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# Plasma modified polymers as a support for enzyme immobilization 1. Allyl alcohol plasma

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**Abstract**

The paper describes deposition of plasma polymerized allyl alcohol on polysulfone film. It is shown that film surface becomes more hydrophilic after plasma treatment independently on presence of argon in a reaction mixture. The chemistry of the new surface layer was established by FTIR-ATR and ESCA spectroscopy. The substrate placed close to the plasma edge was the most hydrophilic but the amount of hydoxyl groups was not the highest there. Presence of argon stabilized the plasma but the deposited layer contained relatively less oxygen-bearing functionalities. The plasma treated polymer was subjected to xylose isomerase immobilization. For this purpose the divinylsulfone method was adapted. The studies revealed no correlation between the surface hydrophilicity and efficiency of immobilization.


*Keywords:* Plasma deposition; Effect of argon; Polysulfone; Xylose isomerase

## 1. Introduction

Immobilization of molecules means in principle any technique that limits their free migration. Many immobilization methods have been developed but they all belong to one of two main types: chemical attachments or entrapment [1]. Chemical attachments mean covalent bonding or reversible adhesion primarily through ionic or hydrophilic interaction. Immobilization most often concerns biologically active agents such as antibodies [2,3], enzymes (such as β-galactosidase [4], glucose isomerase [5–7], invertase [8,9], glucose oxidase [10–12], inulinases [13], endopeptidase bromelain [14], papain [15], acylase [16], urokinaze [17], glucoamylase [18], lactate dehydrogenase [19], insulin [20]), various proteins and peptides (protein A [21,22], protein-4 (BMD-4) [23], oligopeptides [24], fibronectin [25], collagen [26, 27]), polysaccharides (heparin [28–33], dextran [34], hyaluronic acid [35]) and bacteria [1,36]. Immobilization is becoming a leading technology in production of bio- and immunosensors [2,3,11,12,19] and biomaterials [17,20–24,26–30,32,35,37]. A large number of natural and synthetic polymers, for example alginate [12], chitosan [14], cellulose acetate [1] and nitrate [2], PTFE and others fluoropolymers [4,17,24,29,34,35], PE [15], PP [35], poly(HEMA) [22], poly(D,L-lactide) [26], PET [27] polyurethane [28] polysulfone [31], PS [29,30, 35,36] also modified [5], organosilicon polymers [25] and various copolymers [8,10,19–21] have been used as solid substrates for attachement of biomolecules.

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Polysulfone is an excellent material in its chemical stability and mechanical and thermal properties. Its ability to form solid and porous films places it among the materials most frequently used for production of high performance membranes. Due to its low surface energy, however, enhanced protein adsorption takes place, which is believed to be the origin of the fouling of membranes. It proved possible to minimize this unwanted result by hydrophilization of the surface effected mostly by grafting of polar functional groups onto the membrane surface.

Recently plasma techniques have frequently been used to provide materials with surface functional groups that may play the role of "anchoring sites" for biomolecules. From a chemical point of view low-pressure plasma is a very reactive medium containing radicals, ions, and metastable energetic species. They can affect the polymer surface in various ways, depending mainly on the gas they come from. Inert gases (Ar, He) etch the material surface and introduce radicals that, on contact with air, create peroxides, hydroperoxides and various other polar groups. Reactive gases [$N_2$, $O_2$, $NH_3$, $CO_2$, ...] introduce functional groups onto the surface. Plasma of most organic vapours and gases form on the surface a deposit called plasma polymer. The presence of peroxides on the surface after plasma treatment makes possible graft polymerization. All these described processes can occur in the plasma simultaneously and which one dominates is predominantly determined by the gas and plasma conditions used. They all have been used for the task of immobilization: (a) plasma-induced graft polymerization [17,20,24,25,27,28], (b) treatment with nonreactive and reactive gases [2,26,29,35] and (c) plasma polymerization [3,11,15,18,23,34,38].

Plasma processes have many advantages. Among them the following seem to be of special interest: (a) they alter only the surface of the material, with no changes introduced to the bulk properties, (b) they are highly versatile—the same apparatus and class of process can be utilized for completely different applications.

