Synthesis of Polyaniline Using Horseradish Peroxidase Immobilized on Plasma-Functionalized Polyethylene Surfaces as Initiator

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ABSTRACT: The doping of semiconducting conjugated polymers (e.g., polyaniline, PANI) can result in the elimination of the bandgap, leading to high electrical conductivities (comparable to metals). Doped PANI is totally insoluble and thus nonprocessable, which considerably limits its practical applications. Synthesis of PANI using immobilized horseradish peroxidase (HRP) as a catalyst in aqueous solutions can open up additional possibilities for applications through the direct synthesis of controlled-thickness PANI layers on various substrate surfaces. The RF plasma-enhanced surface functionalization of polyethylene and the covalent immobilization of HRP are discussed, and the polymerization of aniline initiated by immobilized HRP is presented. The nature of plasma-grafted surface functionalities on the substrate surfaces and the formation of PANI are demonstrated using X-ray photoelectron spectroscopy and attenuated total reflectance Fourier transform IR spectroscopy. The molecular weight distribution of PANI is evaluated with gel permeation chromatography, and the activity of the immobilized enzyme is monitored using UV spectroscopy. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 369–379, 2003

Key words: plasma-enhanced surface functionalization; immobilized horseradish peroxidase; polyaniline; polyethylene substrates; enzyme activity

INTRODUCTION

Combining organic molecules and cells with microelectronics has created the possibility of new analytical devices (e.g., DNA microchip arrays, biosensors, etc.) with new capabilities. Even novel computer components, based on molecular electronics (e.g., data storage units based on organic molecules), are becoming a reality.

A specific interaction of a targeted analyte with a molecularly designed recognition surface (e.g., immobilized biomolecules including enzymes, oligonucleotides, etc.) generates chemical and/or conformational changes in the resulting surface layers, which are detected and measured by a transducer. The nature of the transducer depends on the changes generated as a result of interactions between the analyte and the biorecognition component. Devices involving electrochemical, optical, mass, and thermal effects are the most common transducers.

Fluorescence-based molecular recognition processes are often preferred over other detection techniques because they offer a noninvasive approach and high sensitivity. Strings of DNA base pairs of a sensor, for instance, immobilized on a substrate in a microarray system, will match and bond specific DNA fragments, which are labeled with a fluorescent compound. Fluorescence is observed at those spots where bonding has occurred. However, the fluorescence labeling is a complex process, and it has to be specific for each target analyte. This considerably limits its application for a simultaneous multianalyte detection procedure.

Development of transducer systems based on electric signals would significantly increase the speed of detection and would allow a direct computer-aided evaluation of the multitudes of signals that are generated simultaneously by different molecular recognition processes. Changes in impedance as a result of interactions between the analyte molecules and the molecular recognition biocomponents of the sensors (e.g., antibodies, enzymes, oligonucleotides, etc.) would permit the design and development of differential electronic detection systems.

The connections between the biomolecules selected for the molecular recognition processes and the signal evaluation system should be achieved using conducting organic molecular wiring (integrated circuitry based on doped, conjugated organic polymers). This would also allow the development of controlled functionalization and subsequent covalent connection of the desired biomolecules to the synthetic metals.

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The era of conducting polymers began with the synthesis of polyacetylene.[1] However, attempts to use this polymer in applications resulted in little success because of its lack of processability and its poor stability to oxidation. To overcome these drawbacks, novel conducting conjugated polymers were synthesized, including polypyrrole, polythiophene, polyaniline (PANI), poly(heteroarylene vinylene)s, and other large conjugated molecules.[2–4]

All these polymers are characterized by the presence of alternating single and double bonds (conjugation), providing a highly delocalized π-electron system along the backbone of the polymer chains. The semiconductor and conductor properties of these materials are related to the overlap of p_z orbitals of double and triple bonds, which leads to well-delocalized valence and conduction bands. The conductivity of these polymers can be enhanced by doping them with appropriate additives (e.g., I_2, SO_3, AsF_5, etc.).

PANI is one of the conjugated polymers that has been investigated for such applications as organic light-weight batteries, microelectronics, optical displays, electromagnetic shielding, antistatic coatings, and so forth. It can be synthesized by oxidizing aniline electrochemically and/or chemically. Depending on the experimental conditions, the resulting PANI can exist in four oxidation states. The doped (e.g., with an acid) conductive PANI requires a linear, head to tail structure and should include both amine and imine bonds (emeraldine salt). However, practical applications of PANI require processability in addition to the electrical characteristics. Attempts made to improve the processability of PANI through polymer–analog reactions and by synthesizing PANI-type structures from aniline derivatives (e.g., sulfonated PANI) resulted in some improvements. However, techniques based on these complex chemistry approaches still require additional purification and separation steps; and many of the resulting PANIs are water soluble only at elevated pH values, where the polymer is in its undoped form.

It was shown as early as 1993[5,6] that electrically conducting high molecular weight unsubstituted and substituted PANI can be synthesized enzymatically by using hydrogen peroxide as an oxidant in a reaction catalyzed by peroxidase at low pH. The resulting polymer is linear; however, it is insoluble in most solvents. Enzymatic techniques were recently developed for the synthesis of water-soluble PANIs from water-soluble aniline derivatives and/or by polyanion (polyelectrolyte) assisted polymerization.[7–9] It was also demonstrated that oxidative free-radical coupling of 2,5-diaminobenzenesulfonate, which was catalyzed by horseradish peroxidase (HRP), can result in the formation of water-soluble PANI with an average molecular weight of around 18,000 Da. The novel PANI is fully sulfonated in comparison to the PANI synthesized through posttreatment of PANI with fuming sulfuric acid, and this makes it water soluble in a wide pH range. It was shown that the polymer can be organized into a multilayer structure by a layer by layer deposition technique. The PANI synthesized enzymatically in the presence of sulfonated polystyrene (SPS) instantly led to the formation of a PANI/SPS complex. Cyclic voltametry studies indicate that the PANI/SPS complex is more stable to oxidation and that the conductivity of the complex increases with the PANI to SPS molar ratio. A conductivity of 0.005 S/cm of the pure complex can be increased to 0.15 S/cm by doping with HCl vapors. It was also emphasized that the enzymatic approach offers enhanced processability, stability, and environmental compatibility.

Nonequilibrium plasma chemistry offers an efficient way for the surface functionalization of organic and inorganic polymeric substrates. The energies of plasma species are comparable with the bond energies of organic compounds and organic compounds containing main-group elements. As a consequence, even inert material surfaces including quartz, Teflon, and so forth can be easily functionalized.[10–12] The functionalization of polymer surfaces represents the first step in the immobilization process of biomolecules and in the development of molecular-recognition processes.

