that comparable initial activities to PPGTR could be obtained if the reduction step by sodium cyanoborohydride was performed (PPTR samples), suggesting the existence of covalent attachment. The strength of each grafting protocol was checked by an optimized washing process and by the control of the absence of measurable release of enzyme during the enzymatic assays.

The different steps of surface treatment have been characterized by XPS spectroscopy analyses. Deduced from these last experiments, an original result highlighted the good linear correlation existing between immobilized trypsin activity and the S 2p atomic ratio. In addition, the S_1 peak corresponding to unoxidized sulfur in protein appeared to be a relevant marker of the active enzyme, demonstrating its special interest for surface enzyme activity characterization. Conversely, the correlation between enzyme activity and the amide peak was very poor.

This work stresses the importance of dry plasma surface chemistry to generate high performance bioactive platforms from commercially available low-cost plastic sheets.

AUTHOR INFORMATION

Corresponding Author

*Mahsa Ghasemi. Phone: +33 144276732. E-mail: mahsa-ghasemi@chimie-paristech.fr. Michel Minier. Phone: +33 144276730. Fax: +33 143260061. E-mail: michel-minier@chimie-paristech.fr.

REFERENCES

(1) Tiefenauer, L.; Ros, R. *Colloids Surf., B* **2002**, *23*, 95–114.
(2) Libertino, S.; Giannazzo, F.; Aiello, V.; Scandurra, A.; Sinatra, F.; Renis, M.; Fichera, M. *Langmuir* **2008**, *24*, 1965–1972.
(3) Kato, M.; Inuzuka, K.; Sakai-Kato, K.; Toyo'oka, T. *Anal. Chem.* **2005**, *77*, 1813–1818.
(4) Conte, A.; Buonocore, G. G.; Bevilacqua, A.; Sinigaglia, M.; Del Nobile, M. A. *J. Food Prot.* **2006**, *69*, 866–870.
(5) Caro, A.; Humblot, V.; Methivier, C.; Minier, M.; Salmain, M.; Pradier, C. M. *J. Phys. Chem. B* **2009**, *113*, 2101–2109.
(6) Caro, A.; Humblot, V.; Methivier, C.; Minier, M.; Barbes, L.; Li, J.; Salmain, M.; Pradier, C. M. *J. Colloid Interface Sci.* **2010**, *349*, 13–18.
(7) Gancarz, I.; Bryjak, J.; Bryjak, M.; Tylus, W.; Pozniak, G. *Eur. Polym. J.* **2006**, *42*, 2430–2440.
(8) Feng, C. L.; Zhang, Z. Z.; Forch, R.; Knoll, W.; Vancso, G. J.; Schonherr, H. *Biomacromolecules* **2005**, *6*, 3243–3251.

