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Preparation of Sulfonated Polysulfone Membrane for Enzymes Immobilisation

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The paper presents a method for the preparation of sulfonated polysulfone in order to receive a matrix to immobilisation of biomolecules. Optimum conditions of the synthesis are described. The phase inversion method was used to prepare a sulfonated polysulfone membrane and then urease was immobilized on the surface of the prepared matrix. Comparison of activity of native urease and the immobilized urease is presented.

K e y w o r d s: sulfonated polysulfone, immobilization of urease, amination of sulfonated polysulfone

1. Introduction

The interest of the application of polymers as a surface to immobilization of substances which are biologically active, such as enzymes or antibodies, in pharmaceutical, chemical, and food industries, as well as in biotechnology is increasing. Immobilized enzymes are used in bioreactors, separating membranes, and to creating of quick enzyme and immunoenzymatic tests.

Polysulfone (PSf) is an important engineering material and common material used in making the membranes. Many reports concerning the modification of PSf to improve its membrane performance are described in the scientific literature. In order to obtain the materials with mechanical resistance and with better chemical proper-

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ties, PSf was copolymerized with polydimethylsiloxane (PDMS) [1, 2]. A surface of the PSf membranes was modified by freeze-fracturing and deep-etching to reduce the fouling of proteins [3, 4].

The modification of polysulfone is mostly based on adding carboxylic, chloromethylic or chlorosulfonic group to the aromatic ring [1, 2, 4, 5].

Polysulfone modified by a reaction with n-buthylolite (*n*-BuLi) and then with 2, 2'-vinylidenedipyridine can be applied to obtain the membranes to processes of ultrafiltration as well as to chelating of trace amount of heavy metals in water solutions [5, 6]. It is used in metallurgy and processes of water purification. The lithated and then carboxylated PSf can also be used in the processes of reverse osmosis and separation courses [7, 8].

The membranes with the modified surface in the chloromethylated reaction are applied to purification of proteins [9], to ultrafiltration [10] as well as to obtaining of ion-exchanging cartridge [11].

The membranes made of the sulfonated PSf are applied in separation processes, biotechnology, energy industry (fuel cell) as well as in biomedical engineering. Another sulfonating factors are described in many different publications: cyclic trimethyl sulfate $(CH_3)_3SO_3$ [12], trimethylsilyl chlorosulfate $(CH_3)_3SiSO_3Cl$ [13], a complex of sulphur trioxide (SO_3) with trietylphosfate (TEP(SO_3)) [12,14] and chlorosulfonic acid [15,16].

PSf which is modified by many reagents in a special sequence can be used to prepare substrate for enzyme immobilization. For instance, proteolitic enzymes can be used to prepare the self-cleaning membranes. This decreases the fouling effect and simultaneously improves stability of the filtration process [16].

The aim of this study was to find conditions for the synthesis of the sulfonated polysulfone in reaction with chlorosulfonic acid in order to receive the matrix to immobilization of biomolecules. Efficiency of immobilization of urease, which was served as a model biomolecules, was studied and compared to the native enzyme.

2. Materials and Methods

2.1. Materials and Apparatus

Polysulfone type Udel 1700 was purchased from Dow Corning, Midland, USA. Dimethylformamide (DMF) and N-methyl-2-pyrrolidone (NMP) were purchased from Sigma-Aldrich and distilled before used and dried over a molecular sieve. Bromothymol blue, *o*-nitrophenol, phenoloftaleine, urea, ethylenodiamine (EDA), dichloroethane (DCE) and potassium bromide (KBr) for the IR were purchased from POCH, Gliwice, Poland. Urease Jack Bean (Type IX) and chlorosulfonic acid were purchased from Sigma-Aldrich. The IR spectrum was made by spectrometer BioRad Excalibur. The spectrophotometric tests were obtained by BioTek Synergy

HT measurements and multiwells spectrophotometer U-3010. pH measurements were made by Mettler Toledo pH-meter.