In the plasma polymerization process the starting monomer is highly degraded. The resulting fragments are scrambled, hence the deposited film is significantly different from that obtained from conventional polymer. No repeating monomeric unit can be found in it. To obtain a deposit that preserves the chemical nature of the monomer it is necessary to apply mild plasma conditions—pulsed plasma at a low power regime and/or positioning of the substrate in the post-discharge area [39].

Plasma polymerization of allyl alcohol was intensively investigated [40–48] and high retention of hydroxyl group (50–70%) [41–43] was confirmed experimentally. This monomer has a sufficiently high vapour pressure to produce a constant flow into the reactor. Due to the presence of a double bond in the molecule its plasma polymerization occurs more readily and faster than deposition of any saturated alcohol [49]. What is also important, aging experiments carried out on plasma polymers of allyl alcohol (up to 350 days) showed no significant change in O/C ratio nor in the relative amounts of the various functional groups [50]. This monomer, however, has rarely been used for polymer surface modification. To the best of our knowledge it has been applied for plasma treatment of polyethylene [51,52] and polypropylene [39] only.

In the present study modification of polysulfone *via* plasma polymerization of allyl alcohol was investigated. Our experience has pointed at critical role of argon in plasma stabilization [53,54]. For this reason this paper compared the effects of plasmas obtained wirh allyl alcohol and its admixture with argon. The presence of hydroxyl groups attached to the polymer surface makes the membrane capable of further modification. The goal of the study was to show some benefits of using modified polysulfone as a support for immobilization of an enzyme (xylose isomerase).

## 2. Experimental

### 2.1. Materials

Polysulfone (PSU) Udel P-1700 was purchased from Amoco, Co., US. Allyl alcohol was supplied by Aldrich Chem. Co. Ltd., England. The solvents used throughout this study were analytical grade *N*,*N*-dimethylformamide (DMF) and chloroform ($CHCl_3$) received from POCh, Poland. Diiodomethane, divinylsulfone (DVS), Lowry Assay Kit and tryptamine were supplied by Sigma Chemical Co, fructose and glucose by Merc, argon—by Linde Gas, Poland. Technical glucose isomerase (Maxazym GI, K8587A) with the specific activity of 3.3 U/mg was kindly donated by Gist-Brocades.

### 2.2. PSU film formation

Polysulfone films were casted from 20% DMF solution onto glass plates and dried 6 h at 120 °C.

### 2.3. Plasma treatment

A microwave plasma generator of 2.45 GHz frequency (Plazmatronika, Poland) was used throughout this study. Plasma was generated in a quartz tube at the top of a reaction chamber. Membranes were attached to the table in the post-discharge area at various distance (10.5, 45.5 and 65.5 mm) from the lower edge of the plasma. Pulsed plasma with a pulse frequency of 125 Hz and 25% of duty time was applied. Plasma parameters were set as follows:

power (counted on continuous plasma): 60–180 W, initial pressure: $5 \times 10^{-3}$ mbar, monomer pressure: 1 mbar,
argon flow rate (when applied): 15 $cm^3$/min, polymerization time: 0–180 s.

### 2.4. Surface characterization

#### 2.4.1. Contact angle measurements

Static contact angles of liquid droplets (ca. 3 μl) were measured using TM 50 System (Technicome SA, France) equipped with Panasonic GL 350 camera. Measurements were carried out on both untreated and plasma-treated gel membranes. Droplets were positioned at different locations on the film surface. An average value of contact angle was calculated from at least 20 independent measurements. Double distilled water and diiodomethane were used as probing liquids; their polar and non-polar components of surface tension were taken after Kuznietsov [55]. From contact angle data the surface tension and its polar and dispersive components were calculated according to harmonic averaging [56].

#### 2.4.2. FT IR measurements

All IR spectra were obtained by the attenuated total reflection (ATR) technique using a Perkin–Elmer System 2000 spectrometer with a horizontal ATR device (Ge, 45°). Sixty-four scans were taken with 4 $cm^{-1}$ resolution.