This contribution reports the direct synthesis and deposition of insoluble PANI thin layers onto polyethylene (PE) substrates using oxidative free-radical polymerization of aniline. The polymerization reaction is catalyzed by HRP immobilized on plasma-functionalized PE surfaces. This technique circumvents the difficult processability associated with conventional PANI and the requirement of a solvent assisted (non-water-soluble PANI) deposition of thin PANI layers for biosensor–transducer applications.

EXPERIMENTAL

Argon and oxygen supplied by Liquid Carbonic were used for the plasma-enhanced decontamination of the reactor. Oxalyl chloride (OC) and 1,2-diamino propane (DP) purchased from Aldrich were selected for chemical derivatization reactions. Dichlorosilane (DS) was obtained from Gelest Co. Fluorescamine was purchased from Molecular Probes Inc., (Eugene, OR).

The HRP (>99.5% HRP, type II; Reinheitszal ratio = 1.9 A_{403nm}/A_{275nm}; activity = 240 U/mg, 1 U will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20°C), *N*-(2-hydroxyethyl) piperazine-*N*′-(3-propanesulfonic acid) (EPPS), and sodium cyanoborohydride were obtained from Sigma and used for the development of the enzyme immobilization reaction. A 1*N* KOH solution (Aldrich) was used to adjust the pH to 8.5–8.7 for the 0.02*M* EPPS buffer solution. Monobasic potassium phosphate (Sigma) was used to prepare buffer solutions (pH 6, adjusted

using 1*N* KOH, pH 4.3). A 30% (w/w) H_2O_2 solution (Aldrich) and pyrogallol (Sigma) were employed for the preparation of enzyme assay reagents. Aniline (>99.5%, Aldrich) was used for the development of polymerization reactions.

To evaluate the relative surface atomic composition, the detailed ESCA C1s, O1s, N1s, Si2p, and Cl2p spectra were acquired for plasma-modified, derivatized, and HRP immobilized samples via a Perkin Elmer Physical Electronics 0 5400 small area ESCA instrument (Mg source; 15 kV; 300 W; pass energy: 89.45 eV; take-off angle of 45°). In order to correct the origin of the surface-charge binding energy shifts, calibrations were performed based on the well-known C1s peak of the binding energies at 285 or 284.4 eV.

Attenuated total reflectance Fourier transform IR spectroscopy (ATR-FTIR) was used to identify the chemical bonds on the functionalized PE surfaces and on the PE surfaces with immobilized HRP (I-HRP). An ATI-Mattson Research Series IR instrument was used, which was provided with a Graseby-Special Benchmark Series ATR in-compartment P/N/11160 unit. All FTIR evaluations were performed under a nitrogen blanket generated from a flow-controlled liquid nitrogen tank. Data were collected in the 600–4000 cm^{-1} range with 250 scans per sample. Because of the very thin nature of the functionalized PE surface layers, differential ATR-FTIR measurements were performed. Plasma-induced surface functionalization (modification) reactions only involve the very top 100 Å surface layers of the processed substrates, while ATR-FTIR reveals information from surface layers as thick as 1–3 μm. As a consequence, the unmodified bulk volume of PE will dominate the IR spectra; therefore, the differential ATR-FTIR technique is needed. The differential spectra resulted from the subtraction of the spectra of untreated PE from those of modified PE.

The absorption intensity of enzyme-generated purpurogallin was monitored using a Spectronic Unicam model UV1 spectrophotometer.

Plasma treatment of PE samples

All plasma-enhanced functionalization reactions were carried out in a cylindrical stainless steel, capacitively coupled (stainless steel disk electrodes, 20-cm electrode diameter, 3-cm gap) plasma reactor equipped with a 40-kHz power supply, which was previously described.[13,14]

In a typical experiment, after the reactor was decontaminated (10 min consecutive oxygen and argon plasma at 200-W power, 250-mTorr pressure, and 6 sccm oxygen and argon flow), the PE substrate was placed onto the lower electrode and the system was evacuated to base pressure level. In the next step the preselected Ar/DS partial pressures were established and the plasma was ignited under the desired experimental conditions for a given time. At the end of the plasma exposure the chamber was evacuated and DP or OC were consecutively distilled into the reactor at 1000 mTorr for 90 min. The evacuation of DP or OC vapors from the chamber was performed for 30 min to remove the nonreacted DP or OC. Finally the PE substrate was removed and stored until analytical evaluations or further reactions were initiated.

The following experimental conditions were employed for plasma-induced functionalization reactions: 20-mTorr base pressure, 100-W power, 180-mTorr Ar partial pressure, 20-mTorr DS partial pressure, and 10- or 30-s plasma exposure time. A vacuum desiccator was used for the storage of plasma-treated substrates.

Identification of primary amine functionalities

The evaluation of DP-based primary amine surface functionalities was performed by the fluorescence labeling technique. The aminated PE substrates were sprayed 3 times with a fluorescamine solution (25 mg of fluorescamine in 100 mL of acetone) by using a Gelman Chromist aerosol propellant attached to a polypropylene bottle. The fluorescence of the substrates was revealed with the aid of a Black-Ray UV lamp (model UBL 21, UVP Inc., San Gabriel, CA) and an FCR-10 photocamera (Fotodyne Inc., Hartland, WI).

Immobilization of HRP

The immobilization of HRP on PE was carried out in a three-step process: Ar/DS plasma assisted implantation of Si—$(Cl)_x$ functionalities, *in situ* gas phase functionalization using DP and OC, and *ex situ* attachment of HRP in solution.

The covalent attachment of HRP onto the functionalized PE surfaces was accomplished according to the following procedure: 120 cm^2 of functionalized PE substrates were dipped for 1 h in 25 mL of HRP solution (60 μg/mL HRP, prepared in 0.02*M* EPPS buffer solution); then 2 mL of sodium cyanoborohydride (0.02*M*) was added, and the samples were kept in this solution for an additional hour. At the end of the procedure the samples were rinsed with buffer and distilled water.

