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A novel plasma-enhanced way for surface-functionalization of polymeric substrates

S. Alvarez-Blanco[2], S. Manolache[2], F. Denes[1,2] (✉)

[1] Biological Systems Engineering and [2] Center for Plasma-Aided Manufacturing, University of Wisconsin-Madison, Madison WI 53706, USA

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Summary

In this contribution a novel, low-pressure RF-plasma-enhanced approach is suggested for the surface functionalization of polymeric substrates. The method takes advantage of the high reactivity Ar-plasma generated free radical sites located on PE substrate surfaces, which can promote under *in situ* conditions, heterogeneous chemical reactions with stable gas-phase molecules, such as 1,3 diamino propane, in the absence of plasma. The presence of covalently attached primary amine functionalities was demonstrated by fluorescamine-labeling technique, further oxallyl chloride-based derivatization, and immobilization of horseradish peroxidase. The existence of specific functionalities on modified PE surfaces at different derivatization stages, was evidenced by ESCA, ATR-FTIR, and enzyme assay tests.

Introduction

The use of non-equilibrium plasma approach for the synthesis of novel thin-layer macromolecular structures and surface modification of various inorganic and organic polymeric substrates, has become a very active research area recently (1–4). Plasma technologies are primarily considered for applications where materials surface characteristics, including composition, functionalities and morphology, have a determinant influence on device performances. Molecular recognition processes based on immobilized synthetic and natural active biomolecules (e.g. immobilized enzymes, anti-bodies, oligonucleotides, etc.) and which will take a leading role in the next future in the development of advanced technologies and analytical techniques (e.g. oligonucleotide libraries, biosensors, etc), have already taken advantage of special characteristics of non-equilibrium plasma processes.

It is recognized, that the first step in the development of devices-based molecular recognition processes is the surface functionalization of selected targets. Active horseradish peroxidase has been already immobilized on acrylic acid and acrylamide radiation-grafted polymer surfaces (5-6). In this work cold plasma-technique has been used for the surface functionalization. Immobilization of bioactive molecules onto synthetic and natural polymeric material surfaces, often require the presence of primary amine functionalities. Early attempts considered for the plasma-enhanced implantation of primary amine functionalities were ammonia discharge

environments. However, due to the extensive fragmentation of NH_3, other saturated, non-saturated and aromatic amines were also used as primary amine group precursors (7–22). Recently it has been shown that hydrazine-RF-plasmas are more adequate in comparison to ammonia discharges for the generation of surface primary amine functionalities on synthetic polymer surfaces (23 and 24).

Non-equilibrium plasma-mediated surface functionalization reactions have their shortcomings. Most of the precursor molecules of the desired surface functionalities (e.g. ammonia, hydrazine, saturated and non-saturated amines, etc.) can undergo plasma-induced, intense fragmentation processes. As a consequence, in addition to the desired functionalities other than those required will be implanted onto the substrate surfaces. These processes are also accompanied by the production of extremely reactive surface-free-radicals, and charged centers, which can induce further active-surface-mediated chemical reactions with the gas-phase plasma components through a variety of pathways. Plasma-generated free radicals (surface and stable, caged free radicals) can also initiate under *in situ* or *ex situ* environments in the absence of plasma, non-specific interactions with target molecules (e.g. biomolecules), which obviously will significantly diminish the molecular recognition capabilities of the modified substrates.

In this contribution the development of a novel plasma-enhanced, two step, *in situ* surface functionalization process is described, based on the high reactivity of surface-species created by inert gas plasma. During the first step free radicals and possibly charged species are generated under inert-gas discharge (e.g. argon-plasma) environments on selected polymeric substrate surfaces, followed by a second step reaction of selected, stable precursor-molecules under vacuum conditions (*in situ*), with the plasma-activated surfaces, in the absence of plasma.