#### 2.4.3. XP spectroscopy

XPS was performed using SPES ESCA system equipped with a Phoibos 100 analyzer and Speclab software. X-rays were generated with a Mg anode at a power of 200 W. A constant take-off angle of 90° with respect to the sample surface was used for all the samples. The base pressure in the analysis chamber was $5 \times 10^{-9}$ mbar. The surface charge effect was neutralized by using the flood gun. A survey spectrum was recorded using a pass energy of 30 eV to determine the elemental compositions of the surfaces. Core level scans were taken for carbon at a pass energy of 5 eV.

### 2.5. Immobilisation of glucose isomerase

#### 2.5.1. Activation step

Hydroxyl groups on the membrane were activated by DVS according to the following procedure. Modified membranes were washed with 1 M sodium carbonate at pH 11 then immersed in 20 $cm^3$ of 1 vol% solution of DVS in sodium carbonate. After 2 h of activation, an excess of activator was washed off several times with distilled water and with solutions of various pH and ionic strength. The activated membranes were put into 25 $cm^3$ of enzyme solution (3 mg of protein in 1 $cm^3$ of 0.5 M carbonate buffer, pH 9) and left for 24 h in 4 °C. The excess of protein was then removed by successive washing with carbonate buffer, pH 9; 0.1 M phosphate buffer and distilled water. In order to block unreacted groups, the membranes were treated with 0.5 M tris-HCl buffer of pH = 8.0 for 12 h.

#### 2.5.2. Enzyme assays

The standard catalytic reactions were carried out in buffered solution of pH 8.0, at 60 °C, and using 0.1 M glucose as the substrate. The pH value of the buffered solution (0.01 M $MgSO_4 \cdot 7H_2O$ and $1.1 \times 10^{-5}$ $Mg(OH)_2 \cdot 3MgCO_3 \cdot 3H_2O$) was adjusted to 8.0 with dilute $H_2SO_4$. After the required incubation time (5–25 min), 100 μl of the reaction mixture was taken for measurement of the concentration of the fructose produced.

Fructose concentrations were estimated spectrophotometrically, according to Taylor's method [57]. The sample was put into 3 $cm^3$ of concentrated HCl and 100 μl tryptamine solution (0.01 mol in 0.1 M HCl). After 15 min of incubation at 60 °C the mixture was transferred into a water bath at 25 °C and kept for additional 40 min; the absorption at 518 nm was then determined. The concentration of fructose was calculated from a standard calibration curve.

One unit of glucose isomerase activity (U) is defined as the amount of enzyme required to liberate 1 μmol fructose in 1 min under the initial reaction rate conditions.

#### 2.5.3. Activity of immobilized preparations

The membrane with immobilized enzyme was put into 25 $cm^3$ of magnesium buffered solution and placed into a reactor thermostated at 60 °C. 25 $cm^3$ of substrate solution was added and after the required time of mixing (1–30 min), a sample of 100 μl was taken for determination of fructose. The activity of the preparation was calculated taking into account only the initial region of reaction, i.e. for less than 5% substrate conversion.

## 3. Results

### 3.1. Surface tension

Measurement of contact angle is a very helpful method for following the progress of plasma treatment. For the untreated polysulfone the polar component of solid surface tension is very small (0.9 mN/m), which indicates the hydrophobic character of this polymer. During plasma deposition of thin film, some polar functional groups were introduced onto the surface. They contributed greatly to surface wettability, expressed numerically as cosine $\Theta$. In all the cases investigated, the polar component of surface tension significantly increased. The data for 60 W plasma when sample was placed 65 mm from the plasma edge are exemplified in Table 1.

Table 1
Dependence of surface tension and both its components on treatment time for polysulfone modified in plasma of 60 W in distance of 65.5 mm from plasma edge

| Plasma medium | Treatment time, s | Surface tension, mN/m | | |
|---|---|---|---|---|
| | | Total | Polar component | Dispersive component |
| None | – | 45.9 | 0.9 | 45.0 |
| AllOH | 30 | 42.5 | 18.9 | 26.2 |
| | 60 | 38.6 | 17.9 | 20.7 |
| | 90 | 38.9 | 13.9 | 25.0 |
| | 120 | 44.5 | 19.4 | 25.1 |
| | 180 | 61 | 35 | 27.0 |
| AllOH/Ar | 30 | 43.3 | 13.6 | 29.7 |
| | 60 | 47.4 | 21.8 | 25.6 |
| | 90 | 58.0 | 31.5 | 26.5 |
| | 120 | 59.1 | 30.3 | 28.8 |
| | 180 | 64.2 | 36.6 | 27.6 |

