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European Polymer Journal 39 (2003) 2217–2224
EUROPEAN POLYMER JOURNAL
www.elsevier.com/locate/europolj

Plasma modified polymers as a support for enzyme immobilization II. Amines plasma

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Received 15 January 2003; received in revised form 14 July 2003; accepted 14 July 2003

Abstract

Polysulfone films were modified by ammonia, *n*-butylamine and allylamine remote plasma using various sample-to-plasma distances. Contact angle measurements, FTIR-ATR and XPS spectroscopy proved the presence of polar, including amine, groups on the modified surface. Presence of argon in the plasma environment made the plasma more stable and in most cases left the surface more hydrophilic but with a lower amount of nitrogen moieties on it. Glucose isomerase was successfully immobilized on the plasma-treated samples. Its activity correlates well with the concentration of C–N bonds on the surface. The highest enzyme activity was achieved for samples treated with allylamine/Ar plasma close to the plasma edge.

Keywords: Polysulfone; Amines plasma; Glucose isomerase immobilization

1. Introduction

Xylose isomerases of microbial origin (classified as D-ksylose ketol-isomerase, EC 5.3.1.5) are intracellular enzymes that catalyse reversible stereospecific conversion of D-xylose to D-xylulose. The enzyme is capable of converting other sugars from aldose to ketose [1] but the highest practical significance has isomerisation of D-glucose to D-fructose [2]. The enzyme is often called glucose isomerase because this process is widely employed on a large scale in production of high-fructose syrup from starch [2,3].

The biocatalyst is considered to be the most costly item, hence intensive efforts have been made to optimise the production of glucose isomerase [4–7] and immobilization procedures as well as continuous operation with immobilized enzyme reactors [1,8–14]. Because of the commercial interest in using glucose isomerase, the enzyme was immobilized by a wide range of the known techniques [2,3].

One of the modern techniques widely investigated in biomedical applications is plasma. It introduces a large amount of various reactive functionalities into the top layer of treated material that can totally change the surface character and also make possible further reaction e.g. immobilization. Plasma techniques are non-specific—to obtain monofunctional surfaces with just one kind of group is rather difficult. It is also not easy to predict which groups will prevail after treatment. The reason for this are the variety of reactions that can take place in plasma simultaneously and the fact that in plasma every substance becomes fragmented and scrambled randomly, depending on plasma medium,

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doi:10.1016/S0014-3057(03)00160-5

apparatus and conditions used [15]. In spite of these disadvantages plasma is commonly used for it has much more merits. It is a versatile method, very fast and efficient, not changing material bulk properties, fulfilling all ecological standards.

Amino groups are usually incorporated on the surface using ammonia or amines in the plasma medium. The former is non-polymer-forming gas, the later ones deposit a plasma polymer layer. Treatment of various materials with ammonia plasma is the subject of many papers [16–28]. Among organic amines allylamine is chosen most often as a plasma medium [20,21,29–39] but plasma polymerization of others, such as *n*-butylamine [29,40], propylamine [33], propargylamine [33], *n*-heptylamine [23,41] 1,3-diaminopropane [42], diaminocyclohexane [20], aminosilanes [43] is also described. Authors of all the papers mentioned confirm the presence of various nitrogen-containing functionalities, among others also amine. Their amount depended very strongy on the plasma apparatus and plasma parameters. Retention of the amine group seemed to be higher when pulsed or remote plasma was used [20,30,31].

Surface amine groups were tested to promote cell growth (adhesion for cell culture) [27,28,31], to change adsorption of proteins [39], to bond biomolecules chemically [22–25,36,40,42,43] and to improve membrane performance [18,20,35] and adhesion to other materials [26].

In the previous work [44] we have applied plasma of allyl alcohol to immobilize glucose isomerase on the surface of polysulfone films; in this paper we describe using ammonia, *n*-butyl amine and allylamine plasma for that purpose.