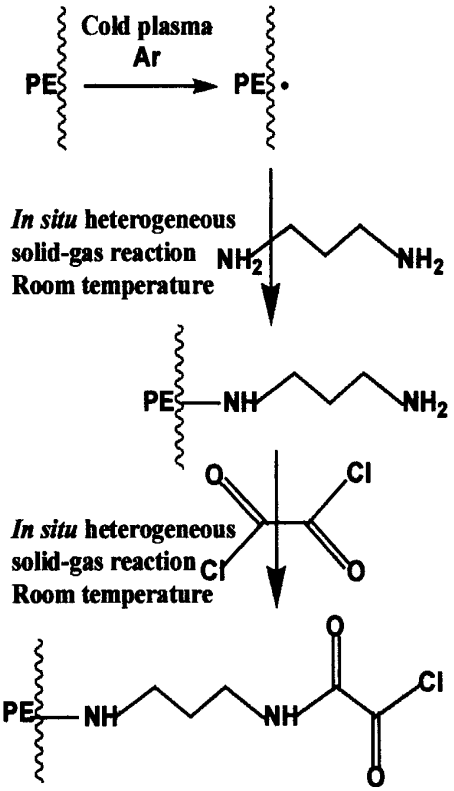
Experimental

Materials and Methods

High purity argon and oxygen, employed for decontamination of the reactor in the presence of plasma, were supplied by Liquid Carbonic. 1,3-Diamino Propane (DP) and oxalyl chloride (OC), selected for the chemical derivatization reactions, were purchased from Aldrich Co. All chemicals used for the enzyme immobilization reactions and assays have been described earlier (3, 4). Fluorescamine was purchased from Molecular Probes Inc., (Eugene, OR). The surface analytical techniques and related experimental conditions, involved into the characterization of modified substrates including, survey and high resolution ESCA, ATR-FTIR, and AFM have been presented before (4).

All plasma-enhanced functionalization reactions were carried out in a cylindrical stainless steel, capacitively coupled (disc-shaped stainless steel electrodes; electrode diameter: 20 cm; gap: 3 cm), RF-plasma-reactor, equipped with 13.56 MHz power supply (4) with pulsing capability. During the plasma-treatments the following experimental conditions were used: Pressure of argon: 200 mT; RF-power dissipated to the electrodes: 100 W; Treatment time: CW-plasma mode: 1 minute; Pulsed-plasma mode: (Duty: 10 % on, 90 % off, Period: 100 ms): 10 minutes; (Duty: 20 % on, 80 % off, Period: 100 μs): 5 minutes.

The immobilization of Horseradish Peroxidase (HRP) was carried out according to the following reaction mechanism:



Results and discussion

Survey and high resolution ESCA data originating from CW and pulsed Ar-plasma treatments of PE substrates (Figure 1) indicate that in all cases a significant oxygen incorporation is generated into the PE substrate surfaces as a result of free-radical-induced, post plasma oxidation mechanisms developed under open laboratory conditions (OLCs). The presence of C–O (286.5 eV), =C=O (288 eV), and =C=C= (284.5 eV) bonds can be identified on modified sample surfaces. It is noteworthy that the plasma exposure mode (CW and pulsed) and the duty characteristics influence the relative ratios of various functionalities and their concentrations (relative peak-surface areas). The highest oxidation levels and =C=O concentrations are associated with the 100 ms, 10% on, pulsed-plasma mode. What is surprising is related to the incorporation of nitrogen under OLCs, into the surface layers of the PE samples. Repeated experiments carried out under identical and similar plasma tool conditions indicate that Ar-plasma generated active sites (e.g. free radical sites) present on PE surfaces, interact with the

Peak	Composition (%)				
	Un	T1	W1	T2	T3
C	97.6	73.6	81.5	80.5	77.5
O	2.4	18.8	16.2	16.3	15.7
N		7.6	2.3	3.8	6.8

Peak	Composition (%)			
	Un	T1	T2	T3
C=C		13.1	10.2	10.1
C-C	93.9	59.2	66.2	66.8
C-N		7.8	5.0	7.4
C-O	6.1	7.5	11.5	7.4
C=O		11.8	7.1	8.0

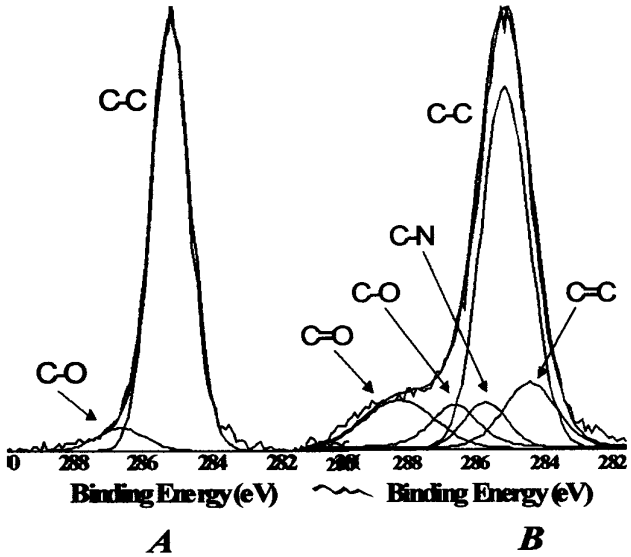
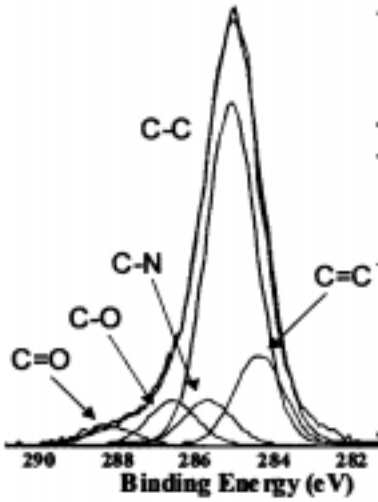


