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EFFECTS OF THE FLOW TYPE ON THE IMMOBILIZATION OF HORSERADISH PEROXIDASE ON POLYMERS

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ABSTRACT

This paper presents the investigation of Horseradish peroxidase (HPR) immobilization on poly-(acrylonitrile–co–acrylamide) and polyamide membranes using three different regimes of a fluid movement: a turbulent, a laminar and an intermediate one. The turbulent movement of the liquid is realized through a magnetic stirrer application. The laminar flow is achieved in a microfluidic system, while the attached amount of enzymes is monitored by a quartz crystal microbalance (QCM). The third type of movement, a fluid movement in an inclined pipe with a varied angle of inclination, is realized in a laboratory made rig. We find that the type of flow influences the immobilized enzyme amount, the time of the immobilization and the structure of the immobilized film. The main conclusion is that the laminar flow favours the process of covalent immobilization most probably because of the orientation of the molecules and their movement parallel to the carrier surface.

Keywords: enzyme immobilization, liquid flow regime, device for biomolecular immobilization, QCM.

INTRODUCTION

Immobilization is an important process in biotechnology during which biomolecules are attached to a carrier. The main reasons for enzyme immobilization are improved stability, restricted mobility of the molecules, and reuse [1]. Often, the final result of enzyme immobilization is widening of pH and temperature range of enzyme activity compared to those of a free enzyme [2]. Research on new enzyme based systems with improved performance is an essential stage in the construction of effective bioreactors [3], successful biofilm formation [4], and the design of more sensitive biosensors [5, 6].

Depending on the application of the enzyme systems, synthetic [7, 8] and natural polymers [9, 10], metal and dielectric nanoparticles [11, 12], sol-gel hybrid materials [13, 14] and a variety of inorganic materials [15, 16] are used as supports. The methods of enzyme molecules attachment include a covalent immobilization, an adsorption, an entrapment, crosslinking and ionic interactions [17]. The diverse properties of the synthetic polymers make them suitable for various biotechnological applications. For example, the presence of -CN and -$CONH_2$ functional groups in poly-(acrylonitrile –co –acrylamide) can be used for an effective covalent immobilization of enzymes [18]. The main advantage of the covalent immobilization refers to the strong attachment which allows the system to be reused many times. The possibility of conformational changes in the enzyme molecules followed by a loss of activity is its disadvantage.

In order for the enzyme molecules to be attached to

a support, a close contact must be established between the molecules and the support. Such contact is achieved through convection and diffusion of enzyme molecules from a moving liquid towards the carrier surface. Furthermore, the movement of the fluid itself exerts shear stress on the molecules attached to the surface. This is why the characteristics of the fluid movement play an important role in the binding process. The influence of the flow regime, and specifically of the shear stress, on the immobilized enzyme activity is the subjects of several papers with contradictory results [19]. Two enzymes, penicillinase and lactate dehydrogenase, are covalently bound to the interior surface of nylon tubes using tubular reactors and a laminar flow [20]. The authors find no effect on the kinetic parameters for shear rates up to 10000 s^{-1} and shear stress up to 73 Pa. The adsorption of the protein b-lactoglobulin to stainless steel surfaces and subsequent rinsing at different flow regimes is studied in ref. [21]. It is reported that the protein amount adsorbed under a turbulent regime is higher than that obtained in a laminar flow. However, to the best of our knowledge, there is no research comparing a covalent immobilization of enzymes at turbulent and laminar flow regimes.

This paper reports an investigation of the immobilization of Horseradish peroxidase (HPR) on two polymers: poly-(acrylonitrile–co–acrylamide) and polyamide membranes using three different regimes of a fluid movement: a turbulent, a laminar, and an intermediate mode. The turbulent movement of the liquid is realized through a magnetic stirrer application. The laminar flow is achieved in a microfluidic system, while the attached enzyme amount is monitored by a quartz crystal microbalance (QCM). The third type of movement, which is similar to a fluid movement in an inclined pipe with a varied angle of inclination, is realized in a laboratory made rig.