(9) Chen, Y. Z.; Yang, C. T.; Ching, C. B.; Xu, R. *Langmuir* **2008**, *24*, 8877–8884.
(10) Biswas, H. S.; Datta, J.; Chowdhury, D. P.; Reddy, A. V. R.; Ghosh, U. C.; Srivastava, A. K.; Ray, N. R. *Langmuir* **2010**, *26*, 17413–17418.
(11) Anastas, P.; Warner, J. *Green Chemistry: Theory and Practice*; Oxford University Press: New York, 1998.
(12) Goddard, J. M.; Hotchkiss, J. H. *Prog. Polym. Sci.* **2007**, *32*, 698–725.
(13) Sano, S.; Kato, K.; Ikada, Y. *Biomaterials* **1993**, *14*, 817–822.
(14) Kang, E. T.; Tan, K. L.; Kato, K.; Uyama, Y.; Ikada, Y. *Macromolecules* **1996**, *29*, 6872–6879.
(15) Schroder, K.; Meyer-Plath, A.; Keller, D.; Ohl, A. *Plasmas Polym.* **2002**, *7*, 103–125.
(16) Meyer-Plath, A. A.; Schroder, K.; Finke, B.; Ohl, A. *Vacuum* **2003**, *71*, 391–406.
(17) Denes, F. S.; Manolache, S. *Prog. Polym. Sci.* **2004**, *29*, 815–885.
(18) Siow, K. S.; Britcher, L.; Kumar, S.; Griesser, H. J. *Plasma Processes Polym.* **2006**, *3*, 392–418.
(19) Arefi-Khonsari, F.; Tatoulian, M. *Plasma processing of polymers by a low frequency discharge with asymmetrical configuration of electrodes*; Wiley −VCH: Weinheim (Germany), 2008; pp 137−174.
(20) Gancarz, I.; Bryjak, J.; Pozniak, G.; Tylus, W. *Eur. Polym. J.* **2003a**, *39*, 2217–2224.
(21) Gancarz, I.; Bryjak, J.; Bryjak, M.; Pozniak, G.; Tylus, W. *Eur. Polym. J.* **2003b**, *39*, 1615–1622.
(22) Jafari, R.; Arefi-Khonsari, F.; Tatoulian, M.; Le Clerre, D.; Talini, L.; Richard, F. *Thin Solid Films* **2009**, *517*, 5763–5768.
(23) Yin, Y. B.; Nosworthy, N. J.; Gong, B.; Bax, D.; Kondyurin, A.; McKenzie, D. R.; Bilek, M. M. M. *Plasma Processes Polym.* **2009**, *6*, 68–75.
(24) Wang, M. J.; Chang, Y. I.; Poncin-Epaillard, F. *Langmuir* **2003**, *19*, 8325–8330.
(25) Bretagnol, F.; Tatoulian, M.; Arefi-Khonsari, F.; Lorang, G.; Amouroux, J. *React. Funct. Polym.* **2004**, *61*, 221–232.
(26) Vartiainen, J.; Ratto, M.; Paulussen, S. *Packag. Technol. Sci.* **2005**, *18*, 243–251.
(27) Boulares-Pender, A.; Prager-Duschke, A.; Elsner, C.; Buchmeiser, M. R. *J. Appl. Polym. Sci.* **2009**, *112*, 2701–2709.
(28) Gan, B. K.; Kondyurin, A.; Bilek, M. M. M. *Langmuir* **2007**, *23*, 2741–2746.
(29) Hirsh, S. L.; Bilek, M. M. M.; Nosworthy, N. J.; Kondyurin, A.; dos Remedios, C. G.; McKenzie, D. R. *Langmuir* **2010**, *26*, 14380–14388.
(30) Ganapathy, R.; Manolache, S.; Sarmadi, M.; Simonsick, W. J.; Denes, F. *J. Appl. Polym. Sci.* **2000**, *78*, 1783–1796.
(31) Ganapathy, R.; Manolache, S.; Sarmadi, M.; Denes, F. *J. Biomater. Sci., Polym. Ed.* **2001**, *12*, 1027–1049.
(32) Alvarez-Blanco, S.; Manolache, S.; Denes, F. *Polym. Bull* **2001**, *47*, 329–336.
(33) Alvarez, S.; Manolache, S.; Denes, F. *J. Appl. Polym. Sci.* **2003**, *88*, 369–379.
(34) Malpass, C. A.; Millsap, K. W.; Sidhu, H.; Gower, L. B. *J. Biomed. Mater. Res., Part A* **2002**, *63*, 822–829.
(35) Conte, A.; Buonocore, G. G.; Sinigaglia, M.; Lopez, L. C.; Favia, P.; D'Agostino, R.; Del Nobile, M. A. *J. Food Prot.* **2008**, *71*, 119–125.
(36) Mutlu, M.; Mutlu, S.; Boyaci, I. H.; Alp, B.; Piskin, E. In *Polymers in Sensors-Theory and Practice*, ACS Symposium Series; American Chemical Society: Washington, DC, 1998; Vol. 690, pp 57−65.
(37) Biederman, H.; Boyaci, I. H.; Bilkova, P.; Slavinska, D.; Mutlu, S.; Zemek, J.; Trchova, M.; Klimovic, J.; Mutlu, M. *J. Appl. Polym. Sci.* **2001**, *81*, 1341–1352.
(38) Abbas, A.; Vercaigne-Marko, D.; Supiot, P.; Bocquet, B.; Vivien, C.; Guillochon, D. *Colloids Surf., B* **2009**, *73*, 315–24.
(39) Matsuda, K.; Harada, H.; Kashiwada, A.; Yamada, K.; Hirata, M. *J. Photopolym. Sci. Technol.* **2003**, *16*, 49–54.
(40) Matsuda, K.; Makino, T.; Kashiwada, A.; Yamada, K.; Hirata, M. *J. Photopolym. Sci. Technol.* **2004**, *17*, 165–170.
