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Investigation of chitosan/xanthan and xanthan/chitosan multilayers on corona charged polylactic acid substrates

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In the present paper polyelectrolyte multilayers (PEMs) deposited on polylactic acid (PLA) substrates were investigated. The substrates were charged in a corona discharge system consisting of a corona electrode (needle), a grounded plate electrode, and a metal grid placed between them. Positive or negative 5 kV voltage was applied to the corona electrode. 1 kV voltage of the same polarity as that of the corona electrode was applied to the grid. Time dependences of the normalized surface potential of PLA substrates were investigated. Layer-by-layer (LbL) deposition technique was applied for multilayer build-up. The LbL deposition was done with the first built-up layer always possessing an electric charge opposite to that of the substrate. In the polyelectrolyte multilayers obtained the enzyme β-galactosidase was immobilized. The biochemical characteristics of β-galactosidase and trans-galactosidase activity were investigated.

Keywords: polylactic acid, corona discharge, polyelectrolyte multilayers, immobilized enzymes, β-galactosidase.

INTRODUCTION

The layer-by-layer (LbL) self-assembly technique received extensively renewed interests as an attractive technique for the production of polyelectrolyte multilayer thin films that are widely used in biomedicine [1], drug delivery [2], food science [3], membranes [4], etc. The LbL method allows very precise control and changes in different physicochemical properties — thickness, charge, hydrophilic–hydrophobic balance [5]. The multilayer built up by the LbL technique is characterized by precisely defined properties, flexible choice of assembled components, and the ability to cover surfaces of any size and geometry. During the self-assembling of polyelectrolytes from solutions, electrostatically bound complexes are formed between the polyelectrolyte functional groups of the oppositely charged that surface and the polymer, leaving excess charges due to charge overcompensation.

In the literature there are several techniques for charging polymer substrates, for example chemical modification [6, 7], plasma treatment [8], corona discharge [9, 10]. The corona discharged is one of the most commonly used methods of materials treatment as it is inexpensive and easy to realise from a technical point of view. In case of a corona discharge the requirement for the substrate is to possess good electret properties and to retain its surface charge long enough for the first polyelectrolyte layer to be deposited.

The immobilization of enzymes into polymer matrices is widely used in biotechnology. This technique makes it possible to increase the functional efficiency of enzyme, enhance the reproducibility of the processes, improve the process control and ensure stable supply of the products in the market [11]. The basic idea of enzyme immobilization is to entrap the protein in a semi-permeable support material, which prevents the enzyme from leaving while allowing substrates, products, and co-factors to pass through [12].

One of the suitable and mostly used natural polymers for enzyme immobilization is chitosan. It is characterized as biocompatible, nontoxic, physiologically inert, and hydrophilic, offers the unique characteristic of a remarkable affinity to proteins and has been widely applied in medicine and biological research. In [13] the authors describe different methods of immobilization of chitosan to the surface of PLA films. The study examines how different kinds of chitosan, deposited through either immersion or electrospinning methods, change the surface properties of a surface treated PLA films. The antibacterial and antifungal properties of chitosan-modified PLA were also investigated. The paper shows that all types of chitosan improve the antibacterial properties of the PLA films.

β-Galactosidases, known also as lactases, are enzymes belonging to glycoside hydrolase families 1, 2, 35, 42 and 59 (GH1, GH2, GH35, GH42 and GH59) [14]. These enzymes catalyze the hydrolysis

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of terminal non-reducing β-D-galactose residues in β-D-galactoside substrates. Microbial sources of β-galactosidases are bacteria (*Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Escherichia*), yeasts (*Kluyveromyces*, *Sterigmatomyces*) and fungal producers (*Aspergillus*) [15]. In [16] the size effect on the properties of enzyme immobilization was investigated by using chitosan macroparticles and nanoparticles. In this study β-galactosidase was used as a model enzyme. It was established that the different sizes and porosities of the particles modify the enzymatic load, activity, and thermal stability of the immobilized biocatalysts. The thermal stability was improved for macroparticles, especially under reactive conditions (presence of lactose) in comparison with the free enzyme.

In [17] alginate–chitosan core-shell microcapsules were prepared as a novel biocompatible matrix system for β-galactosidase enzyme immobilization where the catalyst is confined to either a liquid or solid core and the transport properties of the substrate and product are dictated by the permeability of the shell. Such the biological agent is protected in the inner biocompatible alginate core and the outer chitosan shell dictates the transport properties.