Figure 1. Survey ESCA data and C*1s* functionalities of untreated (A) and Ar-plasma treated T1 (B) polyethylene. T1: 100 ms period, 10% on, 10 min treatment time; T2: 100 μs period, 20% on, 5 min treatment time; T3: CW, 1 min treatment time; W1: T1 after washing; Un: untreated.

molecular nitrogen of air, and covalently incorporate the nitrogen atoms into the surface layers of PE. The highest relative nitrogen atomic- and C–N concentrations are also generated similarly to the oxygen-based functionalities, under the longest duty period and shortest plasma-on conditions (100 ms, 10% plasma on). However, the relative surface nitrogen atomic concentration decreases dramatically after washing the modified PE-substrates with water, and fluorescamine labeling technique indicates the presence of nitrogen-based functionalities other than the primary amine groups. It is suggested that imine group formation might be responsible for the nitrogen incorporation; these functionalities undergo hydrolysis in the presence of moisture with the generation of surface =C=O groups and NH_3. The generation of oxygen- and nitrogen-based functionalities on Ar-plasma-exposed PE substrate surfaces under OLCs, indicate, that extremely reactive sites (e.g. free radical sites) are created as a result of interaction of Ar-plasma species with the surfaces which confine the discharge.



Peak	Composition (%)	
	T1	T2
C	79.0	78.2
O	15.4	14.6
N	5.6	7.2

Peak	Composition (%)	
	T1	T2
C=C	10.7	16.6
C-C	68.1	63.5
C-N	8.2	8.4
C-O	8.8	8.2
C=O	4.2	3.3

Figure 2. Survey ESCA data and non-equivalent C *1s* functionalities of Ar-plasma treated and subsequently *in situ* DP-functionalized PE. T1: 100 ms period, 10 % on, 10 min treatment time; T2: CW, 1 min treatment time.

PE substrate surfaces resulting from *in situ*, post plasma amination of Ar-plasma-modified samples using DP, show the presence of C–O, =C=O and C–N bonds (Figure 2). The reduced relative oxygen atomic concentration and C=O peak surface area indicate that the Ar-plasma-induced free radical sites were quenched by the DP molecules. It should be noted that the relative nitrogen atomic concentration and the relative concentration of C–N functionalities are comparable to the highest nitrogen contents and C–N concentrations of the Ar-plasma- and OLCs-exposed samples. However, ESCA analysis cannot distinguish imine from primary amine functionalities, while fluorescamine-labeling technique allows the identification of primary amine functionalities. Comparative images generated from fluorescamine labeled

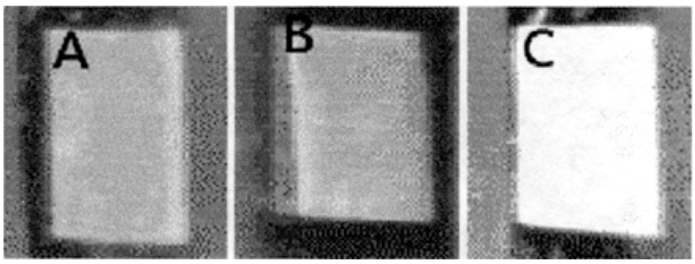


Figure 3. UV flourescence image of untreated (A), Ar-plasma-treated (B) and Ar-plasma-treated and *in situ* aminated (C) PE.

PE, Ar-plasma-treated PE and Ar-plasma-treated and *in situ* aminated PE clearly indicates that fluorescence is solely associated with the aminated substrates (Figure 3). These allow us to conclude, that plasma-generated free radicals can covalently anchor DP under *in situ* conditions, and generate primary amine functionalities on PE surfaces.