EXPERIMENTAL

Chemicals

Peroxidase from horseradish (HRP) Type I, (EC. 1.11.1.7), CH_3OH (p.a.), 99 % periodic acid (H_5IO_6), 99,8 % ethylene glycol, 99.85 % acetic acid, a polyamide membrane and methoxybenzene were purchased from Sigma-Aldrich. $NaH_2PO_4.2H_2O$ (p.a.) and $Na_2HPO_4.12H_2O$ (p.a) used for a phosphate buffer and 30 % H_2O_2 were products of Merck. O-dianisidine (3,3`- dimethoxybenzidine) and a dialysis membrane were provided by Serva. The synthetic polymer of poly-(acrylonitrile-co-acrylamide) was obtained in the laboratory. 99.8 % N,N-Dimethylformamide (DMF) was also used.

Carriers and immobilization conditions

The laboratory made rig (immobilizer) was run by a DC motor with a motor controller ensuring a smooth adjustment and a stable motor speed. A plexiglas disk with a diameter of 30 cm was fixed to the motor axis. Sixteen holders for sample containers were situated regularly along the disk circumference. The containers were glass or plastic bottles of a different volume depending on the experimental conditions. The motor was fixed to a solid base, while its height could be adjusted. Such a construction allowed the simultaneous immobilization of two or more different proteins or other types of biomolecules on different carriers and/or for different durations. A scheme of the immobilizer is shown on Fig. 1.

HRP was chosen in our experiments as a model enzyme. It was immobilized onto two types of supports: granules of poly-(acrylonitrile–co–acrylamide), a synthetic polymer, with a diameter between 1 mm and 2 mm obtained under laboratory conditions using the method described in ref. [22], and polyamide membranes from Sigma-Aldrich. The immobilization was realized in 20 mL plastic bottles of a diameter of 25 mm. Each bottle contained 1.0 g of the carrier and 5 mL of oxidized and dialyzed solution of HPR with a concentration of 0.5 mg/mL. The immobilizer as described above was rotated at a speed of 15 rpm. In one of the experiments sets a 10 mm magnetic bar placed in the bottles was rotated with a speed of 690±15 rpm (measured by a stroboscope) by the magnetic stirrer. In both sets of experiments the immobilized enzyme amount was measured after 1 h, 2 h, 6 h, 8 h, 12 h, and 24 h.

Carbohydrate residues oxidation

Oxidation of carbohydrate horseradish peroxidase residues was carried out using the Zaborsky and Ogletree method [23] (0.4 mL 4.10^{-5} M periodic acid in 20 mL 5.10^{-2} M acetic buffer, pH = 5.6 in dark). The unreacted periodic acid was removed with 0.025 mL ethylene glycol. After oxidation and neutralization, the enzymes were dialyzed using a dialysis membrane immersed in 5.10^{-2} M acetic buffer of pH = 5.6 for 24 h and T = 4°C.

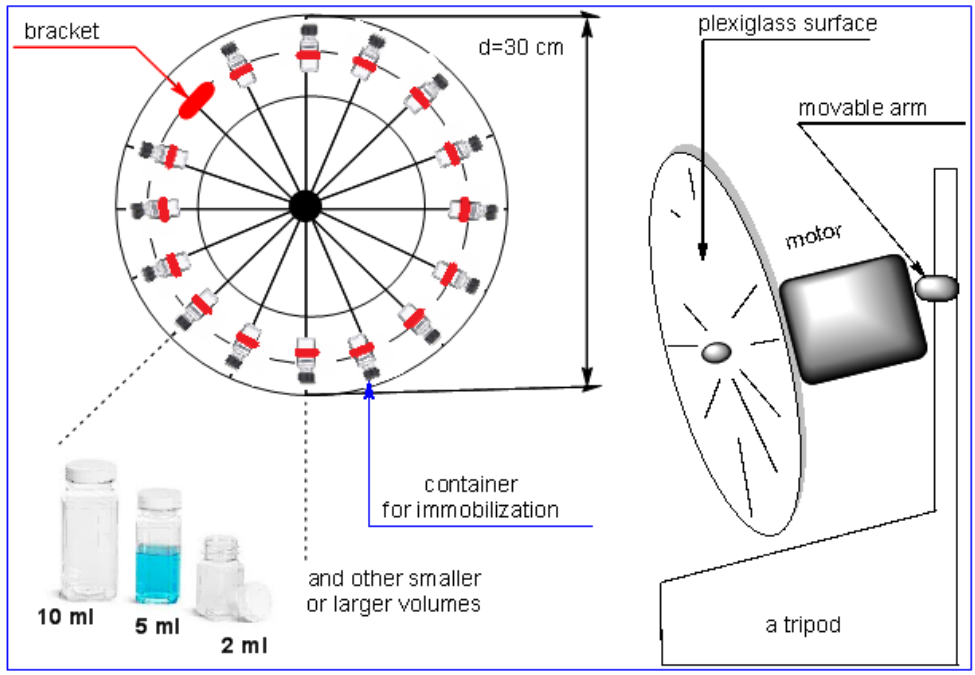

Fig. 1. Scheme of the immobilizer.