2.2. Chemical Modification of Polysulfone

0.6 M solution PSf in DCE and 1M solution $ClSO_3H$ were prepared in the same solvent. The reaction was carried out in a tri-neck flask supplied with mechanical stirrer, thermometer, and dropper with pressure equalizing pipe, where the right capacity of the sulfonating factor was introduced by a syringe with a steel needle. The solution of acid was dropped to the being stirred PSf solution (approx. 30 minutes.) The deposit of the product immediately started to precipitate. After the dropping process, the reaction mixture was left with stirring for approximately 24 hours. Then, not reacted acid was neutralized by adding about 20 mL of 1M NaOH solution in methanol. The solvents were evaporated in a vacuum drier. Such precipitate was recrystallized from DMF.

Titration was used for detecting the amount of sulfonic groups per mer of the polymer [17]. For this purpose the solution of product in DMF was prepared. Titration was conducted with the use of a standard solution of NaOH with phenolphthalein as an indicator. The degree of substitution is defined as the number of sulfonic groups per mer of the polymer. It has been designated by the formulae:

$$SP = \frac{444 \times C_{NaOH} \times V_{NaOH}}{W - 81 \times C_{NaOH} \times V_{NaOH}} \times 100\% \quad (1)$$

where: SP – degree of substitution, C_{NaOH} – concentration of NaOH (mmol/mL), V_{NaOH} – volume of NaOH used for titration of the sample (mL), W – weight of sample (g), 444 – the molecular weight of PSf, 81 – molecular weight of a $–SO_3H$ group.

2.3. The Preparation of Membranes

In order to obtain the membrane the solution of sulfonated polysulfone (SPSf) in DMF with concentration 28% was prepared in a beaker with a capacity of 50 mL. Such prepared solution was being poured out at an aluminum or a glass plate. The solution was distributed evenly at the surface of the plates. The foils were coagulated in 40ºC for about 24 hours.

2.4. Urease Immobilization

The reaction of sulfonation of polysulfone (SPSf) with EDA has been conducted in the temperature of 22ºC, under the atmospheric pressure for 1 h. 5% solution of diamine in a 0.1M buffer $NaOH/NaHCO_3$, pH 9 was used in this reaction. After the reaction the foils have been rinsed with distillated water in order to remove

unbounded diamine. The reaction with 1% water solution of glutaraldehyde was conducted in the ambient conditions. The result of these reactions was the substrate ready to immobilization of the enzymes. The reaction with proteins was conducted in the temperature of 4ºC. The properly prepared foils were placed in the 1% urease solution in the 0.01M Na_2HPO_4/KH_2PO_4 buffer, pH 5 for about 20 hours. After the reaction foils were rinsed with the 0.01M Na_2HPO_4/HCl buffer, pH 5 with 20 mL portions. The foils were immersed in the buffer for 5 minutes and stirred. The rinsing was repeated three times.

3. Results and Discussion

A set of reactions in 4 different temperatures was conducted by using the different proportions of reagents (Table 1). As it could be expected the larger excess molar chlorosulfonic acid in relation to the polysulfone degree of substitution (SP) is higher.

Large excess of chlorosulfonic acid, applied in reactions 2–3 in the relation to polymer probably influenced on its degradation caused by hydrolysis of the ether bounds. As a result of this process, a fragile product soluble in water has been produced, from which it was not possible to obtain a permanent, flexible membranes capable of reacting in aqueous solution.

Due to the lowering temperature of the process, the reducing excess of chlorosulfonic acid and the prolonging time of the instilling reagent, degradation of the polymer has decreased, the physical properties of SPSf has improved and the high degree of substitution has been enabled. (Table 1. Products: 1a, 1b and 1c).

Table 1. The conditions of the chlorosulfonation of polysulfone and the degree of substitution of the product obtained by titration and elementary analysis

Product	Temperature of the reaction [ºC]	The molar ratio of the reagents $ClSO_3H$: PSf	Solubility in water	The Degree of Substitution – SP [%]	
				titration analysis	elementary analysis
1	22 ÷ 24	1:1	-	0.020	–
2	22 ÷ 24	2:1	+	0.026	–
3	22 ÷ 24	3:1	+	0.290	–
4	22 ÷ 24	4:1	+	0.320	–
5	22 ÷ 24	5:1	+	0.440	–
1a	**7**	**1:1**	**–**	**0.210**	**0.45**
2a	7	2:1	–	0.020	–
1b	**0**	**1:1**	**–**	**0.110**	**0.35**
2b	0	2:1	+	0.300	–
1c	**–10**	**1:1**	**–**	**0.210**	**0.43**
2c	–10	2:1	–	0.230	–