Aminated substrates exposed under vacuum conditions to oxallyl chloride (OC) vapors, show the presence of specific chloroacide/carboxilic functionalities [–NH–C(O)–*C(O)–Cl; 289.2 EV)] on PE surfaces (Figure 4). The very low chlorine atomic concentrations associated with the OC-modified substrates can be explained by the hydrolysis of chloroacide groups in the presence of moisture.

Peak	Composition (%)	
	T1	T2
C=C	7.8	9.7
C-C	53.2	45.2
C-N	15.8	18.4
C-O	14.4	16.3
C=O	2.8	3.3
NH-C*O-CO	3.4	3.1
NH-OC-C*OOH	2.6	3.9

Peak	Composition (%)	
	T1	T2
C	73.2	71.8
O	15.4	14.4
N	9.5	11.8
Cl	1.9	2.0

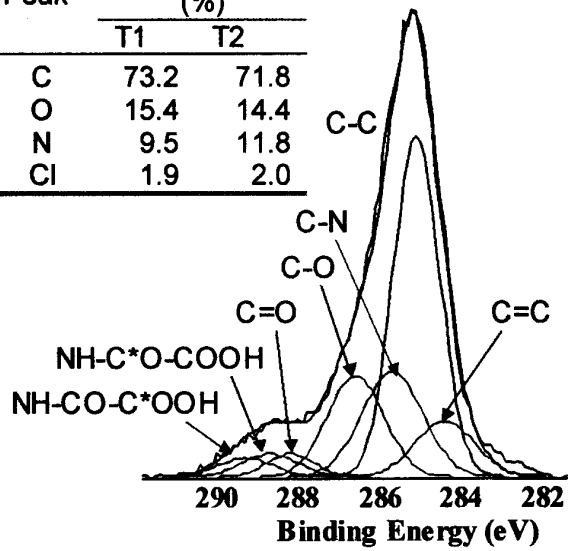


Figure 4. Survey ESCA data and non-equivalent C*1s* functionalities of Ar-plasma treated and *in situ* DP and subsequently OC functionalized PE. Ar-plasma conditions: T1: 100 ms period, 10 % on, 10 min treatment time; T2: CW, 1 min treatment time.

Results from Differential ATR-FTIR analysis of PE–Ar, PE–NH_2, and PE–NH–OC surfaces (Figure 5) substantiate the findings of ESCA data. The HR IR spectra were investigated in the wavenumber regions of 950–1450 cm^{-1} (Figure 5 A), 1450–1750 cm^{-1} (Figure 5 B) and 2900–3700 cm^{-1} (Figure 5 C), where C–O–C groups, secondary amines with internal hydrogen bonding (Amide I), carboxyl ions, and NH stretching absorptions of secondary amines and amides, and bonded –OH stretching absorptions respectively usually appear (25, 26). The presence of oxygen / carbon- and nitrogen / carbon-based linkages can be identified on the functionalized substrates. The low-wavenumber region of the IR diagram (Figure 5 A) shows the presence of a strong bimodal vibration (1000 and 1100 cm^{-1}) indicating the formation of C–O–C linkages as a result of argon-plasma treatment of PE substrates. The intensity of this absorption is significantly diminished as a result of amination mechanism, owing to the recombination of DP with the Ar-plasma generated free radicals. This absorption pattern can also be identified in the spectra of aminated substrates. The aminated PE samples exhibit in the middle spectral region (Figure 5 B), the characteristic 1650 cm^{-1} and 1575 cm^{-1} absorptions of Amide I, C=O groups and of NH deformation vibrations of primary and secondary amines. The existence of a strong 1660 cm^{-1} absorption in the spectrum of OC-treated PE is indicative for the presence of keto-form secondary