Determination of enzyme activity and content of protein

Peroxidase activity was determined spectrophotometrically (VWR UV-1600 PC Spectrophotometer, Germany) at $\lambda = 460$ nm using H_2O_2 as a substrate. The latter was prepared by diluting 1 mL of 30% hydrogen peroxide to 100 mL with distilled water. Immediately prior to the start of the experiment 1 mL of this solution was further diluted in 50 mL of 0.2 M potassium phosphate buffer with pH of 7.0. One unit of an enzyme activity was the equivalent of the conversion of one micromole of hydrogen peroxide per minute at 25°C and pH of 7.0. The total protein content was determined by the modified Lowry method [24] using bovine serum albumin as a standard.

Thin polymer film deposition onto the quartz resonator

Thin polymer films were obtained by a spin coating. The solution was prepared by dissolving 0.1 g poly-(acrylonitrile-co-acrylamide) in 6 mL DMF and 2 mL methoxybenzene. A sufficient amount of the liquid was placed on the larger of the gold electrodes covering the whole surface area of the resonator and then the sample was rotated at 2000 rpm for 60 s. The thickness of the polymer film measured by surface plasmon spectroscopy (results not shown) was 25 nm.

Scanning Electron Microscopy

The morphology of the obtained structures was observed with a dual beam scanning electron/ focused ion beam system LYRA I XMU, TESCAN after the samples were coated with a conductive thin gold film.

FT-IR

FT-IR transmission spectra were recorded by Brucker Tensor 27 Spectrometer with a scanner velocity of 10 kHz using a MCT detector. The average results of 64 scans with a resolution of 1 cm^{-1} are shown on Fig. 2.

QCM in situ immobilization of HRP

A quartz crystal microbalance was used to monitor the amount of protein attached to the sensor surface during the laminar liquid flow in a microfluidic system. Sensor chips from Attana A100 (Attana AB, Sweden) were used. The resonators were made using AT-cut quartz with gold electrodes with a nominal resonance frequency of 10 MHz. These resonators oscillated in a thickness-shear mode with a displacement vector parallel to the plate surface. The cell volume was approximately 4 µl, while the electrode surface was 16 mm^2. According to the Sauerbrey equation [25] a linear relationship exists between the mass Δm deposited onto the electrode and the change of the resonance frequency Δf:

$$\Delta m = -C\Delta f \quad (1)$$

as long as Δm is less than 2 % of the quartz plate mass. In our case, constant C was equal to 0.7 ng/Hz. The oscillation frequency was recorded every second by a frequency counter from the beginning of the liquid flow introduction to the cell. It was stored in a computer and displayed on the computer monitor for convenience. The periodically oxidized and dialyzed solution of 0.5 mg/mL HRP was run through the microfluidic system at a velocity of 6 mL/h.

RESULTS AND DISCUSSION

The oxidation of the carbohydrate residues (approximately 18 % as described in Materials and Methods) provides achieving a covalent immobilization of HPR molecules on both poly-(acrylonitrile-co-acrylamide) and polyamide membranes without blocking the active catalytic enzyme centres [23, 26].

FT-IR spectra

The presence of a covalent bond may be indirectly proved by the existence of specific absorption lines in FT-IR spectra. The free enzyme spectrum has already been discussed in ref. [27] and Fig. 2(a) shows that our results are similar. The FTIR spectra of the copolymer prior to and after the enzyme immobilization are shown in Fig. 2(b). The two sharp absorption peaks at 2243 cm^{-1} and 1690 cm^{-1} are attributed to stretching of –CN and $–CONH_2$ groups, respectively, and are related to the covalent binding between the enzyme and the polymer [22]. The bands at 3365 cm^{-1} and 3463 cm^{-1} correspond to N-H bond stretching and indicate the presence of acrylamide in the polymer chain. The absorption band at 530 cm^{-1} is due to the bond C-C≡N. The 1075 cm^{-1} band is assigned to CH vibration mode. The absorption band at 1250 cm^{-1} is due to the bending mode of methane – (CH) group coupled with the rocking mode of the methylene (CH_2) group. The bands that appear at 1220 cm^{-1} - 1270 cm^{-1}, 1345 cm^{-1} -1375 cm^{-1}, and 1465 cm^{-1} are assigned to C-H vibration modes [22, 27].