The purpose of the present paper is to investigate the immobilization of the β-galactosidase enzyme in chitosan/xanthan and xanthan/chitosan multilayers deposited on corona charged polylactic acid substrates.

EXPERIMENTAL DETAILS

Poly(lactide) substrates formation

Poly(DL-lactide) (PDLA) (inherent viscosity 0.55-0.75 dL/g), purchased from Lactel Absorbable Polymers (USA), was used for the preparation of the biodegradable substrates. The substrates were prepared by dissolving of 2 grams of PDLA in 100 ml of chloroform. The solution was poured in petri dishes and dried at 35°C for 48 hours until the evaporation of the solvent. Then the PDLA substrates were kept in an exicator at room temperature at relative humidity (RH) of 54 % for 24 hours.

Corona charging and surface potential measurement of the samples

The substrates obtained were charged in a corona discharge, in order to achieve positive or negative electric charge on their surface. The charging of the samples in a corona discharge was carried out by a conventional corona triode system (fig. 1), consisting of a corona electrode (needle), a grounded plate electrode and a grid placed between them.

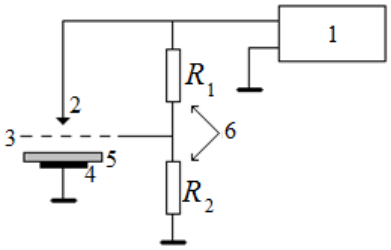

Fig. 1. Scheme for obtaining electrets: 1. high voltage source; 2. corona electrode; 3. grid; 4. plate grounded electrode; 5. sample on a metal pad; 6. voltage divider.

The distance between the corona electrode and the grid was 10 mm and the distance between the grid and the grounded plate electrode was 3mm.

Charging of the electrets was performed under relative humidity of (45% - 50%), room temperature and atmospheric pressure for 1 minute. Positive or negative 5 kV voltage was applied to the corona electrode. A voltage of 1 kV of the same polarity as that of the corona electrode was applied to the grid. After charging, the initial surface potential of the samples V_0 was measured. Electrets' surface potential was measured by the method of the vibrating electrode with compensation [18] and the estimated error was better than 5%.

Layer-by-layer deposition of chitosan/xanthan multilayers

Chitosan (low molecular mass, degree of deacetylation > 75%) and xanthan gum were purchased from Sigma-Aldrich. They were used without further purification or characterization. The layer-by-layer deposition technique was applied for multilayer build-up. For LbL assembling process 0.1% w/v chitosan and 0.05% w/v xanthan solutions of acetate buffer (pH 5 and ionic strength 0.1 M) as solvent were prepared.

1 g/L β-galactosidase was dissolved in the chitosan solution just before the deposition process. The deposition was done by the dip-coating process. The first built-up layer always possesses opposite to the substrate electric charge. A programmable slide stainer (Poly Stainer IUL, Spain) was used with the following program: 15 min dipping process – adsorption from the first polyelectrolyte solution, 5 min washing step in the acetate buffer, 15 min dipping process - adsorption from the second polyelectrolyte molecules of opposite charge; 5 min washing in the same acetate buffer. The procedure was repeated until obtaining

the desired numbers of even layers (xanthan/chitosan or chitosan/xanthan). After the deposition of the last layer the film was dried in hot air. The produced PEMs structures were stored in an exicator at 55% RH.

Enzyme activity

Commercial fungal β-galactosidase (from *Aspergillus niger*) was used in the current kinetic studies. One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the release of 1 μmol.min^{-1} glucose at 37 °C and pH 5.0. The influence of the substrate concentration on the initial velocity of the enzyme reaction was studied in the range of 0.01 M – 1.30 M lactose. β-Galactosidase activity was studied in the presence of lactose – 1%, 5%, and 10% and mixtures of chitosan (0,1%) and lactose (1%, 5%, and 10%). The concentrations of the released glucose were determined enzymatically [19]. Protein concentration was assayed by the method of Bradford [20]. All the analyses were performed at least in triplicate. Programmable scientific calculation "CITIZEN" SRP-45N and SigmaPlot 12.0 (Systat Software, Inc) were used for data analysis.