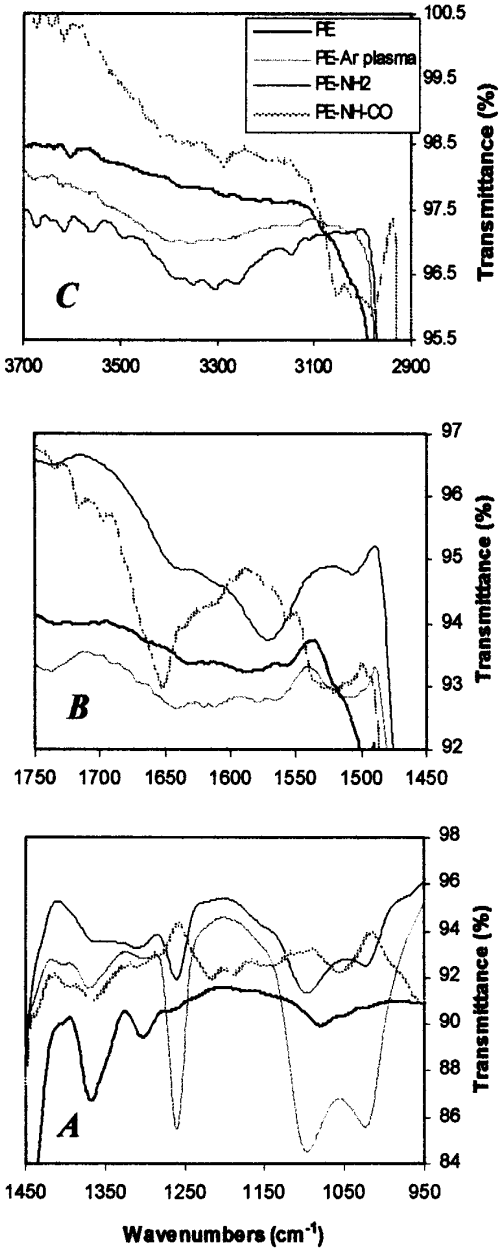


Figure 5. ATR-FTIR data of untreated PE, and differential ATR-FTIR data of Ar-plasma treated, Ar-plasma treated and subsequently DP fucntionalized (PE-NH_2) and Ar-plasma treated ar DP and OC functionalized (PE-NH-CO) PE.

stretching mode of secondary amides. These findings substantiate the results of ESCA investigations that PE–NH–$(CH_2)_3$–NH–OC(O)–C(O)–OH chains have been generated on PE substrate surfaces.

The comparative IR spectra of amides ($R–C(O)^-–NH^+$), that allow us to suggest that the reaction between the primary amine functionalities and the OC underwent successfully. The broad vibration zone (3100 – 3600 cm^{-1}) (Figure 5 C) associated both with Ar-plasma treated PE and Ar-plasma-treated and consecuti-vely *in situ* animated PE, can be related to the presence of OH groups, and to the implantation of primary amine functionalities as a result of the PD-mediated process. A strong multimodal vibration present in the spectrum of OC-modified substrates, in the 2950–3150 cm^{-1} wavenumber zone, has been assigned to the presence of bonded OH stretching mode of –COOH functionalities and to the bonded

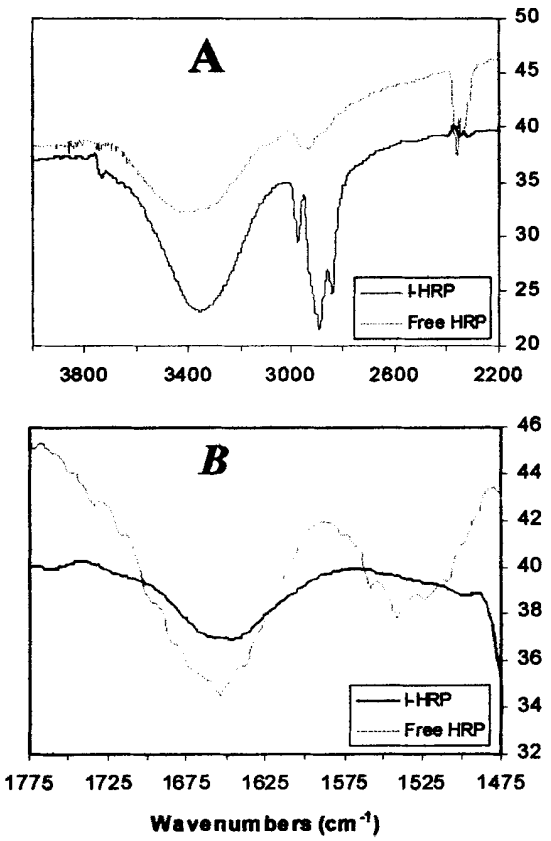


Figure 6. ATR-FTIR data collected from free HRP and differential ATR-FTIR from I-HRP.

free HRP and thoroughly washed and vacuum-dried I-HRP (Figures 6), reveal the existence of strong absorption zones, centered around 3400 and 1675 cm^{-1} which indicates that the HRP was immobilized on functionalized PE surfaces.