The concentration of the HRP on the PE sample surfaces was evaluated using UV–visible absorption spectrometry. Calibration curves were prepared using various concentrations of free enzyme to measure the concentration of HRP in the EPPS buffer solutions before and after the immobilization step. The absorption intensity changes at 402 and 281 nm were recorded [Fig. 1(A,B)]. As shown in Figure 1(B), the HRP absorption data follow the Lambert–Beer law agreement. For estimating the enzyme concentration, the

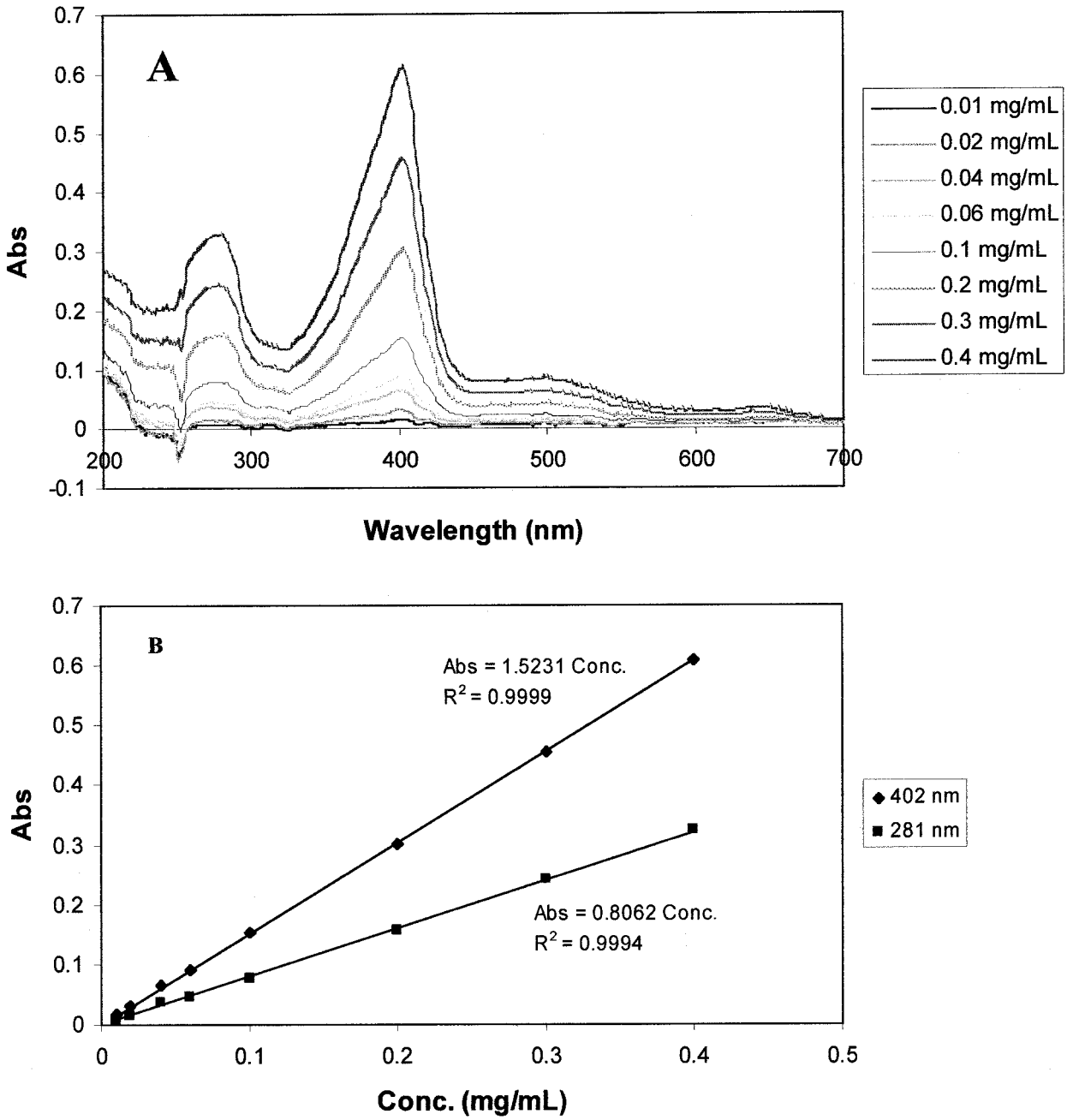


Figure 1 The calibration curves to determine the concentration of HRP: (A) the absorbance versus the wavelength for different enzyme concentrations and (B) the calibration curves at 281 and 402 nm.

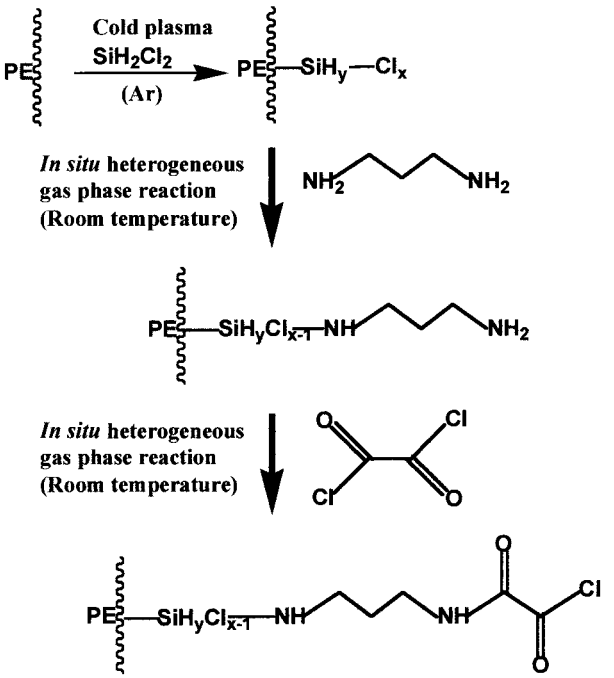
absorption at 402 nm was employed, because of the higher absorptivity at this wavelength. The surface bound HRP was calculated from the difference in the two absorption measurements before and after HRP surface immobilization.

Evaluation of activity of I-HRP

The activity of the immobilized enzyme was estimated using a standard assay test,[15] according to the following reaction:

$$H_2O_2 + \text{pyrogallol} \xrightarrow{\text{HRP}} H_2O + \text{purpurogallin}$$

Three milliliters of the Sigma test solution [containing specific amounts of distilled water, potassium phosphate buffer (pH 6), hydrogen peroxide, and pyrogallol] were added to a cuvette in the spectrophotometer, and the absorbance was recorded for 5 min at 420 nm. The absorption intensity of the enzyme-gen-



Scheme 1 The surface functionalization of PE substrates.

erated purpurogallin was used to evaluate the relative activities of free and surface bound HRP (I-HRP). PE samples (2.7-cm^2 surface area) with I-HRP were placed for 5 min into one of the quartz cuvettes of the spectrophotometer containing the Sigma test solution. The PE samples were then removed and the absorbance of the resulting purpurogallin solutions was determined. Because a portion of the nascent purpurogallin was adsorbed onto the PE samples, imparting them with an intense yellow coloration, the absorption of colored PE samples versus that of the HRP-immobilized PE was also recorded. These absorption measurements were compared to those of purpurogallin generated by an identical amount of free HRP (81 μg of HRP added to the cuvette).

Polymerization of aniline by I-HRP

The free and I-HRP catalyzed polymerization of aniline was carried out at room temperature according to the polymerization procedure described earlier,[9] using identical amounts of HRP. In a typical polymerization reaction, the PE I-HRP substrate was introduced into a solution of 1.8 mg of aniline in 200 mL of 0.1*M* potassium phosphate buffer (pH 4.3) after which a stoichiometric amount of H_2O_2 was added to initiate the addition polymerization process. In order to minimize the inhibition of HRP, which might result from an excess of hydrogen peroxide, a diluted H_2O_2 (0.002*M*) solution was added dropwise in increments over 1.5 h. The reaction mixture was kept under stirring for an additional 1.5 h.