2. Experimental

2.1. Materials

Polysulfone (PSU) Udel P-1700 was purchased from Amoco Co., USA. Technical glucose isomerase (Maxazym GI, K8587A) with the specific activity of 3.3 U/mg was kindly donated by Gist-Brocades. Gaseous ammonia and argon were supplied by Linde gas, Poland. Allylamine was received from Fluka AG (Buschgs, Switzerland), *n*-butylamine from Aldrich Chem. Co., Ltd, diiodomethane, glutaraldehyde, Lowry Assay Kit and tryptamine from Sigma Chemical Co, fructose and glucose from Merck. Chloroform and other of analytical grade chemicals were purchased from POCH, Gliwice, Poland.

2.2. PSU films

Polysulfone films were casted from 20 wt.% chloroform solution onto glass plates and dried for 6 h at 120 °C. Their thickness was about 50 μm. From the film, disc of 78 mm in diameter were cut.

2.3. Plasma treatment

A microwave plasma generator of 2.45 GHz frequency (Plazmatronika, Wrocław, Poland) was used throughout this study. Pulsed plasma with pulse frequency 125 Hz and 25% of duty time was applied. Plasma was generated in a quarz tube on the top of a reaction chamber. A PSU sample was attached to a table, the position of which could be easily changed in the reactor. Treatments were performed at three distances from the lower edge of plasma – 65, 46 and 10.5 mm. The others parameters of plasma treatment are given in Table 1.

2.4. Surface characterization

2.4.1. Contact angle measurements

For evaluation of PSU surface free energy after plasma treatment, static contact angles of water and diiodomethane were measured. TM 50 System (Technicome S.A., France) equipped with a Panasonic GL 350 camera was used. At least 20 readings were made on each modified film. Surface tension and its polar and dispersive components were calculated according to harmonic averaging [45]. Polar and dispersive components of surface tension for selected liquids were taken after Kuznietsow [46].

Table 1
Plasma media and parameters used for PSU films treatment

Plasma medium	Plasma power (W)	Treatment time (min)	Argon flow (cm^3/min)	Final pressure in the reactor (mbar)
Ammonia	120	3	0	1
Ammonia/argon	60	3	10	1
n-Butyl amine	120	1	0	2
n-Butyl amine/argon	60	3	10	2
Allylamine	120	0.5	0	2
Allylamine/argon	60	3	10	2

2.4.2. Fourier transform infrared spectroscopy

Spectra were obtained by the total reflection (ATR) technique, using Perkin–Elmer System 2000 spectrometer with horizontal ATR device (Ge, 45°). 64 scans were taken with 4 cm^{-1} resolution.

2.4.3. XP spectroscopy

XPS was performed using a SPES ESCA system equipped with Phoibos 100 analyzer and Speclab software. The X-rays were generated with a Mg anode at a power of 200 W. The constant takeoff angle of 90° with respect to the sample surface was used for all samples. The base pressure in the analysis chamber was 5×10^{-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. Immobilization of glucose isomerase

2.5.1. Activation and immobilization step

The activation step for amino groups was done with glutaraldehyde. Two modified and cut up membranes were equilibrated in 0.1 M phosphate buffer (pH 7.0), then placed into 20 cm^3 of 5 vol.% glutaraldehyde in the buffer. After 2 h shaking, the excess of activator was washed off several times with distilled water and phosphate buffer. Activated membranes were put in 25 cm^3 of enzyme solution (3 mg of protein per 1 cm^3 in phosphate buffer) and stirred for 2 h. The excess of protein was washed off successively with buffers: 0.1 M phosphate buffer, pH 7.0; 0.1 M phosphate buffer with 0.5 M NaCl, pH 7.0; 0.1 M acetate buffer, pH 5.0, and finally distilled water. In order to block unreacted active groups, the pieces of membranes were suspended in 0.5 M tris–HCl buffer, pH 8.0 and stored for 24 h at 4 °C.