(41) Lahari, C.; Jasti, L. S.; Fadnavis, N. W.; Sontakke, K.; Ingavle, G.; Deokar, S.; Ponrathnam, S. *Langmuir* **2010**, *26*, 1096–1106.
(42) Tebeka, I. R. M.; Silva, A. G. L.; Petri, D. F. S. *Langmuir* **2009**, *25*, 1582–1587.
(43) Brack, N.; Lamb, R.; Pham, D.; Turner, P. *Surf. Interface Anal.* **1996**, *24*, 704–710.
(44) Yin, Y. B.; Bax, D.; McKenzie, D. R.; Bilek, M. M. M. *Appl. Surf. Sci.* **2010**, *256*, 4984–4989.
(45) Achyuta, A. K. H.; Stephens, K. D.; Lewis, H. G. P.; Murthy, S. K. *Langmuir* **2010**, *26*, 4160–4167.
(46) Freije, J. R.; Mulder, P.; Werkman, W.; Rieux, L.; Niederlander, H. A. G.; Verpoorte, E.; Bischoff, R. *J. Proteome Res.* **2005**, *4*, 1805–1813.
(47) Liu, J. Y.; Lin, S.; Qi, D. W.; Deng, C. H.; Yang, P. Y.; Zhang, X. M. *J. Chromatogr., A* **2007**, *1176*, 169–177.
(48) Stigter, E. C. A.; de Jong, G. J.; van Bennekorn, W. P. *Anal. Bioanal. Chem.* **2007**, *389*, 1967–1977.
(49) Sakai-Kato, K.; Kato, M.; Toyo'oka, T. *Anal. Chem.* **2003**, *75*, 388–393.
(50) Temporini, C.; Perani, E.; Mancini, F.; Bartolini, M.; Calleri, E.; Lubda, D.; Felix, G.; Andrisano, V.; Massolini, G. *J. Chromatogr., A* **2006**, *1120*, 121–131.
(51) Cingoz, A.; Hugon-Chapuis, F.; Pichon, V. *J. Chromatogr., A* **2008**, *1209*, 95–103.
(52) Arefi-Khonsari, F.; Kurdi, J.; Tatoulian, M.; Amouroux, J. *Surf. Coat. Technol.* **2001**, *142*, 437–448.
(53) Ruckenstein, E.; Guo, W. *Biotechnol. Prog.* **2004**, *20*, 13–25.
(54) Schwert, G. W.; Takenaka, Y. *Biochim. Biophys. Acta* **1955**, *16*, 570–575.
(55) Chen, B.; Pernodet, N.; Rafailovich, M. H.; Bakhtina, A.; Gross, R. A. *Langmuir* **2008**, *24*, 13457–13464.
(56) Ghasemi, M.; Minier, M.; Tatoulian, M.; Arefi-Khonsari, F. *Langmuir* **2007**, *23*, 11554–11561.
(57) Isgrove, F. H.; Williams, R. J. H.; Niven, G. W.; Andrews, A. T. *Enzyme Microb. Technol.* **2001**, *28*, 225–232.
(58) Mikulec, L. J.; Puleo, D. A. *J. Biomed. Mater. Res., Part A* **1996**, *32*, 203–208.
(59) Achstetter, T.; Ehmann, C.; Wolf, D. H. *Arch. Biochem. Biophys.* **1981**, *207*, 445–454.
(60) http://www.nuncbrand.com/en/page.aspx?ID=10251 (accessed 2010).
(61) Barrias, C. C.; Martins, C. L.; Miranda, C. S.; Barbosa, M. A. *Biomaterials* **2005**, *26*, 2695–2704.
(62) Blattler, T. M.; Pasche, S.; Textor, M.; Griesser, H. J. *Langmuir* **2006**, *22*, 5760–5769.
(63) Tyan, Y. C.; Liao, J. D.; Jong, S. B.; Liao, P. C.; Yang, M. H.; Chang, Y. W.; Klauser, R.; Himmelhaus, M.; Grunze, M. *J. Mater. Sci.: Mater. Med.* **2005**, *16*, 135–142.
(64) Yi, F.; Li, Q.; Guo, Z. X.; Yu, J. *J. Appl. Polym. Sci.* **2006**, *99*, 1340–1345.
(65) Cavalleri, O.; Gonella, G.; Terreni, S.; Vignolo, M.; Pelori, P.; Floreano, L.; Morgante, A.; Canepa, M.; Rolandi, R. *J. Phys.: Condens. Matter* **2004**, *16*, S2477–S2482.
(66) Willey, T. M.; Vance, A. L.; van Buuren, T.; Bostedt, C.; Terminello, L. J.; Fadley, C. S. *Surf. Sci.* **2005**, *576*, 188–196.
(67) Zerulla, D.; Chasse, T. *Langmuir* **1999**, *15*, 5285–5294.
(68) Kirchner, U.; Vogt, R.; Natzeck, C.; Goschnick, J. *J. Aerosol Sci.* **2003**, *34*, 1323–1346.
(69) Leiros, H. K. S.; Brandsdal, B. O.; Andersen, O. A.; Os, V.; Leiros, I.; Helland, R.; Otlewski, J.; Willassen, N. P.; Smalas, A. O. *Protein Sci.* **2004**, *13*, 1056–1070.
(70) Gappa-Fahlenkamp, H.; Duan, X.; Lewis, R. S. *J. Biomed. Mater. Res., Part A* **2004**, *71A*, 519–527.
(71) Minier, M.; Salmain, M.; Yacoubi, N.; Barbes, L.; Methivier, C.; Zanna, S.; Pradier, C. M. *Langmuir* **2005**, *21*, 5957–5965.
(72) Yamada, K.; Nakasone, T.; Nagano, R.; Hirata, M. *J. Appl. Polym. Sci.* **2003**, *89*, 3574–3581.
(73) Fears, K. P.; Latour, R. A. *Langmuir* **2009**, *25*, 13926–13933.