The dramatic change of ratio of both surface tension components—the drop of the dispersive and the rise of the polar component—took place within the first 30 s of treatment. After that time $\gamma_s^d$ stayed almost constant while $\gamma_s^p$ seemed steadily to increase, though much scatter in the collected data is observed. There are several possible reasons for this phenomenon:

- appearance of errors in determination of both surface tension components. The error estimation for the data juxtaposed in Table 1 lies in the range of 1.7–2.1 and 0.6–3.1 for $\gamma_s^d$ and $\gamma_s^p$, respectively,
- continuous change of surface character being a result of many simultaneous processes that may take place in plasma, e.g. deposition, etching,
- instability of the plasma. Especially it is the case for allyl alcohol plasma where the highest scattering of data was noted.

The highest values of total surface tension and its polar component are observed after the longest treatment time investigated.

The same dependence on time is observed for all three chosen distances from the plasma edge. For simplicity, the alterations of surface tension with geometry and plasma power are shown in Figs. 1 and 2 respectively. From the given data one can discover at least two relationships. Firstly, total surface tension of modified PSU is higher when the sample-to-plasma distance is shorter. This is mainly on account of the polar component for the dispersive part seems to have a constant value. Secondly, neither the total surface tension nor its components are very sensitive to plasma power. The last relationship is valid independently of sample-to-plasma distance. To learn more about the nature of polar groups introduced some additional studies were needed. The plasma treated surfaces were analyzed by FTIR-ATR and XP spectroscopies.

### 3.2. FTIR-ATR spectroscopy

FTIR-ATR spectra of modified samples show a broad band between 3500 and 3000 $cm^{-1}$ that can be assigned to hydrogen-bonded hydroxyl groups. Their concentration seems to increase with treatment time for both plasma media—AllOH (Fig. 3a) and AllOH/Ar (Fig. 3b). This effect is most pronounced for smaller sample-to-plasma distances (Fig. 4). What it really means is that

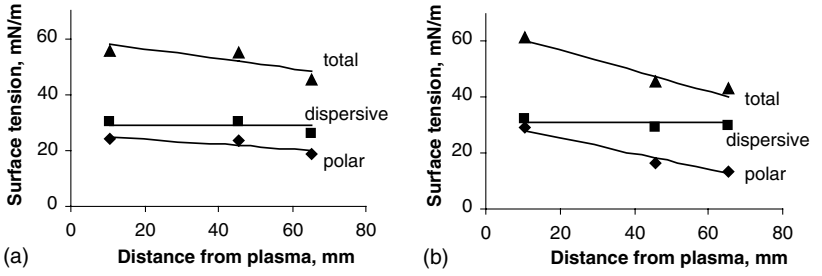


Fig. 1. Surface tension of modified polysulfone surfaces. Effect of sample-to-plasma distance: (a) allyl alcohol plasma and (b) allyl alcohol/argon plasma. Plasma parameters: power—60 W, treatment time—30 s.

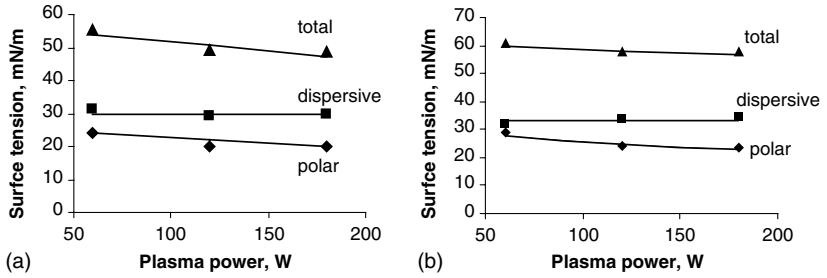


Fig. 2. Surface tension of modified polysulfone surfaces. Effect of plasma power: (a) allyl alcohol plasma and (b) allyl alcohol/argon plasma. Plasma parameters: sample-to-plasma distance—10.5 mm, treatment time—30 s.