2.5.2. Enzyme assays

The standard catalytic reactions were carried out at pH 8.0, 60 °C, using 0,1 M glucose solution as the substrate. The pH value of the buffered solution (0,01 M $MgSO_4\cdot7H_2O$ and $1{,}1\times10^{-5}$ $Mg(OH)_2\cdot3MgCO_3\cdot3H_2O$) was adjusted to 8.0 with dilute H_2SO_4. After the desired time (up to 30 min) of incubation 100 μl of the reaction mixture was sampled for measurement of the produced fructose.

Fructose concentration was estimated spectrophotometrically, according to Taylor's method [47]. The sample of 100 μl was put into 3 cm^3 of concentrated HCl and 100 μl tryptamine (0.01 M in 0.1 M HCl). After the incubation at 60 °C for 15 min the mixture was put into a water bath at 25 °C for an additional 40 min, then the absorption was determined at 518 nm. The concentration of fructose was calculated from a standard 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. Protein concentration was measured by Lowry's method.

2.5.3. Activity of immobilized preparations

Two cut up membranes with immobilized enzyme in 25 cm^3 of buffer were placed in a thermostated reactor and the temperature was maintained at 60 °C. Then 25 cm^3 of substrate at the same temperature was added. After the desired time of mixing (from 5 up to 30 min) the 100 μl of sample was taken and fructose concentration was immediately determined. The activity was calculated on the basis of points from the initial region of the reaction (less than 5% conversion of the substrate).

3. Results and discussion

3.1. Conditions of plasma treatment

The first step in the process of immobilization through covalent bonding is to generate the same reactive, functional groups in the material to be a support for biomolecules. We decided to bond the chosen enzyme—isomerase—through the amine groups generated on the polysulfone surface, followed by reaction with glutaraldehyde. Ammonia, *n*-butyl amine and allylamine were chosen as plasma media to introduce these groups. Ammonia is non-polymer-forming gas which etches the polymer surface and introduces nitrogen functionalities. The remaining two media are known to deposit a thin layer of plasma polymer. However, they caused some problems. Plasma of these organic vapors was not stable, seemed to twinkle and after a short time died out. The plasma parameters shown in Table 1 were then chosen for the best plasma stability and admissible treatment time. The mentioned problems were avoided when argon was added to the plasma environment and modification with such gas mixtures was also performed.

The modification was performed in remote-plasma conditions therefore the sample-to-plasma edge distance was taken as a variable plasma parameter.

Ammonia and amine plasmas are known to leave polymer surfaces with dominant nitrogen-containing moieties such as amine and amide groups but some oxygen moieties were also detected, mainly due to post-reactions after exposing samples to the air [16,18,19]. All these groups were expected to make the surface more hydrophilic and measurements of surface tension were performed to prove this.

3.2. Surface tension

On the surface of all modified samples the contact angle of water and diiodomethane were measured and both the polar and dispersive component of the surface tension were calculated. The results are shown in Table 2. Polysulfone is a rather hydrophobic polymer with surface tension equal to 45.9 mN/m in which polar forces have a very small contribution (0.9 mN/m). Ammonia plasma seems not to influence the total surface tension significantly but it dramatically changes the ratio between the polar and dispersive components. The polar component values become comparable with the dispersive ones. What is more, distance from the plasma and addition of argon to ammonia seems to matter very little.

n-Butylamine plasma surprisingly makes polysulfone surface more hydrophobic—the surface tension values, though increasing a little when plasma-to-sample distance becomes smaller, do not reach the value for unmodified polysulfone. The polar component, however, is 7–13 times higher than for PSU. Argon changes the situation only for the closest distance from the plasma but it has a very significant influence—the polar component reaches the highest value in this experiment series value (72 mN/m) and indicates a very polar surface.

The surface tension of polysulfone film set at distances of 65 and 46 mm from allylamine plasma—as for butylamine—is lower than before treatment. Close to the plasma and with argon contribution, the modified surfaces became very hydrophilic, surface tension values being higher than 70 mN/m with the polar component much higher than the dispersive.