Comparison of the amount of the immobilized enzyme in the magnetic stirrer and the immobilizer

Immobilization on poly-(acrylonitrile-co-acrylamide)

The main goal of our study is to determine the relationship between the immobilization time and the quantity of the bonded HPR on one hand and the effect of the fluid movement type on the other. Fig. 3 presents the average amount of immobilized protein of three independent measurements at room temperature as a function of the duration of the mixing in the immobilizer and in the magnetic stirrer. It can be seen that the quantity of the immobilized protein in both rigs starts to saturate after the sixth hour. This is due to the exhaustion of the immobilization capacity of the polymer and/or a possible obstruction (steric) effect among the enzyme molecules. After the eighth hour, only insignificant changes in the amount of the immobilized protein are registered – about

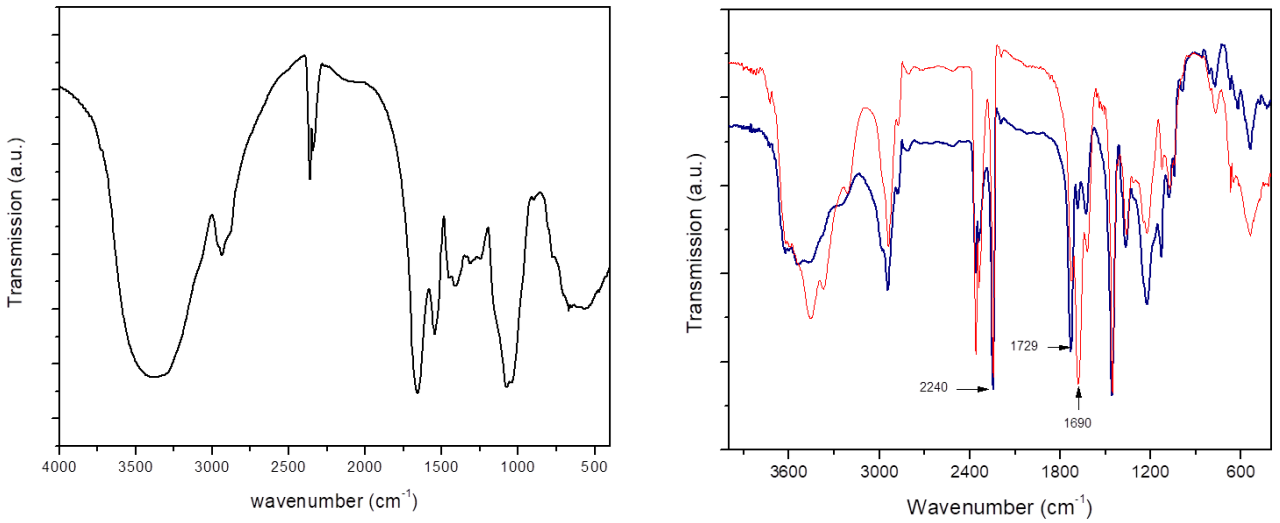

Fig. 2. (a) FT-IR spectrum of the free enzyme, (b) FT-IR spectra of the polymer and the polymer with the attached enzyme.

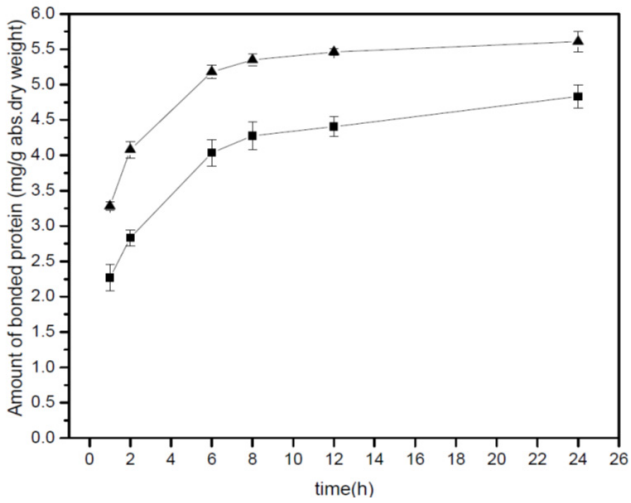

Fig. 3. Dependence of the immobilized protein on time using a magnetic stirrer (squares) and the immobilizer (triangles).