The molecular weight distribution of the PANI synthesized using free and I-HRP catalysts was evaluated using a GPC instrument (Beckman Instruments) provided with a UV detector (TSK 4000 SW column, 7.5-mm internal diameter, 300-mm length, 10 μm particle size, Agilent Technologies). For all molecular weight estimations, the absorptions were recorded at 280 nm. Potassium phosphate buffer solution (pH 4.3) was used as the solvent.

RESULTS AND DISCUSSION

Surface functionalization of the PE substrates was accomplished according to the reaction mechanism sequences shown in Scheme 1.

Identification of the functionalities on the modified PE surfaces was carried out after each derivatization step. The relative surface atomic compositions of PE, Ar plasma-treated PE, and Ar/DS plasma-treated and *in situ* DP-functionalized PE (PE-NH_2) are presented in Table I. The low relative oxygen atomic concentration of unmodified PE (2.4%) and the significantly increased oxygen content of the Ar plasma-treated and open laboratory conditions (OLCs) exposed PE (20%) is indicative of the development of oxidation reactions initiated by the plasma-generated free radicals on PE surfaces.

The Ar/DS plasma-modified PE exposed to DP under *in situ* conditions exhibits a lower oxygen concentration (16.4%) than the Ar plasma-treated PE. This allows us to suggest that some of the free-radical sites covalently reacted with DP. However, the presence of a relatively high oxygen concentration can probably be related to the hydrolysis under OLCs of plasma implanted, $SiCl_x$ groups. For example, it was observed that the N/Si atomic ratio (1.64) after the reaction with DP is significantly smaller in comparison to the theoretical value (N/Si = 2) predicted by a —Si—NH —$(CH_2)_3$ —NH_2 structure. This suggests that not all Si —Cl bonds were involved in the post plasma amine formation reactions. (Some may have been involved in hydrolysis.)

TABLE I
Survey ESCA Data from PE, Ar Plasma-Treated PE, and Ar/DS Plasma-Treated and *In Situ* DP-Functionalized PE (PE—NH_2)

	Composition (%)			
Peak	Untreated PE	Ar Plasma-treated PE	PE—NH_2	DP (theoro)
C1s	97.6	80.0	57.3	40
O1s	2.4	20.0	16.4	—
N1s	—	—	13.3	60
Si2p	—	—	8.1	—
C12p3	—	—	4.9	—

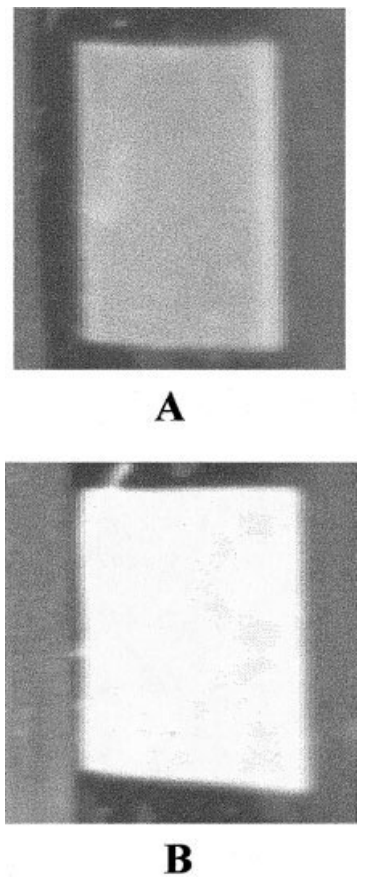


Figure 2 The UV fluorescence images of (A) untreated and (B) Ar/DS plasma-treated and DP-functionalized PE after the fluorescamine labeling technique.

The presence of primary amine functionalities on the PE-NH_2 surfaces is also demonstrated by fluorescence measurements performed on fluorescamine-labeled substrates [Fig. 2(A,B)]. One can observe that fluorescence is associated solely with the PE-NH_2 surfaces. The PE does not show any measurable optical emission.

Based on the fact that stable primary amine functionalities were immobilized onto the PE surfaces as a result of the reaction between the $SiCl_x$ groups and DP [to form PE—$Si(OH)_x$—NH—$(CH_2)_3$—NH_2] chains, the possible peak assignments for C1s, N1s, Si2p, and O1s ESCA spectra can be suggested. The C1s spectrum [Fig. 3(A)], which is fitted with seven peak components, shows the presence of C—Si and C═C (284.4 eV), C—C (285 eV), C—N (285.6 eV), —CH_2—$NH_3^+Cl^-$, C—O (286.2 and 286.5 eV), and C═O (288 eV) bonds. The N1s spectrum [Fig. 3(B)] allows us to suggest that C—N, Si—N, and C—$NH_3^+Cl^-$ functionalities are present in the modified PE surface.

It should be emphasized that the assignments employed in fitting the C1s, N1s, Si2p, and O1s spectra into individual functionalities must be considered only as approximate and suggestive. In fact these curve-fitting approaches undoubtedly represent an oversimplification of various atom groups present in the surface layers as a result of functionalization reactions. However, all high resolution peak assignments were performed according to recommended values for C, Si, N, and O atom containing polymers (Table II).[16–23]

More information on the involvement of Si in the structure of the functionalized PE surface layers is given by the Si2p high resolution diagram [Fig. 3(C)]. It is suggested that in addition to Si—C (100.7 eV) and Si—N (101.4 eV) peaks, Si—O (102.2 eV) and O—Si—O (103 eV) functionalities can also be identified. This is not surprising because of the extreme moisture sensitivity of $Si(Cl)_x$ groups. Chlorine atoms (not shown) are present in the modified surface layers as ionic (C—$NH_3^+Cl^-$, 197.7 eV) and covalent (C—Cl, 200.6 eV) functionalities. The relative low surface atomic Cl concentration (Table I) is indicative of the fact that Si—Cl groups of the Ar/DS plasma-treated PE were consumed by the amination reactions, by dehydrochlorination processes, and by the hydrolysis of SiCl groups under OLCs. The curve-fitted O1s ESCA spectrum of aminated PE surfaces shows the possible existence of two major Si and O based peaks, including Si—O (531.4 eV) and Si—O—Si (532 eV) in addition to the low surface area O and C related peaks, such as C═O (532.3 eV), C—O (532.8 eV), and C—C(O)—*O—C [533.6 eV; Fig. 3(D)]. The presence of low surface area C- and O-based peaks and the existence of high surface area Si- and O-based peaks can be related to the development of post-plasma *ex situ* oxidation and hydrolysis processes. It can be suggested that most of the oxygen atoms are involved in Si—O bonds in the modified surface layers of PE.