3.3. Spectroscopic studies

Infrared attenuated total reflection (IR-ATR) is a routine surface technique. The sampling depth of FTIR-ATR is dependent on wavelength and for PSU is in the range of 800 nm (for 850 cm^{-1}) to 170 nm (for 4000 cm^{-1}). This is two orders of magnitude greater than the thickness of plasma modified layer, for plasma treatment changes only the outer few nanometers of material. This difference implies that in the IR spectra new bands resulting from the modification are expected to be of very low intensity.

In the spectra of almost all the samples examined the lower intensity of PSU-origin bands is observed. It would suggest that the layer of new material is deposited on the PSU surface. This is obvious in the case of *n*-butyl amine and allylamine, for they are both plasma polymerizable reagents. Ammonia however is a reactive, non-polymerizable gas and the deposit in that case might come from material etched by plasma.

In all spectra also a clear thought wide band of low intensity appears in the wave number range between 3600 and 3100 cm^{-1}. There are many functional groups that absorb in that frequency region, among them certainly amine, that we need to experiment further with. For ammonia plasma (Fig. 1a) the intensity of that band seems to be independent of sample distance from the plasma edge and not sensitive to argon presence

Table 2
Influence of plasma medium and sample-to-plasma distance on surface tension of modified polysulfone

Plasma medium	Sample distance from plasma (mm)	Surface tension (mN/m)		
		Polar	Dispersive	Total
Ammonia	65	22.2	26.4	48.6
	46	22.9	27.0	49.9
	10.5	23.0	26.4	49.4
Ammonia + argon	65	25.5	29.5	55.1
	46	26.6	29.3	55.9
	10.5	26.9	29.4	56.3
n-Butyl amine	65	6.7	32.4	39.1
	46	6.6	33.5	40.1
	10.5	11.9	33.5	45.4
n-Butyl amine + argon	65	7.1	29.0	36.2
	46	8.7	29.2	37.9
	10.5	52.0	20.3	72.3
Allylamine	65	4.1	35.0	39.2
	46	5.9	35.1	41.1
	10.5	48.2	24.0	72.2
Allylamine + argon	65	40.2	31.0	71.3
	46	42.7	30.5	73.2
	10.5	45.8	29.9	72.7

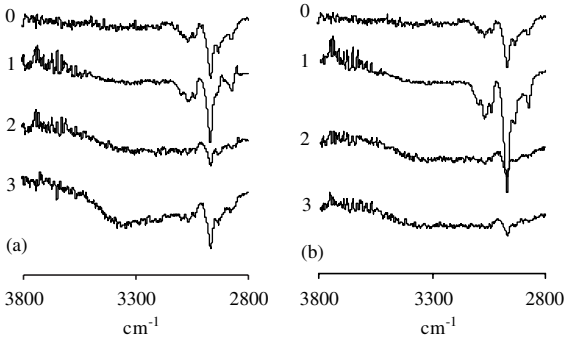

Fig. 1. The chosen bands of FTIR-ATR spectra of ammonia (a) and ammonia/Ar plasma treated polysulfone in various distances from plasma: 0—PSU, 1—65 mm, 2—45 mm, 3—10.5 mm.

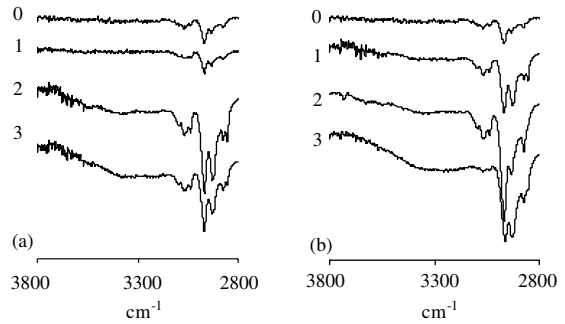

Fig. 2. The chosen bands of FTIR-ATR spectra of *n*-butylamine (a) and *n*-butyl amine/Ar plasma treated polysulfone in various distances from plasma: 0—PSU, 1—65 mm, 2—45 mm, 3—10.5 mm.