In order to determine the amount of immobilized enzyme on the multilayer films, an enzymatic assay of β-galactosidase with ONPG was conducted. The samples were placed in glass beakers and a mixture of 1500 μl of ONPG solution (ionic strength 2.0 mM) and 900 μl of deionized water was added. The samples were left in a water bath at 37 °C and at 30 min and 60 min 800 μl of the reacted solution was taken from them and mixed with 4 ml of sodium carbonate solution (with ionic strength 1M) to stop the reaction. The absorption of the samples at 405 nm was measured using a spectrophotometer. The test was repeated several times at 24 h increments to measure the remaining activity of the samples after repeated use.

RESULTS AND DISCUSSION

Time storage influence on the electrets surface potential decay

The dependences of normalized surface potential V/V_0 on the time of storage under room conditions for positively and negatively charged PDLA films were investigated for 360 minutes. The surface potential was measured once every 10 minutes except for the first 20 minutes when it was measured more often (once in 2 minutes) because the charge was rapidly decaying. After this period, steady state values of the surface potential were established for all investigated samples. Time dependences of the normalized surface potential for positively and negatively charged PDLA films are presented in Fig. 2.

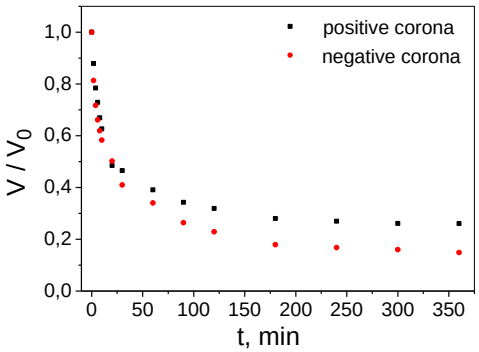

Fig. 2. Time dependences of the normalized surface potential for positively and negatively charged PDLA electret films.

Each point in the figure is a mean value from 6 samples. The calculated standard deviation was better than 5 % from the mean value with confidence level 95 %.

The experimental results presented in Fig. 2 show the following features:

- For all investigated samples the values of the normalized surface potential are initially decaying exponentially for the first 50 minutes and then are slowly decreasing and are practically stabilized to the 360 minute.

It was established that the value of the electret surface potential depends on the amount of trapped charges in the different localized surface states of the samples. In the initial period of time after the corona charging, the surface potential rapidly decreases. This is due to the release of the weakly captured charges from the shallow energy states. Then the surface potential stabilizes to a steady state value caused by the tightly captured charges in the deep energy traps. Similarly, exponential decay with subsequent slow linear reduction of the electrets charge was observed in [21].

- The steady state values of the normalized surface potential for the samples charged in a positive corona are higher than those for the samples charged in a negative corona.

During the corona discharge in air, at atmospheric pressure, different types of ions are deposited on a sample. Therefore, charging in a corona discharge depends on the corona polarity [22, 23]. In case of a positive corona the ions are mainly $H^+(H_2O)_n$ and those for a negative corona - CO^{3-}. Those ions are bound in traps of various depths and they are released from them depending on the surrounding conditions.

Effect of type of PEMs on immobilization and enzyme activity

The enzyme activity of 6 to 8 samples of each of the multilayer film configurations was measured on the 30th and 60th minute after submersion in the reactive agent for several consecutive days (displayed as roman numerals in the following Tables). After the 60th minute, the samples were removed from the remaining solution and left to dry. On the following day, the measurements were repeated with fresh reactive solution.

From the results presented in Tables 1 and 2 can be seen that in the case of enzyme immobilization in a multilayer film with 4 polyelectrolyte layers the negatively charged PLA films show 30 % higher activity of the enzyme β-galactosidase compared to the ones with positive charge. In case of 8 layers the efficiency of immobilization is considerably better, without significant difference in activity between the positively and negatively charged PLA films. The initial activity of the 8 layer PLA films is around 25% higher than that of the 4 layer films. The degree of storage of the activity up to 48 hours is also higher and it is about 40 % and 50 % of the initial activity (Tables 3 and 4).

Table 1. Enzyme activity (in units/ml enzyme) of positively charged PLA films with 4 layers.