The results of the elementary analysis differ from the results obtained on the titration (Table 1). The likely cause of these differences is a two-step mechanism of this process, in result of which there are two products [18].

$$PSf\text{-}H + ClSO_3H \rightarrow PSf\text{-}SO_3H + HCl \quad (2)$$

$$PSf\text{-}SO_3H + ClSO_3H \rightleftarrows PSf\text{-}SO_2Cl + H_2SO_4 \quad (3)$$

In the first phase a derivative of PSf came in the acid form. With the increase of excess of acid it becomes acidic chloride. Both products were maintained in a state of dynamic equilibrium.

To make a qualitative analysis of the product, a IR spectroscopy was utilized. The IR spectrum of the substrate and of the particular products of the sulfonating reactions are shown in Fig. 1.

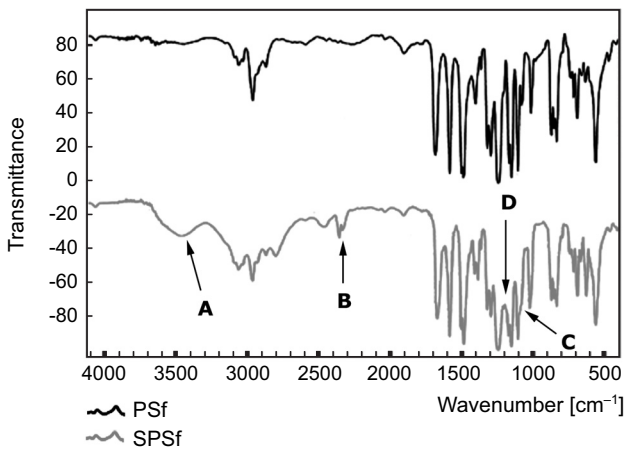

Fig. 1. IR spectrum for polysulfone (upper graph) and sulfonated polysulfone (bottom graph) – comparison A – 3460 cm^{-1} – broad band for vibration stretching groups –OH SO_3–H; B – 2350 cm^{-1} – peaks –OH in –SO_3H; C – 1080 cm^{-1} – group –SO_3H – characteristic peak for vibration stretching groups – SO_2 in sulfonic acid; D – 1205 cm^{-1} – peak corresponding to vibration stretching groups –SO_2 in sulfochlorides (R–SO_2Cl)

In order to confirm the qualitative course of the amination of SPSf, a IR spectrum was made. The spectrum of the product and the substrate (in this case SPSf) were compared similarly to the previous case. By analyzing the spectrum, the reduction of intensity of the peaks characteristic for –OH group and the emergence of the peaks corresponding to –NH_2 group (Fig. 2) can be observed.

In conclusion, the analysis of IR spectrum has confirmed the qualitative course of the aminating reaction.

The characteristic peaks for the primary and secondary amines as well as for the associated –NH groups appeared. As a result of the SPSf/EDA reaction, the partly

prepared surface to immobilization was obtained. This process did not have influence on the foil properties. After the reaction they were still resilient and had tensile strenght. The product of such quality might be subjected to further reaction.