2 % between the eighth and the twelfth hours and about 8 % between the twelfth and the twenty fourth hours. The shapes of the two curves are similar but the amount of the immobilized enzyme in the immobilizer is always greater than that in the magnetic stirrer: by about 30 % during the first two hours and by about 13 % after 24 h. Fig. 3 also shows that the optimal duration of the immobilization in both rigs is about 8 h. The comparison of the results obtained with the magnetic stirrer and the immobilizer is carried out on the ground of three experiments.

The relationship between the relative activity of the immobilized enzyme and its catalytic activity is shown in Table 1. The last column shows that the relative activity in the immobilizer is 18 % higher than that recorded in case of the magnetic stirrer, a result consistent with the higher amount (by 17 %) of the immobilized protein in the immobilizer compared to that in case of the stirrer.

Immobilization on polyamide membranes

Another set of experiments on immobilization of HPR in both the immobilizer and magnetic stirrer is carried out on polyamide membranes under identical conditions. The results in Table 2 reveal a trend similar to that obtained with poly-(acrylonitrile-co-acrylamide) granules. The amount of the enzyme attached to the membrane in the immobilizer is 17 % and 10 % higher compared to the amount in the magnetic stirrer after 6 h and 12 h of immobilization, respectively.

Immobilization in the magnetic stirrer and the immobilizer

The immobilization conditions in case of a magnetic stirrer and in an immobilizer application are identical during the experiments. The only exception refers to the fluid movement. The movement of the liquid in case of a magnetic stirrer is three dimensional, and depends on the bar angular frequency, the fluid viscosity and the volumes of both the vessel and the bar itself. A characteristic funnel is formed at the centre of the vessel [28, 29] due to the simultaneous radial and vertical movement of the fluid. The mixing frequency in case of a magnetic stirrer application is chosen to be 690±15 Hz (measured by a stroboscope). An optimal movement of the polymer balls at this frequency and those close to it is observed. At lower frequencies, the funnel is not observed and the mixing is not effective enough, while at higher ones the fluid integrity breaks off. The motion of particles lighter than those of water (as in our case) in the case of a magnetic stirrer application is described in ref. [29]. The main conclusions drawn in this paper are: i) the dynamics of such particles when a vortex is present is that of a chaotic attractor; ii) the drag force is more complex than the Stokes one because of the high Reynolds number and

Table 1. Catalityc properties of enzyme immobilized in the magnetic stirrer and in the immobilizer on poly-(acrylonitrile-co-acrylamide).

	1h	*2h*	*6h*	*8h*	*12h*	*24h*
Enzyme activity U/mg by immobilizer	22.8	29.1	36.6	39	39.8	42
Enzyme activity U/mg by magnetic stirrer	17.3	20.1	29.7	31.5	31.7	34.5
Relative activity, % by immobilizer	33.4	42	53	56	56	59
Relative activity, % by magnetic stirrer	25	29	43	45	46	50
Enzyme activity of free HRP, 69 U/mg						

Table 2. Amount of bonded protein on polyamide membrane in mg/g absolutely dry weight.

	2 h	*6 h*	*12 h*
Immobilizer	4.89	6.19	6.40
Magnetic stirrer	4.83	5.06	5.52

iii) the role of the turbulence is essential.

In the immobilizer, the fluid movement is governed by the gravitational and centripetal forces. The diameter of the circle is 30 cm and the speed of rotation is 15 rpm. Under these conditions, the centripetal acceleration is 0.37 m/s^2, representing less than 4 % of the gravity acceleration. This allows the fluid movement in the immobilizer to be treated as a fluid movement in an inclined pipe without taking into account the centripetal acceleration. At small angles such movement is described as smooth and undisturbed (i.e. close to a laminar one), while at large ones it forms 3D structures, including solitary waves (solitons) [30]. Most of the time the movement of the fluid we observed is close to the first type – smooth and close to laminar.