Because of the fact that some of the primary amine functionalities might appear in the —$NH_3^+Cl^-$ ionic form, the efficiency of the formation of amide bonds in the OC functionalization step could be diminished. Only the free primary amine functionalities will probably undergo amide group formation reactions according to the reaction mechanisms demonstrated in Scheme 2.

The presence of —C(O)—OH groups at the end of spacer chains is clearly evidenced by the C1s spectrum of the Ar/DS plasma-treated and DO/OC-functionalized PE [Fig. 4(A)]. The existence of other characteristic C-, Si-, N-, and O-based functionalities is also suggested to be present in the spectrum of Ar/DS plasma-treated and *in situ* DP- and subsequently OC-functionalized PE (PE—NH—CO). However, because of the complex chemical structure of the thin layers generated by the modification of PE surfaces, the assignments considered in fitting the spectra into individual functionalities must be considered only as a suggestion.

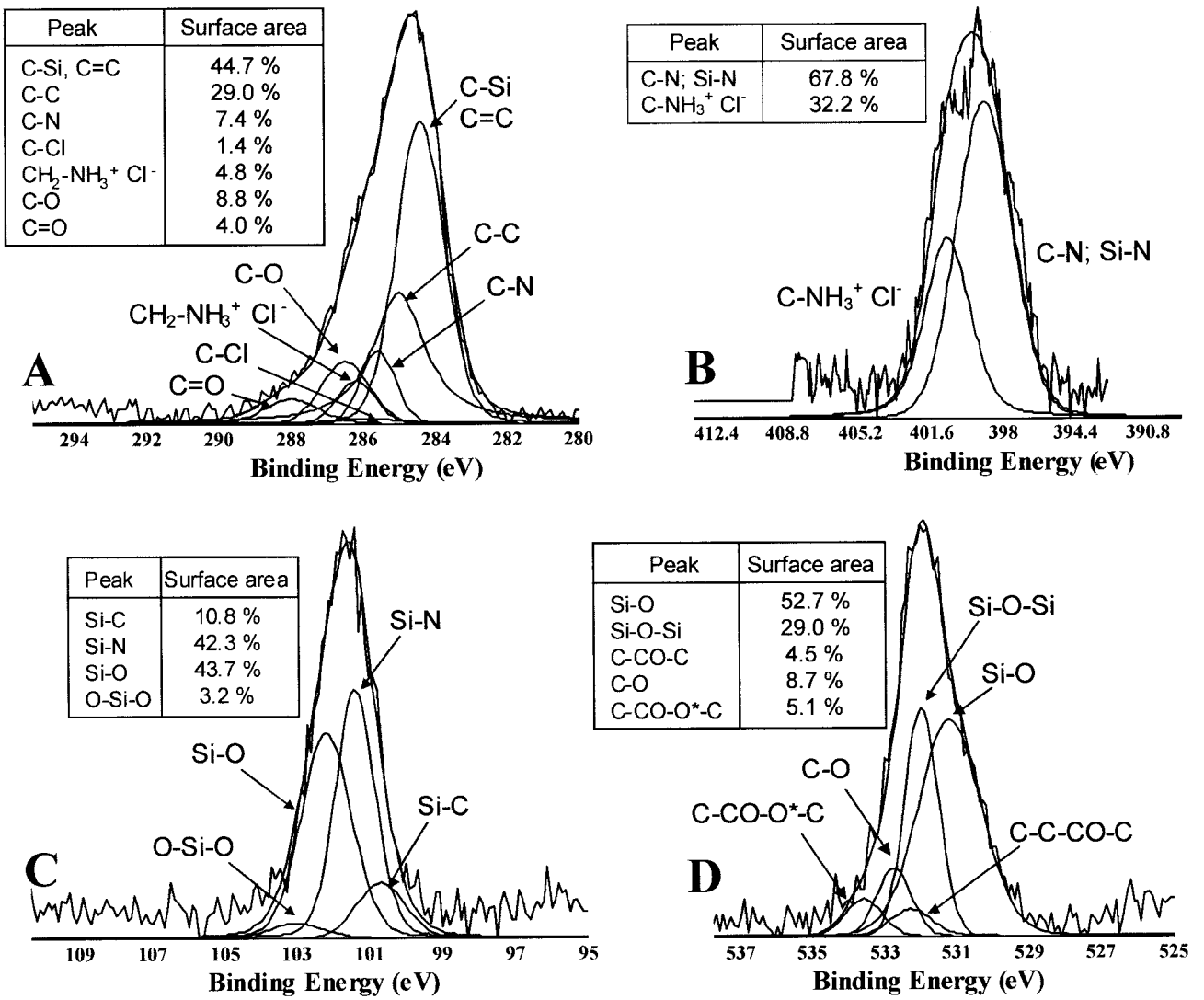


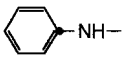
Figure 3 The (A) C1s, (B) N1s, (C) Si2p, and (D) O1s functionalities recorded from Ar/DS plasma-treated and subsequently *in situ* DP-functionalized PE.

The N1s diagram of PE—NH—CO has a bimodal pattern [Fig. 4(B)]. The peaks were assigned for —C—*NH^+—C(O)—$COOH^-$ (402.1 eV) and Si—N and N—C (401 eV) functionalities. The presence of both ionic and covalently linked chlorine atoms was also evidenced by the high resolution Cl_2p spectra of —CH_2—$NH_3^+Cl^-$ (198.5 eV), —C—$^+NH_2$—C(O)—C(O)—OH (Cl^-), and C—Cl (200.6 eV, spectrum not shown).