PLA positive 30 min				
	I	II	III	IV
1	0.045	0.021	0.013	0.015
2	0.048	0.024	0.013	0.017
3	0.041	0.016	0.007	0.011
4	0.035	0.014	0.013	0.014
5	0.064	0.025	0.016	0.016
6	0.038	0.013	0.009	0.015
7	0.058	0.017	0.014	0.015
8	0.058	0.022	0.011	0.013
PLA positive 60 min				
	I	II	III	IV
1	0.010	0.015	0.009	0.010
2	0.015	0.020	0.008	0.008
3	0.013	0.022	0.007	0.007
4	0.013	0.008	0.007	0.009
5	0.019	0.025	0.010	0.010
6	0.010	0.011	0.007	0.001
7	0.015	0.016	0.008	0.010
8	0.014	0.015	0.007	0.007

This is probably explained by the type of cross-linking that occurs after the specific treatment of the gel, created from two polysaccharides with different electric charge. In addition it is possible that the two polysaccharides interact with each other as a consequence of the interaction of the electric charges, creating so called "pockets", in which the molecule of the enzyme is physically positioned. This type of physical immobilization gives the enzyme spatial freedom and access of the molecules of the substrate to its active center.

During the study of the activity of the enzyme up to 48 hours, after the first reaction it was discovered that around 30% of the initial activity of the enzyme is retained in both types of 8-layer films.

Table 2. Enzyme activity (in units/ml enzyme) of negatively charged PLA films with 4 layers.

PLA negative 30 min			
	I	II	III
1	0.065	0.027	0.019
2	0.071	0.016	0.013
3	0.053	0.022	0.015
4	0.065	0.025	0.015
5	0.063	0.028	0.018
6	0.058	0.042	0.019
PLA negative 60 min			
	I	II	III
1	0.008	0.010	0.008
2	0.008	0.012	0.009
3	0.006	0.016	0.011
4	0.011	0.015	0.010
5	0.018	0.015	0.010
6	0.011	0.019	0.011

Table 3. Enzyme activity (in units/ml enzyme) of positively charged PLA films with 8 layers.

PLA positive 30 min				
	I	II	III	IV
1	0.078	0.083	0.061	0.048
2	0.067	0.057	0.044	0.039
3	0.062	0.034	0.030	0.024
4	0.071	0.062	0.054	0.047
5	0.062	0.043	0.032	0.026
6	0.066	0.057	0.033	0.029
7	0.065	0.047	0.033	0.032
8	0.062	0.031	0.026	0.024
PLA positive 60 min				
	I	II	III	IV
1	0.020	0.031	0.024	0.022
2	0.015	0.024	0.019	0.016
3	0.010	0.020	0.014	0.012
4	0.018	0.025	0.024	0.022
5	0.010	0.021	0.013	0.013
6	0.013	0.024	0.016	0.015
7	0.012	0.022	0.014	0.014
8	0.007	0.019	0.012	0.008

Table 4. Enzyme activity (in units/ml enzyme) of negatively charged PLA films with 8 layers.

PLA negative 30 min				
	I	II	III	IV
1	0.080	0.051	0.031	0.032
2	0.071	0.051	0.028	0.031
3	0.034	0.040	0.022	0.030
4	0.065	0.035	0.022	0.028
5	0.061	0.030	0.021	0.027
6	0.061	0.026	0.021	0.025
7	0.055	0.023	0.016	0.024
8	0.054	0.012	0.013	0.022
PLA negative 60 min				
	I	II	III	IV
1	0.023	0.020	0.030	0.016
2	0.014	0.017	0.025	0.015
3	0.012	0.017	0.022	0.015
4	0.012	0.016	0.020	0.014
5	0.010	0.014	0.018	0.013
6	0.010	0.014	0.015	0.013
7	0.010	0.011	0.014	0.011
8	0.009	0.010	0.010	0.011

This percentage is obtained by comparing the top left column (column I at 30 min) of each of the 8-layer films with the bottom right column (column IV at 60 min) of the same table, as these are the first and last measurements of the enzyme activity of each sample. The results for 4-layer PLA films also show some retention of the activity, but the lower initial activity limits the level of retention of both positive and negative films. This is why we decided to concentrate on the results of the 8-layer PLA films.

The results obtained demonstrate the prospect of this immobilization method in multilayer films for reactions in aseptic conditions during 48 hours. A possible reason for the reduction in activity can be the change in the reaction conditions, which affects the access of the substrate to the enzyme, contained in the inner layers of the multilayer films. Another factor that needs to be taken into account is the partial dissolving of the upper layers of the films, placed in a buffer solution with pH 5.0 for an extended amount of time.

CONCLUSION

For the first time effective immobilization of the enzyme β-galactosidase in an eight-layer film, based on the interaction between the two electrostatically charged polysaccharides chitosan and xanthan, was demonstrated. In conclusion, the results of the experiments show on one hand the prospect of the developed method of immobilization of β-galactosidase in multilayer films and on the other the need for optimization of the immobilization conditions depending on the specific application.

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