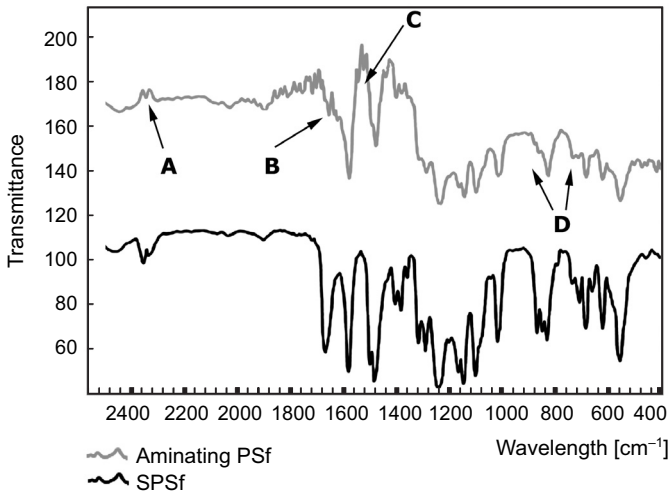

Fig. 2. Spectrum IR SPSf (bottom graph) and product of SPSf amination (upper graph) – comparison. A – 2350 cm^{-1} – loss of characteristic peaks for the stretching vibration of –OH groups into $–SO_3H$; B – 1660 – 1610 cm^{-1} – deformating oscillation of –NH groups characteristic for primary amine; C – 1550 cm^{-1} – deformating oscillation of –NH groups characteristic for secondary amine; D – 900 cm^{-1}, 730 cm^{-1} – deformating oscillation of associated –NH groups

The reaction of SPSf with glutaraldehyde was also confirmed, similarly to the case of the analysis of the earlier products, by IR spectroscopy. On the made spectrum, the peaks characteristic for –CHO groups, –C=N groups and decreasing of intensity of the peaks corresponding to the primary amines (Fig. 3) can be observed.

The result of the reaction with glutaraldehyde was getting a ready base for the enzyme immobilization. The glutaraldehyde concentration used in the reaction affects significantly the physical properties of the foil. Its high concentration (5 and 2.5%) increases stiffness and fragility of the membrane. Best results have been obtained for the foil of a low thickness after reacting in a 1% solution of modifier.

The reaction with the enzyme has been conducted at 4˚C, in the 0.01M Na_2HPO_4/HCl buffer with pH 5. The properly prepared foils were placed in the protein solution for approximately 20 h. After the reaction of urease, the foils were rinsed three times by 0.01M buffer Na_2HPO_4/HCl of a pH 5. The finished foils with the immobilized urease were examined in order to estimate the amount of immobilized urease. Urease catalyzes the reaction of decomposition of urea (4), as a result of which the pH changes.

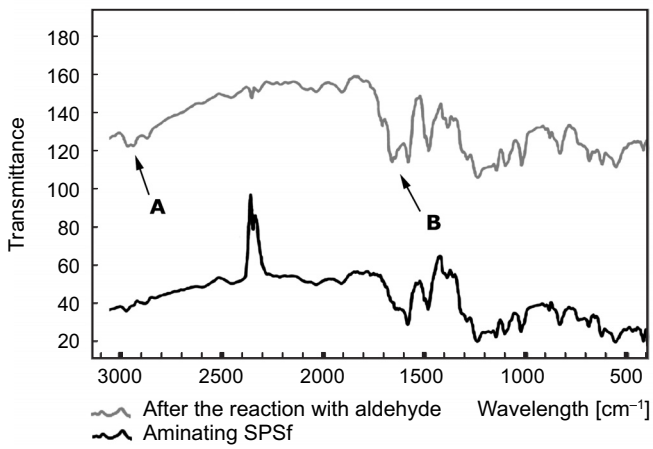

Fig. 3. Spectrum IR of aminating SPSf (upper graph) and a product of the reaction with glutaraldehyde (bottom graph) – comparison. A – 2950 cm^{-1} – vibration extending – C-H groups in aldehydes. B – 1660 cm^{-1} – vibration extending –C=N groups

$$H_2N\text{-}CO\text{-}NH_2 + H_2O \xrightarrow{\text{urease}} 2\ NH_3 + CO_2 \qquad (4)$$

The reaction of decomposition of urea catalyzed by urease (5) was used in the study of spectrophotometry. Changes of the absorption of the radiation were registered in 620 nm. In each series of the measurements the results obtained for the immobilized urease were compared with those of the urease standards (Fig. 4).