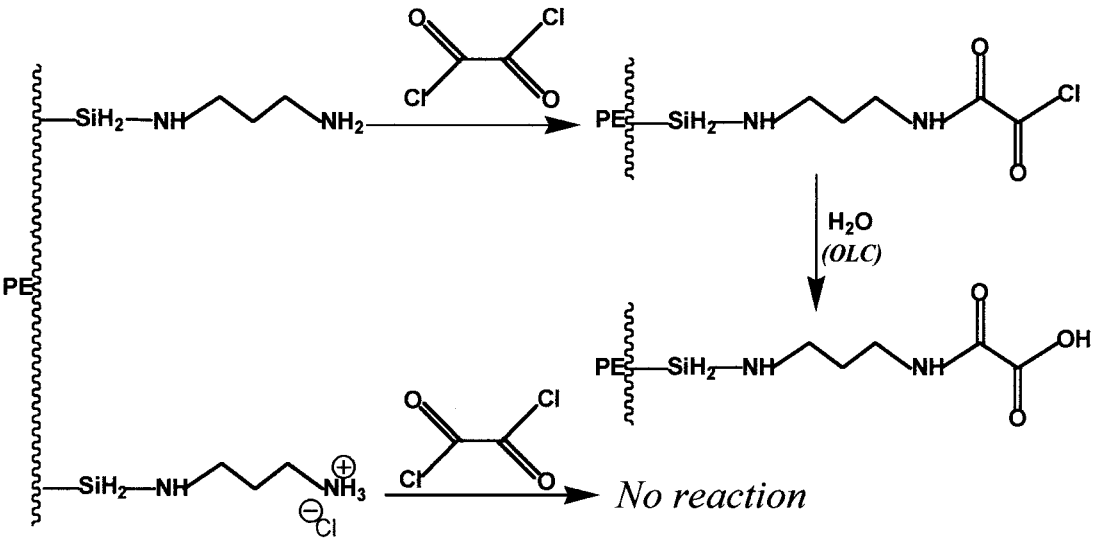
The relative surface atomic composition of I-HRP onto functionalized PE surfaces and free HRP (Table III) are comparable, which is indicative of the fact that the enzyme was successfully immobilized on the modified PE. The presence of low Si and Cl concentrations in the I-HRP indicates a very thin layer of covalently linked HRP and thus detection of the underlying Si and Cl atoms. Because of the extreme complexity of the HRP structure and the additional contributions from the underlying substrate, detailed functional group assignments for X-ray photoelectron spectroscopy spectra of HRP or I-HRP are not possible.

Differential ATR-FTIR analysis was performed on PE—NH_2, PE—NH—OC, and I-HRP surfaces. The high resolution IR spectra were investigated in the wavenumber regions of 1500–1700 and 2800–3700 cm^{-1}, where secondary amines with internal hydrogen bonding (amide I), carboxyl ions, and NH stretching absorptions of secondary amines and bonded —OH stretching bands, respectively, usually appear.[24,25] These absorptions should also be present in the PE—NH_2, PE—NH—OC, and I-HRP structures. As is well known, PE does not have any significant absorption in the 1500–1700 cm^{-1} range and above 3000 cm^{-1}. The IR spectra of PE—NH_2 are shown in Figure 5(A,B). The presence of characteristic primary (3250–3450 cm^{-1}) and secondary amine (3300–3500 cm^{-1}) stretching and deformation vibration regions (primary amines: 1580–1650 cm^{-1}; secondary amines:

TABLE II
High Resolution Peak Assignments in Literature

	Linkage	Model Compound	Binding energy (eV)	Reference
C1s	C—C	Polyethylene	285.0	16, p 54
		Polypropylene	285.0 and 285.2	16, p 56
	C═C	Poly(*cis*-butadiene)	284.7	16, p 64
		Poly(*cis*-isoprene)	284.7	16, p 68
	C—O	Poly(ethylene glycol)	286.5	16, p 84
		Poly(propylene glycol)	286.4	16, p 86
	C—O	Poly(vinyl methyl ketone)	287.97	16, p 98
		Poly(methyl isoprenyl ketone)	287.92	16, p 102
	C—N	Poly(ethylene imine) Poly(2-ethyl-2-oxazoline)	285.6–285.8	16, p 182, 194
	C—NH_3^+ Cl^-	Poly(allylamine hydrochloride) Poly(vinylbenzyltrimethyl ammonium chloride)	285.9–286.2	16, p 202, 208
	—NH—C*O—COOH	Oxamic acid	288.6	
	—NH—CO—C*OOH	Oxamic acid	289.3	
		Aldrich PANI	284.7	17
		PANI	284.54	18
		Aldrich PANI	285.9	17
		PANI	285.80	18
N1s	N—Si	Hexamethyldisilazane	398.0–398.5	19, 20
	N—C	Poly(ethylene imine)	399.1	16, p 182; 19–21
	Cl^- NH_3^+ C	Poly(allylamine hydrochloride) Poly(vinylbenzyltrimethyl ammonium chloride)	401.5–402.1	16, p 202, 208 19–21
Si2p	Si—C	Tetramethylsilane-plasma deposited layers	100.7–101.5	19–22
	Si—N	Hexamethyldisilazane-plasma deposited layers	101.4	19–22
	Si—O	—	101.4–102.5	19–22
	Si—$(O—C)_2$ and Si—$(O—Si)_2$	—	102.2–104.6	19–22
O1s	C═C—O	—	531.2	16
	C═O	Poly(vinyl methyl ketone) Poly(vinyl ethyl ketone) Poly(methyl isopropenyl ketone)	532.3–532.6	16, p 98, 100, 102
	C—O	Poly(propylene glycol) Poly(tetramethylene glycol) Poly(vinyl methyl ether)	532.8	16, p 86, 88, 90
	C(O)—*O—C	Poly(methyl acrylate) Poly(D,L-lactide) Poly(3-hydroxybutyrate)	533.6–533.8	16, p 114, 136, 138
	Si—O	Hexamethyldisiloxane	531.4	23
	Si—O—Si	Poly(dimethylsiloxane)	532	16, p 268
C12p3	C—C	Poly(vinyl chloride)	200.64	16, p 240
		Polychloroprene	200.63	16, p 248
	C—NH_3^+ Cl^-	Poly(allylamine hydrochloride) Poly (vinylbenzyltrimethyl ammonium chloride)	197.7	16, p 202, 208

1490–1580 cm^{-1}) confirms that the reaction between the plasma implanted $SiCl_x$ surface functionalities and DP was successful. The extension of the primary amine, broad stretching absorption close to 3100 cm^{-1} and the presence of a strong 1500–1600 cm^{-1} deformation region can be attributed to the existence of amine hydrohalide structures.[24,25] In the case of PE—NH—OC structures, the IR diagrams [Fig. 5(A,B)] reveal the existence of a unimodal C═O (amide I: 1654 cm^{-1}) vibration, a strong —C(O)—O^- (1610 cm^{-1}) absorption, and a broadband absorption in the 1500–1550 cm^{-1} region that was assigned to NH deformation [amide II band, keto form of secondary amides: R—C(O^-)—NH^+—] vibration [Fig. 5(A)]. The broad bimodal absorption band extending between 2950 and 3200 cm^{-1} [Fig. 5(B)] can be related to the presence of bonded —OH stretching of COOH (in most of the cases the main peak is located near 3000



Scheme 2 The amide group formation reactions.

cm^{-1}) and to bonded NH stretching of secondary amides, respectively.

The comparative IR spectra of free HRP and thoroughly rinsed and vacuum-dried I-HRP [Fig. 6(A,B)] reveal the existence of identical absorption zones, which validate the earlier conclusion that HRP was successfully immobilized on functionalized PE surfaces. The small differences noted in the absorption peak areas of PE-HRP relative to HRP might be related to the very thin layer nature of the I-HRP and to the presence of a nonuniform enzyme coating.