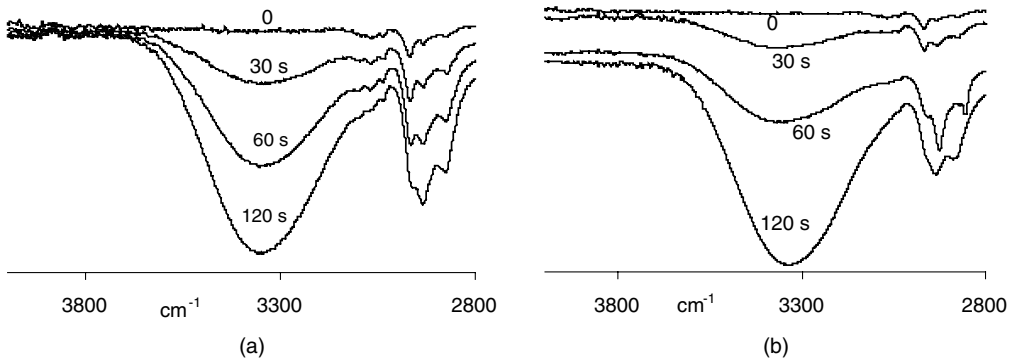


Fig. 3. The chosen bands of FTIR-ATR spectra for various plasma polymerization time: (a) allyl alcohol plasma and (b) allyl alcohol/argon plasma. Plasma parameters: sample-to-plasma distance—10.5 mm, power—60 W.

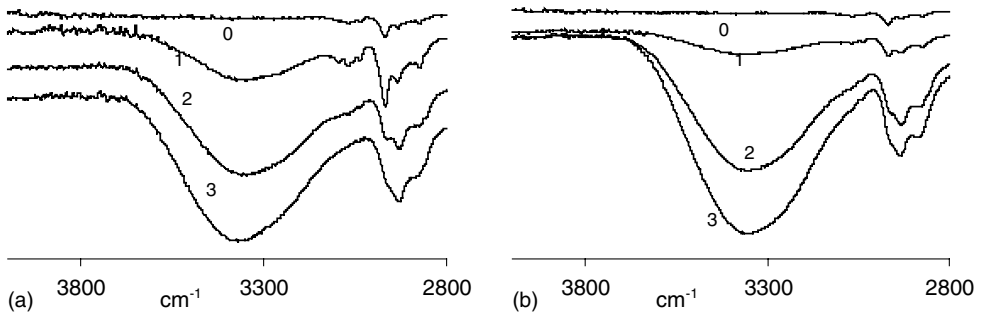


Fig. 4. The chosen bands of FTIR-ATR spectra for various sample-to-plasma distance: (a) allyl alcohol plasma and (b) allyl alcohol/argon plasma. 0 = virgin PSU, 1—65.5 mm, 2—45.5 mm, 3—10.5 mm. Plasma parameters: power—60 W, treatment time—30 s.

thickness of the deposited on the membrane plasma polymer layer grows with treatment time. This is in accordance with the most reports on plasma polymerization of organic compounds.

### 3.3. XPS analysis

XPS survey scans of all modified membranes show the presence only of carbon and oxygen and no signs of sulfur (Table 2). Hence, it is clear that the whole surface is covered with the plasma deposited layer of thickness greater than the penetration depth of photoelectrons. The O/C molar ratio is much higher than for polysulfone and depends on certain plasma parameters; it is much higher for AllOH (where it reaches the range of poly(allyl alcohol), for which it equals 0.33) than for plasma formed from AllOH/Ar (Table 2).