Immobilization onto the QCM resonator

In order to investigate the dependence between type of the fluid movement and the enzyme immobilization, the dynamics of the enzyme immobilization onto the polymer film is registered using QCM. A thin film of poly-(acrylonitrile-co-acrylamide) is cast onto the resonator by a spin coating. A 0.5 mg/mL HRP solution (a concentration value used in the previous experiments) is run through the system at a speed of 100 μL/min. Fig. 4 shows the resonance frequency change due to the enzyme immobilization. One can see that the frequency change is insignificant after 2 h. This shows that the immobilization process is over. Using Eq. (1) one can calculate that the enzyme amount immobilized onto the resonator is about 100 ng or 7 ng/mm^2. Since the molecular weight of HRP is 44 kDa, this means that approximately one molecule is attached to 10 nm^2 of the surface. This is consistent with the size of HPR molecule (4.0 x 6.7 x 11.7 nm^3) [31] and means practically that a monolayer of HPR is formed on the polymer surface thus confirming that the immobilization process is over. The immobilized enzyme amount on the resonator obtained by us is equal to 7 ng/mm^2. It can be compared with HPR adsorbed quantity on Si measured in ref. [31] by ellipsometry. That value is 3 ng/mm^2. The atomic force microscopy measurements of their samples reveal that a part of Si surface remains uncovered by the enzyme. Taking into account this fact and also the different measuring techniques used, the agreement between the two results can be considered very good.

Due to the cell design and the small fluid velocities, the flow in the QCM cell is laminar [32, 33]. For example, it is calculated in ref. [33] that the maximum fluid velocity at the centre of the cell is v_{max} = 3.9 mm/s. The volume flow is 100 μL/min as in our experiments. Since the cell height is 200 μm, it is easy to calculate that the Reynolds number is less than 1. The shear stress τ at the surface in case of a laminar flow between two parallel plates can be calculated by the formula:

$$\tau = \frac{4\mu}{h} v_{max} \qquad (2)$$

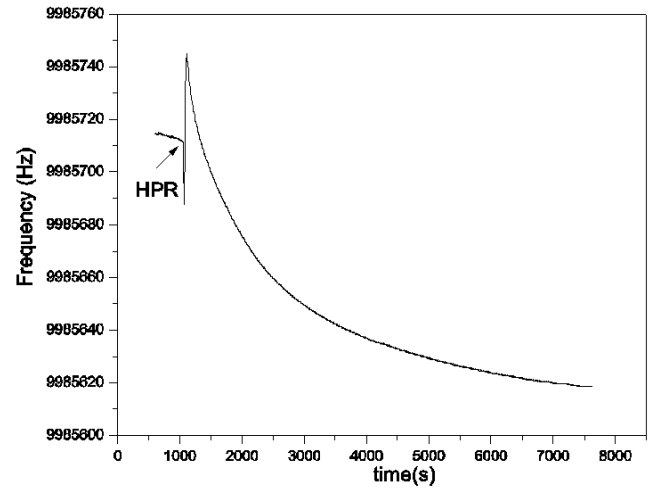

Fig. 4. Change of the resonant frequency of the QCM during HRP immobilization on poly-(acrylonitrile–co–acrylamide); the arrow shows the moment when from deionized water of the enzyme solution.

where μ is the dynamic viscosity (10^{-3} Ns/m), and h is the distance between the plates. Substituting the corresponding values in Eq.(2), we calculate the shear stress to be 8 Pa. Since the height of the cell we use is was about 100 μm, while the other dimensions are identical with those in ref.[34], we can conclude that the shear stress in the QCM cell we use is about 16 Pa at a flow rate of 6 mL/h. Even smaller values of shear stress are calculated in ref. [33] for other QCM cells. The shear stress effect on the immobilization of penicillinase and lactate dehydrogenase in a laminar flow is studied [20] and it is found that shear stress up to 73 Pa has no influence on the immobilization rate. Therefore, we conclude that the shear stress in a QCM cell doesn't affect the immobilization process.

Structure of the enzyme layers

The different type of the fluid movement leads not only to a different amount of enzyme immobilized on the carrier but also to a different structure of the protein layers. SEM images (unpublished results) reveal that the polymer surface is slightly wavy and without pores. Such a structure doesn't allow enzyme molecules to penetrate into the carrier volume and the immobilization occurs only at the fluid - polymer interface. Fig. 5a shows SEM images of the enzyme layers formed in case of a magnetic stirrer application, in the immobilizer (Fig. 5b), and in the QCM system (Fig. 5c). It can be seen that the layer grown in the immobilizer is smooth and evenly distributed over the surface following the carrier topography, while the film grown in presence of a magnetic stirrer is inhomogeneous with enzyme congregations and pores. The structure of the immobilized enzyme film in the immobilizer is similar to the one obtained in the QCM system thus confirming that the fluid movement in the immobilizer is laminar most of the time but also that a laminar movement or one close to it results in an immobilized protein film much smoother than that obtained under turbulent fluid conditions.