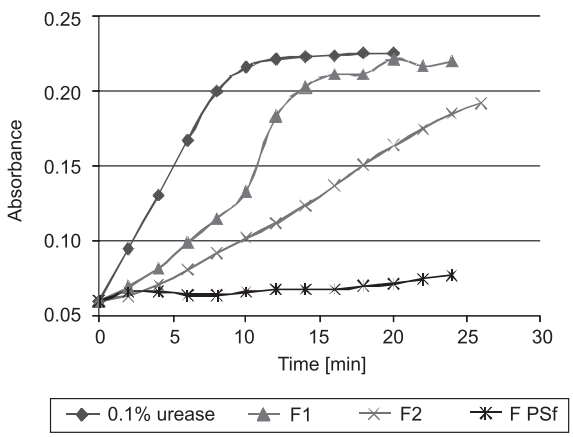

Fig. 4. Efficiency of urease immobilization on different substrate (different temperature of chlorosulfonation: F1 7°C; F2 0°C) – comparison with standard urease and polysulfonic membrane (F PSf)

In the successive analysis, the volume of the used urea was reduced to 40 μL. The aim of this treatment was checking the behavior of enzyme with a less excess of the substrate. This proved enough to get a change in absorbance similar to the changes resulting from using a greater amount of excess of the substrate. This is a promising signal since the enzyme test response, even in case of the very little substance concentration in a sample, demonstrates its sensitivity. The foils F1 (temperature of chlorosulfonation 7°C) and F3 (temperature of chlorosulfonation –10°C) with two standards (Fig. 5) have been compared in these studies.

The response time for the F7 sample was comparable to the time for 0.05% of urease solution (9 min), the longest time of reaction can be observed for the F-10, while the inclination of the curve for both foils was comparable and intermediate between the value for the standards.

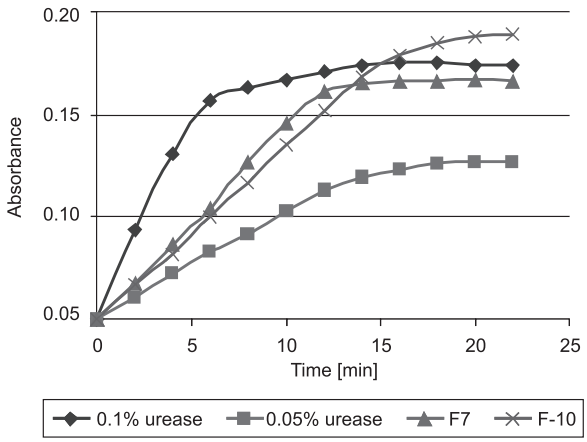

Fig. 5. Efficiency of urease immobilization on different substrate F7 and F-10 in comparison with urease standard (pH 2)

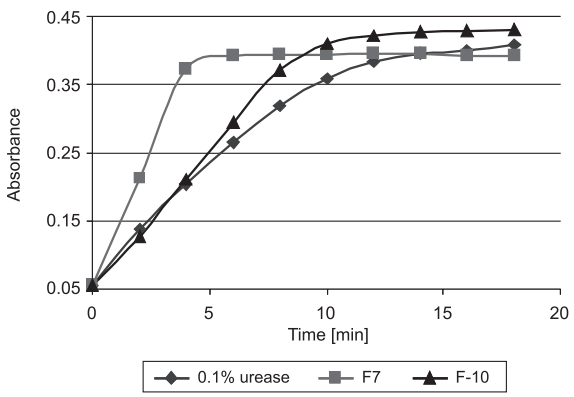

Fig. 6. The graph of dependence between absorbance and reaction time for foils F7 and F-10 with use of 0.1M urea solution

There were used different types of excess of the substrate while examining the reaction of decomposition of the urea in the ambient temperature.

For less excess of the substrate the shortest response time of the reaction was gotten for the F7 foils (Table 1)

4. Conclusion

In this work the conditions for sulfonation of polysulfone were chosen. The water-insoluble sulfonated polysulfone derivative with a high degree of substitution was obtained. The phase inversion method was used to make the membrane from the synthesized sulfonated polysulfone. In order to obtain the membrane with the good physical properties the reaction with 5% ethylenediamine in the 0.1M carbonate buffer pH 9 and then with 1% glutaraldehyde should be carried out. Higher concentration of the glutaraldehyde results in destruction of the SPSf foils. Urease was immobilized on the matrix surface with high efficiency, which was comparable with the native urease (~70000 units/g solid) in concentration of 0.1% by weight. That is adequate 6 units/cm^2 of the membrane.

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