Figure 7, exhibits 3D AFM images of PE, of Ar-plasma-treated and consecutively, *in situ* aminated PE, and that of PE bearing the immobilized HRP. It can be observed that the relatively smooth surface- morphology of PE is replaced by cone-type topographies, as a result of the derivatization process. It is suggested that the etching reaction mechanisms, and which accompany the Ar-plasma treatments are responsible for the formation of cone-type struc-tures. The attachment of the HRP to the functionalized PE surface did not change significantly the specific surface topography (The dimensions of HRP-molecules relative to the surface roughness of aminated PE are negligible).

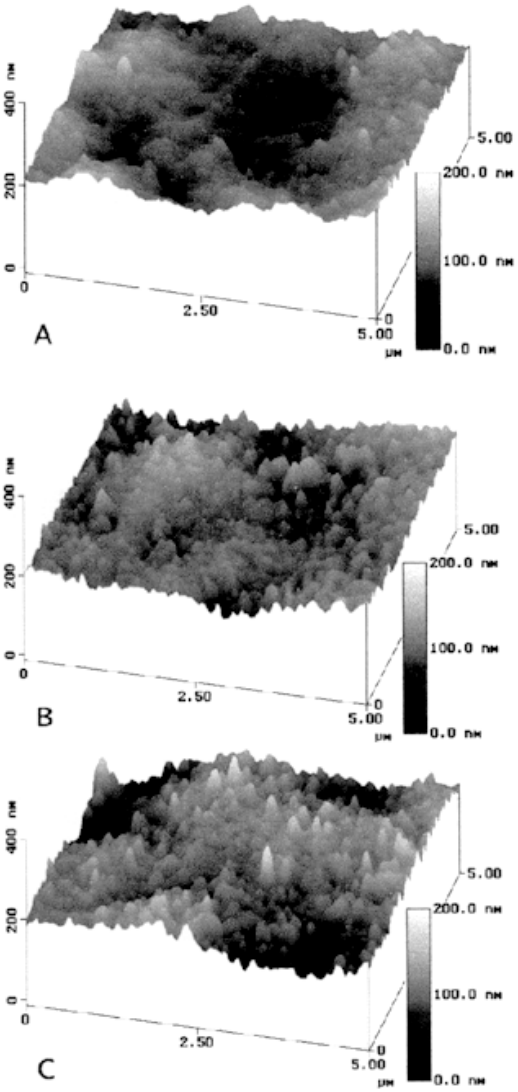


Figure 7. AFM images of untreated (A), aminated (B) PE and aminated PE bearing immobilized enzyme (C).

The activity of I-HRP relative to the free enzyme was evaluated by monitoring the UV absorption intensity of purpurogallin generated by the free and immobilized HRP (27) during the assay (Figure 8). The diagrams resulted from normalized enzyme concentration indicate that the I-HRP retained only a fraction of the activity of the free enzyme. However, due to the fact that a part of the enzyme-generated purpurogallin is adsorbed onto the PE surface during the assay, and that the amount of HRP hardly can be quantified and related to the UV absorption, the evaluation of the activity of I-HRP has a qualitative character only.

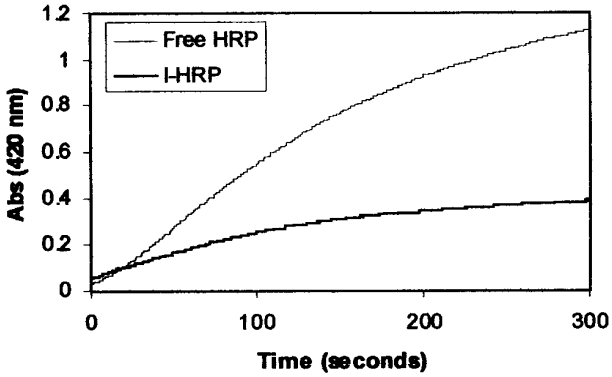


Figure 8. Activity of free and immobilized HRP evaluated as the absorption intensity of the enzyme-generated purpurogallin.

Conclusions

It was demonstrated that inert-plasma-generated free radical

sites can promote chemical reactions with stable gas-phase molecules under *in situ* environments in the absence of plasma. ESCA, ATR-FTIR, fluorescence labeling technique, and the activity of the immobilized HRP clearly demonstrate that the covalently linked primary amine functionalities can be involved into further derivatization reactions, and the resulting spacer chain molecule bearing chloroacide end-groups can immobilize active HRP. This novel procedure opens up a very convenient, efficient way for the surface functionalization of polymeric substrates for biotech applications.

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