There are several possible reasons explaining the different amounts of immobilized protein and the different times of immobilization observed for the three types of fluid movement. They refer to the concentration of the enzyme molecules near the surface, the shear stress exerted by the fluid on the immobilized molecules, the average time spent by a molecule near the surface (average residence time), the effects due to the orientation of

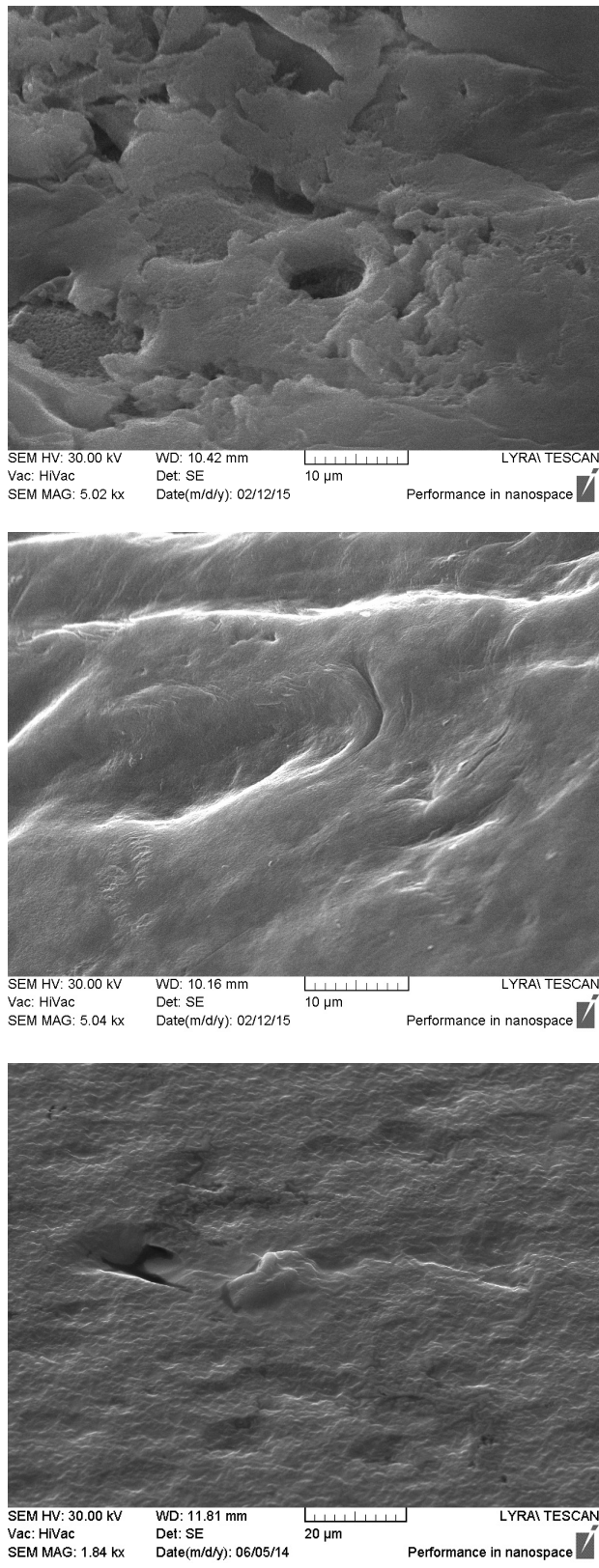

Fig. 5. SEM images of the enzyme layers formed in (a) the magnetic stirrer, (b) in the immobilizer, and (c) – in the QCM microfluidic system.

the molecules in relation to the surface.

The immobilization process in a laminar flow is modelled with a stationary boundary layer through which the molecules are transported towards the walls only by molecular diffusion [34, 35]. In this layer, the mass transport rate to the surface is determined by the layer's thickness (generally thought to be a few microns), the diffusion coefficient and the bulk concentration of the molecules. In contrast, under turbulent conditions, turbulent mixing dominates the mass transfer towards the wall and higher mass transfer rates are observed [35]. Thus, it is highly unlikely that the concentration of the molecules near the surface in case of a magnetic stirrer during the turbulent regime is smaller than that in a laminar flow, such as in QCM experiments.