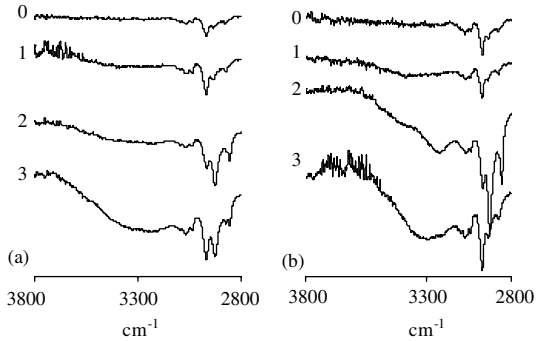

Fig. 3. The chosen bands of FTIR-ATR spectra of allylamine (a) and allylamine/Ar plasma treated polysulfone in various distances from plasma: 0—PSU, 1—65 mm, 2—45 mm, 3—10.5 mm.

(Fig. 1b) in the plasma gas. In the case of *n*-butylamine plasma (Fig. 2a) the band of 3600–3100 cm^{-1} seems to be absent for the sample far from plasma but it appears and increases with the smaller distances. The influence of Ar in the plasma is also small—band intensity seems to be higher (Fig. 2b). For all examined parameters of allylamine plasma wide bands in the range mentioned are observed (Fig. 3a) and the effect of argon addition to the plasma gas is evident (Fig. 3b). For most samples modified close to the plasma edge low intensity bands at $\approx$1650 and $\approx$2200 cm^{-1} could be observed. They can be attributed to =C=N– and –C≡N– groups respectively.

XPS analysis was used to determine the surface compositions of chosen modified PSU samples. In virgin PSU the only elements detected by XPS were carbon, oxygen and sulfur (Table 3). After plasma treatment

Table 3
Concentration of elements on surface of plasma treated PSU

Plasma medium	Sample-to-plasma distance (mm)	Atomic concentration (%)				O/C	N/C
		C	O	S	N		
None (PSU)	–	88.8	9.1	2.1	0	0.102	0
NH_3	10.5	67.9	6.5	0.3	25.3	0.096	0.373
	65	65.9	18.5	1.0	14.6	0.281	0.221
NH_3/Ar	10.5	73.9	13.1	0.8	12.2	0.177	0.165
	65	70.5	17.0	1.4	11.1	0.241	0.157
$BuNH_2$	10.5	78.0	7.2	0.4	14.4	0.092	0.185
	65	78.2	14.8	1.7	5.3	0.189	0.068
$BuNH_2$/Ar	10.5	86.9	2.8	0	10.3	0.032	0.118
	65	78.7	13.7	1.4	6.2	0.174	0.079
$AllNH_2$	10.5	77.5	2.3	0.8	19.4	0.030	0.250
	65	76.6	11.6	1.8	9.9	0.151	0.129
$AllNH_2$/Ar	10.5	79.8	3.8	0	16.4	0.048	0.205
	65	80.0	4.4	0	15.6	0.055	0.195

nitrogen is detected on all surfaces. Its concentration is always higher for samples treated close to the plasma edge. For most cases argon reduces the amount of nitrogen on the surface; only far from the plasma edge is the situation for polymerizable gases different. Allylamine plasma, both with and without argon, seems to introduce more nitrogen functionalities than *n*-butylamine plasma. This is in agreement with the well known statement that compounds with double bonds polymerize more easily in plasma than do saturated ones. Sulfur concentration is lower than in PSU and for a few samples is not present at all. The thickness of the plasma polymer deposits is then higher than the penetration depth of XPS. The much higher O/C ratios for samples treated at a large distance from the plasma suggest a larger amount of radicals created in such condition than for samples close to the plasma. These radicals react with air after exposure of the sample to atmosphere, so increasing the concentration of oxygen functionalities.