The concentration of HRP bound to PE sample surfaces, which was calculated from the absorption difference measurements from HRP, was found to average 0.3 μg/cm^2 of HRP. The activity of I-HRP relative to the free enzyme was evaluated by monitoring the UV absorption intensity of purpurogallin generated by the free HRP and I-HRP during the assay test (Fig. 7). The amount of free enzyme employed was equal to that estimated to be immobilized on the PE. As shown in Figure 7, purpurogallin is generated faster by the free HRP than by the immobilized one. However, it is not possible to quantify the differences in reactivity between the free and bound HRP in view of the strong and rapid adsorption of purpurogallin to the PE substrate. This adsorption not only reduces the solution phase concentration of purpurogallin but may also inhibit the enzyme activity. However, it is significant to note that the I-HRP is still catalytically active as shown by the progressive increase in purpurogallin concentration after a prolonged reaction time.

Aniline was polymerized using both free and immobilized enzymes as initiators, and the structures and molecular weight distributions of the resulting PANIs were compared. The intrinsic spectrum of PANI (Fig. 8) could be resolved into three peaks at different binding energies (284.7, 285.9, and 286.5 eV).

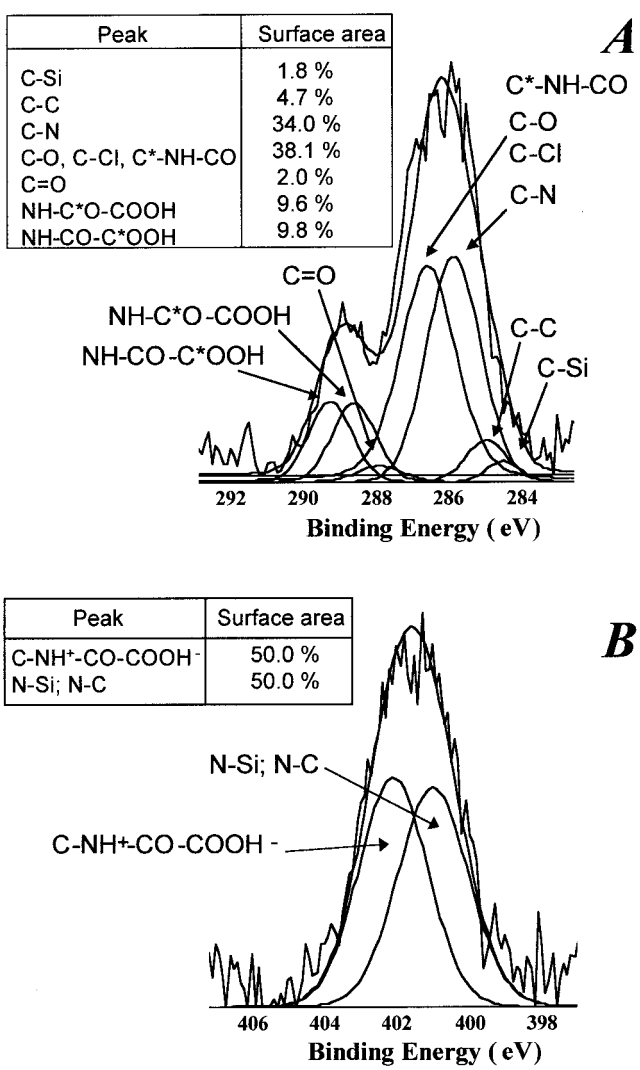


Figure 4 The (A) C1s and (B) N1s functionalities recorded from Ar/DS plasma-treated and *in situ* DP- and subsequently OC-functionalized PE.

TABLE III
Survey ESCA Data from Free and Immobilized HRP

	Composition (%)	
Peak	Free HRP	Immobilized HRP
C1s	62.8	58.6
O1s	27.3	25.9
N1s	9.9	10.0
Si2p	—	4.0
C12p3	—	1.6

The peak at 284.7 eV was attributed to C═C, and C—C bonds, while the peak at 285.9 eV was due to the presence of C—N bonds. The broad higher energy peak (287.8 eV) can probably be related to C═O groups. Carbonyl functionalities have been observed in PANI samples, regardless of the synthetic route.[17,18] The existence of C═O bonds can be related to the development of oxidation reactions of PANIs during the polymerization reactions or under OLCs after the synthesis. The relative ratios of C and N atoms involved in the enzyme-synthesized PANI structure (free HRP: C/N = 2.03; I-HRP: C/N = 2.19) are comparable to that resulting from the theoretical structure of PANI (C/N = 2).

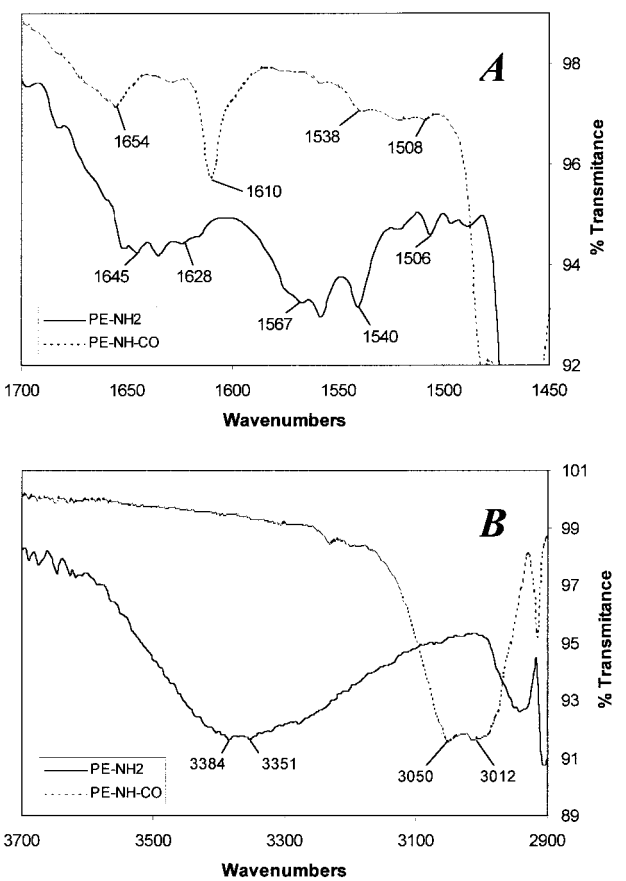


Figure 5 The differential FTIR data collected from Ar/DS plasma-treated and subsequently *in situ* DP-functionalized PE (PE-NH_2) and from Ar/DS plasma-treated and *in situ* DP- and subsequently OC-functionalized PE (PE—NH—CO).