When immobilized on the surface, the molecules are exposed to the shear stress caused by the fluid flow. Several papers deal with the influence of this stress on the immobilized enzymes [19, 20, 36]. Inactivation of enzyme molecules and bond breaking are considered possible shear stress consequences. Inactivation is not an issue in our case because the enzyme activity is proportional to the amount of immobilized enzyme in the magnetic stirrer and in the immobilizer (Table 1). Breaking the covalent bond between the enzyme molecule and the matrix requires more energy (approximately equal to 10^5 cal/mol) than that of inactivation [19] and thus bond breaking is even less probable than enzyme inactivation under the conditions of our experiments. An indirect proof that shear forces cannot influence the immobilization process in our case is the comparison of our results with those reported in ref. [21]. An increment of adsorbed proteins on the surface is observed there in a turbulent flow compared to the case of a laminar one. Since covalent bonds are much stronger than those due to van der Waals forces, shear forces cannot be the reason for the smaller amount of the enzyme deposited on the surface at a turbulent flow in our experiments.

Let us now consider the possible influence of the residence time of the molecules near the surface. If this time is short enough the molecule may not have time to settle down on the surface. However, the chemical bond forms very quickly – the time is similar to that required by an electron to make a tour around the atom. In addition, the argument based on the comparison with the adsorption mechanism [21] is also true.

The discrepancy between our results and those reported in ref. [21], where the adsorbed protein amount increases in a turbulent flow, merits further discussion. One possible explanation is that, unlike the covalent bond, the physical adsorption is due to van der Waals forces. The latter arise from electric multipole – multipole interactions but no chemical bond is formed. Apart from being much weaker than covalent bonds, they depend less on the orientation of the molecules, especially in case of large molecules such as those of the enzymes. The covalent bonds are highly directional and also, as in the current case, may be formed only between specific atomic groups. This means that the orientation of the enzyme molecules plays a much more important role in our experiments than in case of a simple adsorption. Molecules in a flow are subject to rotation due to angular momenta arising from shear forces. This leads to molecules predominantly aligned with the longest axis along the streamlines in a laminar flow (this alignment cannot be perfect, however, because of the Brownian motion). On the other hand, in a turbulent flow, eddies of various sizes are superimposed onto the mean flow and such predominant orientation of molecules is very unlikely. We believe that the differences in the orientation of the molecules near the surface caused by the dissimilar nature of the laminar and turbulent flows may explain the variation in the immobilization process of HPR under different flow conditions.

CONCLUSIONS

This paper reports results obtained in the investigation of the fluid movement effect on the enzyme covalent immobilization process on two polymers: poly-(acrylonitrile–co–acrylamide) and polyamide membranes. Three types of a fluid movement are studied: a turbulent (with a magnetic stirrer application), a laminar (in a microfluidic system and QCM) and a third one that is considered an intermediate one. The third type is similar to that in an open tube with a variable angle of inclination, which is laminar at a small angle and complex at higher ones. It is realized in a laboratory made rig (an immobilizer) described in the paper. The results show that for different durations the immobilization efficiency in the immobilizer is typically with 10 % - 23 % higher than that observed in a magnetic stirrer presence for both carriers. Furthermore, higher immobilization percentage is obtained during the first six

hours demonstrating that the process in the immobilizer is faster. The immobilization time varied from 2 h in the QCM to typically 8 h - 12 h in the other two rigs. The SEM pictures reveal that the structure of the HRP film obtained in the new device is smooth and similar to that formed in the QCM where the fluid movement is completely laminar. Those obtained with a magnetic stirrer have a very irregular and uneven surface.

Several reasons explaining the behaviour registered are considered leading to the conclusion that the fluid movement type most probably affects the enzyme molecules orientation near the surface and thus the process of covalent bonding. In a laminar movement, the molecules are predominantly aligned by the shear forces with the longest axis along the surface and their movement is parallel to the surface. It seems that this creates favorable conditions for the formation of highly directional covalent bonds.

We hope that this study will contribute to the optimization of the enzyme covalent immobilization process. It demonstrates that the fluid movement must be taken in consideration during the interaction between the protein molecules in the fluid and the carrier along with the carrier type, the temperature, pH and several other factors.

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