High resolution ESCA multiplay scans of C1s peaks were used to obtain quantitative results for functional group compositions of modified surfaces. However, the exact determination of the nature of the incorporated groups is not easy for many signals coming from CN, CS or CO groups can be overlapped. Typical deconvolution of the C1s XPS spectrum is shown in Fig. 4 and results of such operations for modified samples are in Table 4. Chemical assignments for deconvoluted peaks were based on binding energies quoted in the literature [33,34,42,48–50]: 284.5—C–C, 285.6—C–S, C–N, 286.6—C–O, C═N, 287.8—C═O, O═C–NH, 288.5—C≡N.

As can be seen from Table 4, the amount of C–N bonds, the most interesting to us, is always higher for smaller sample-to-plasma distance. Most of the oxygen incorporated in the polymer by plasma seems to take the form of C–O bonds (C–OH, C–O–C); relatively bigger amounts of C═O and C≡N are observed for surfaces modified by ammonia plasma.

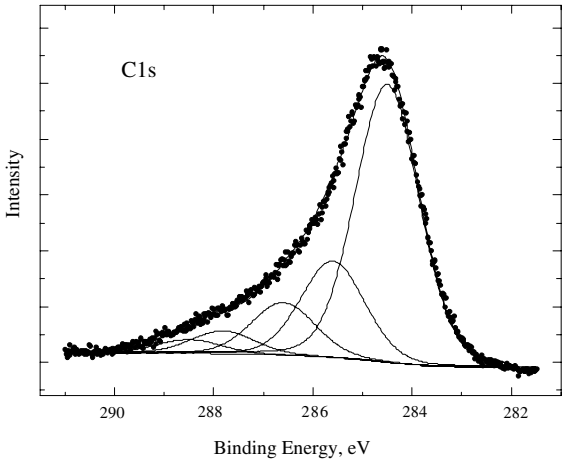

Fig. 4. The example of XPS narrow scans of C 1s peak for PSU after modification. Plasma parameters—ammonia plasma, 65 mm from plasma edge.

3.4. Immobilization

The presence of the enzyme on the polymer surfaces was confirmed by FTIR-ATR spectroscopy (Fig. 5). A few new bonds, at 3187.8, 1630.0 and 1554.5 cm^{-1} that appeared in the spectrum can be attributed to NH stretching, NH deformation and C═O stretching bonds of protein, respectively.

Table 4
Presence of carbon in various chemical functionalities

Plasma medium	Sample-to-plasma distance (mm)	eV[a]/relative fraction (%)				
		284.5	285.6	286.6	287.8	288.5
NH_3	10.5	55.0	20.1	17.3	4.4	3.1
	65	60.5	18.8	12.2	5.3	3.2
NH_3/Ar	10.5	61.9	27.8	6.8	3.3	0.2
	65	67.3	18.3	9.3	3.5	1.7
$BuNH_2$	10.5	59.2	24.8	11.3	4.7	0.02
	65	71.3	16.8	10.0	0.6	1.3
$BuNH_2$/Ar	10.5	71.2	22.1	5.1	1.6	0.0
	65	71.1	17.3	9.2	1.9	0.5
$AllNH_2$	10.5	57.1	20.0	18.4	2.0	2.5
	65	64.5	18.6	14.4	1.4	1.1
$AllNH_2$/Ar	10.5	65.5	23.1	8.9	2.4	0.0
	65	71.1	18.2	7.3	0.03	2.4

[a] Peak assignment according to the text.

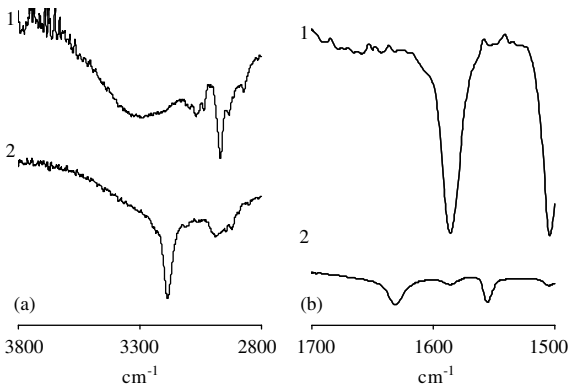

Fig. 5. An example of chosen FTIR-ATR spectra regions for PSU sample after amine plasma modification (1) and enzyme immobilization (2). Plasma parameters—allylamine/Ar, 10.5 mm from plasma edge.