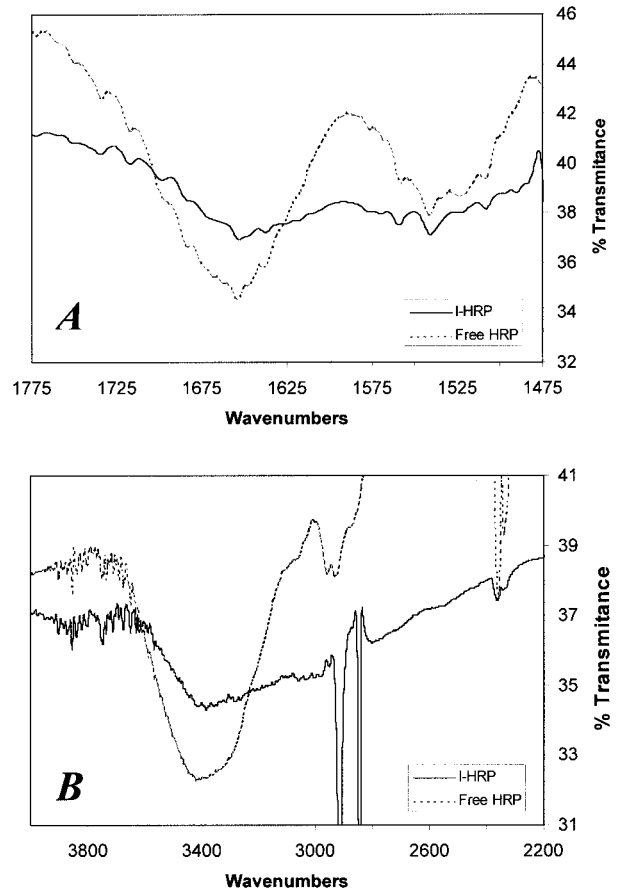


Figure 6 The differential FTIR data collected from I-HRP and FTIR data collected from free HRP.

Figure 9 provides molecular weight data of PA synthesized using HRP and I-HRP as initiators. One can observe that similar molecular weight distribution patterns result, regardless of whether the enzyme is in the free form or immobilized on PE. The trimodal GPC curves indicate a molecular weight distribution range

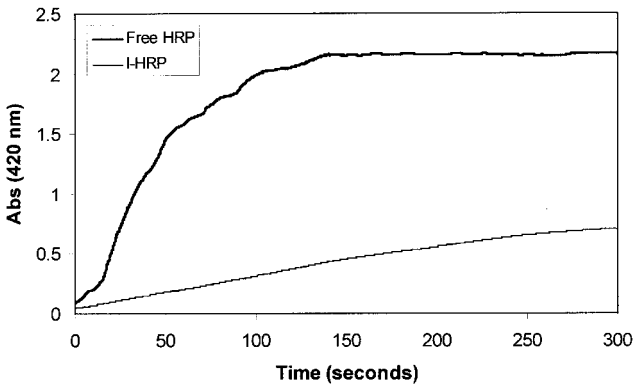


Figure 7 The activities of free and I-HRP evaluated as the absorption intensity of the enzyme-generated purpurogallin.

between the limits of 7,000 and 32,000, which is comparable to the molecular weight distribution values reported by other laboratories using free HRP initiated polymerization of aniline. It should be mentioned that a blank run of the solution containing only the free HRP did not show the presence of any high molecular weight compounds.

CONCLUSIONS

HRP was successfully immobilized onto plasma-functionalized PE surfaces and used for the synthesis of PANI in the presence of hydrogen peroxide. The presence of I-HRP and the spacer-chain molecules involved in the immobilization process were evidenced by ESCA, ATR-FTIR, and chemical (fluorescence) derivatization techniques. The molecular weight distribution of PANIs synthesized by free HRP and I-HRP reaction mechanisms were evaluated using GPC. It was found that both enzyme assisted polymerization reactions resulted in PANI macromolecular structures with comparable molecular weight distributions. These investigations open up additional ways for the generation of thin PANI layers on various substrate surfaces, with potential applications for the development of biosensors. It is also important to note that the surface modification process described in this article is one general utility that should be usable for successful surface immobilization of biomolecules, including other enzymes.

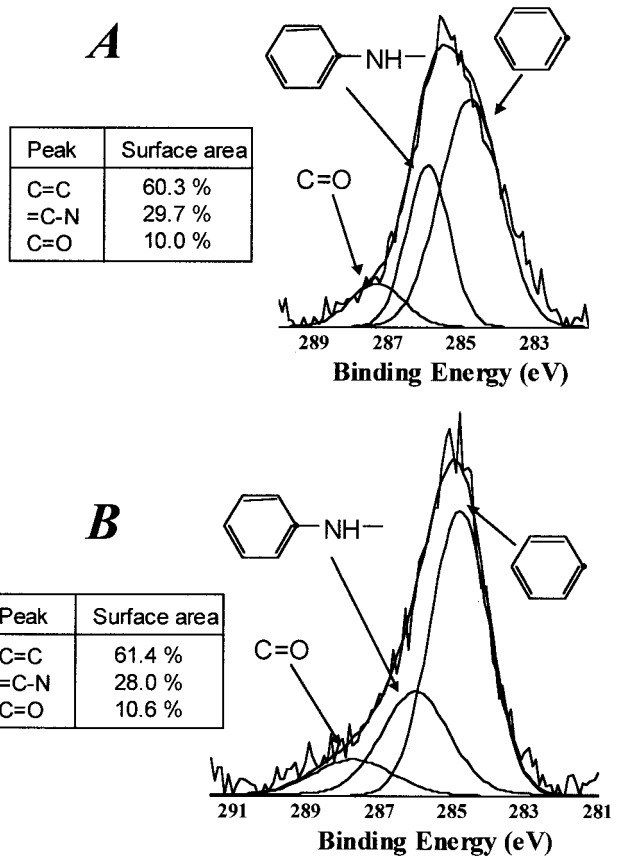


Figure 8 The C1s functionalities recorded from PANI synthesized in the presence of (A) free HRP and (B) I-HRP.

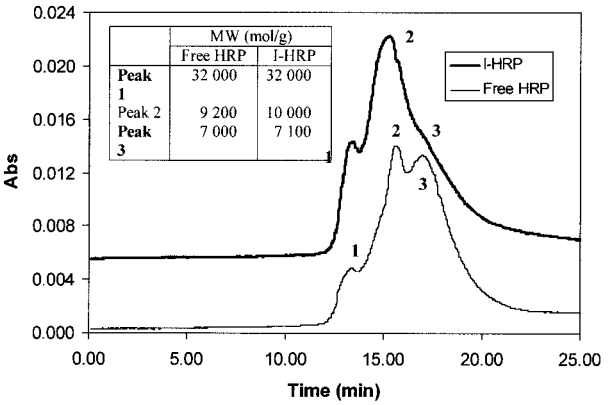


Figure 9 The molecular weight distribution of PANI synthesized by free HRP and I-HRP evaluated using GPC.

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