A narrow scan of C 1s region allows the detection of some carbon functional groups created during the plasma treatment. Deconvolution of the spectra was

Table 2
XPS elemental analysis of plasma treated PSU films

| Plasma treatment | Distance from plasma, mm | Molar concentration, % | | | O/C, % |
|---|---|---|---|---|---|
| | | C | O | S | |
| None (PSU) | – | 88.8 | 9.1 | 2.1 | 10.2 |
| AllOH | 10.5 | 71.5 | 28.5 | 0 | 39.9 |
| | 65.5 | 75.8 | 24.2 | 0 | 31.9 |
| AllOH/Ar | 10.5 | 87.0 | 13.0 | 0 | 14.9 |
| | 65.5 | 78.8 | 21.2 | 0 | 26.9 |

Table 3
Results of deconvolution of the C 1s XPS peak—presence of carbon in various chemical functionalities

| Plasma treatment | Distance from plasma, mm | Relative fraction, % | | | | |
|---|---|---|---|---|---|---|
| | | 284.8 eV | 286.4 eV | 287.1 eV | 287.6 eV | 288.6 eV |
| | | C–C–C | C–OH | C–O–C | C=O | C(O)O |
| AllOH | 10.5 | 47.7 | 34.6 | 8.1 | 5.9 | 3.8 |
| | 65.5 | 47.7 | 40.1 | 4.1 | 5.0 | 3.2 |
| AllOH/Ar | 10.5 | 71.6 | 17.1 | 11.2 | 0 | 0 |
| | 65.5 | 64.7 | 18.6 | 16.8 | 0 | 0 |

done according to the commonly used method. The five contributions were assumed to assign the carbon bonding [44]. The results are seen in Table 3. Higher concentration of OH groups were observed for the plasma polymer deposited in the case of larger sample-to-plasma distances, though the differences are not very high. Retention of alcohol functionality for the allyl alcohol plasma seems to be twice as large as for systems including argon. Also, in the absence of argon more carbonyl groups are created.

The observed results are somewhat unexpected. One would think that the presence of argon in the plasma would create more radicals on the surface, which in turn in reaction with air would result in an increase in the number of oxygen-bearing groups, e.g., carboxyl and carbonyl functionalities. The data obtained deny that hypothesis. They clearly show, however, that the mechanism of plasma polymerization is different in the presence of argon from that in its absence.

### 3.4. Enzyme immobilization

Modified membranes with OH surface groups seemed to be an excellent support for enzyme immobilization. We selected glucose isomerase (catalyst in the conversion of D-glucose into D-fructose) as the target enzyme, as it has practical usage in the food industry. Additionally it shows satisfactory chemical resistance and is active over wide ranges of temperature (45–65 °C) and pH (6.5–8.5) [58]. Testing of its immobilization and activity is relatively fast and easy. DVS was applied as a coupling agent between the hydroxyl groups on the surface and the amine groups of the enzyme. Calculated activities of immobilized enzyme for substrate modified at various distance from plasma are shown in Table 4.

Table 4
Activity of enzyme immobilized on plasma modified PSU membranes in relation to sample-to-plasma distance. Other plasma parameters: power—60 W, time—30 s

| Membrane distance from plasma, mm | Enzyme activity, U | |
|---|---|---|
| | AllOH plasma | AllOH/Ar plasma |
| 65.5 | 16.7 | 13.8 |
| 45.5 | 17.3 | 14.1 |
| 10.5 | 29.2 | 24.8 |

It is clearly seen that the most profitable substrate for immobilization is material modified close to the plasma edge. In these conditions the polar component of surface tension also takes the highest values (Fig. 1). However, a simple correlation between these two results does not exist. Also, enzyme activity does not follow the content of hydroxyl groups calculated from XPS. The reason for this is not known at the present stage of investigation.

## 4. Concluding remarks

Plasma modified polysulfone film can be successfully used for immobilization of enzymes. The surface layer resulting from allyl alcohol deposition in various con-

ditions is enriched with oxygen-bearing functional groups. These functionalities may serve as anchoring-sites for immobilization of enzymes. The use of argon to make the plasma stable is not effective when one considers the DVS method for enzyme immobilization. In the presence of argon the deposit is not so rich in oxygen and the concentration of hydroxyl groups on the surface is not so high as in the case of plasma using pure allyl alcohol. To obtain a support suitable for immobilization one should apply plasma without argon and keep the substrate close to the plasma edge. Such conditions offer deposits enriched with hydroxyl groups that in turn could be used for immobilization.

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