The immobilized enzyme showed some activity (Table 5). The highest value (35.2 U) was achieved for allylamine/Ar plasma and sample close to the plasma edge. The relationship between activity and sample-to-plasma distance is clear—making the sample closer to the plasma edge increases enzyme activity, which means more enzyme immobilized on the surface. For ammonia and allylamine plasmas the presence of argon seems to be favourable.

We tried to correlate the activity of immobilized enzyme with sample surface tension, polar component of surface tension, nitrogen content in the sample or C–N bond concentration. This last value can be easily calculated knowing amount of sulfur in the sample (Table 3) and assuming that 285.6 eV peak comes from C–N and C–S bonds (Table 4). The calculated correlation coefficients were 0.65, 0.60, 0.57 and 0.77 respectively. The only correlation that seems to be reasonable is the last one (Fig. 6a). It looks much better if we calculate separately for each gas; then the correlation coefficients are: 0.97 for ammonia, 0.95 for *n*-butylamine and 0.90 for allylamine (Fig. 6b). This would prove that attribution of the peak at 285.6 eV to CN and CS bonds is generally correct but the way in which the surface reacts with enzyme depends on the source of the active groups. There might be a connection here with the accessibility of these groups.

Table 5
Dependence of immobilized enzyme activity on plasma parameters

Plasma medium	Sample-to-plasma distance (mm)	Activity (U)	Plasma medium	Sample-to-plasma distance (mm)	Activity (U)
NH_3	10.5	15.2	NH_3/Ar	10.5	20.1
	46	11.3		45	15.7
	65	8.9		65	13.6
$BuNH_2$	10.5	28.2	$BuNH_2$/Ar	10.5	20.9
	46	13.5		45	10.9
	65	15.0		65	10.3
$AllNH_2$	10.5	31.2	$AllNH_2$/Ar	10.5	35.2
	46	15.7		45	20.0
	65	13.4		65	18.3

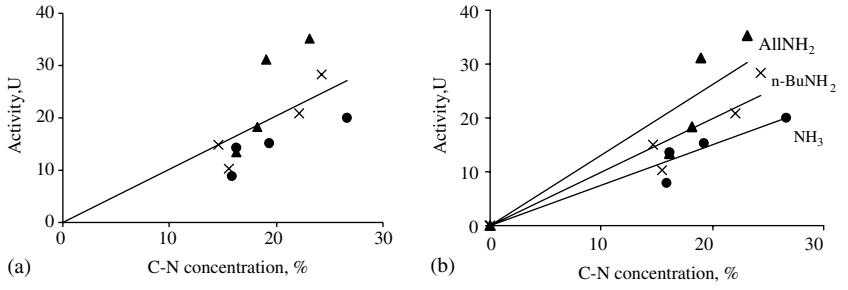

Fig. 6. The correlation between immobilized enzyme activity and concentration of C–N bonds (from XPS) for all modified samples (a) and for three amines separately (b).

4. Conclusions

Plasma of ammonia changes dramatically the participation of the polar component of the surface tension of polysulfone; this effect seems to be independent of the plasma parameters. The same effect is observed for *n*-butyl amine and allylamine plasmas and in this case it seems to be greatest for argon presence in plasma gas and for samples close to the plasma edge.

FTIR-ATR and XP spectroscopy confirm the presence of nitrogen-containing moieties on the modified surfaces. Plasma acting close to the sample introduces more nitrogen and less oxygen to the surface; also more nitrogen is in the form of C–N groups.

Glucose isomerase was successfully immobilized on all plasma-treated samples; its activity was highest for samples modified with allylamine/Ar plasma close to the plasma edge.

Acknowledgement

The financial support of Center of Biomonitoring, Biotechnology and Protection of Ecosystems of Lower Silesia, is greatly appreciated.

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