Effect of β-galactosidase immobilization on properties of chitosan/xanthan and xanthan/chitosan multilayers

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Abstract. Polyelectrolyte multilayers (PEMs) deposited on non lyophilized and lyophilized polylactic acid (PDLA) substrates were investigated. The substrates were charged in a corona discharge. The charging of the substrates was carried out by means of a conventional corona triode system consisting of a corona electrode (needle), a grounded plate electrode and a grid placed between them. Positive or negative 5 kV voltage was applied to the corona electrode and 1 kV voltage of the same polarity as that of the needle - to the grid. The dependences of the normalized surface potential on the storage time for positively and negatively charged non lyophilized and lyophilized PDLA substrates were studied. The results obtained show that the values of the normalized surface potential of non lyophilized substrates decay faster than those of the lyophilized ones. For multilayer build-up layer-by-layer (LbL) deposition technique was used. The first built-up layer always possesses an electric charge opposite to that of the substrate. PEMs with different number of layers (4 or 8 layers) were obtained. In the polyelectrolyte multilayers obtained the enzyme β-galactosidase were immobilized. The enzyme activity of each multilayer configuration was measured. The experimental results show that, in case of enzyme immobilization in multilayer films with 8 layers the efficiency of immobilization is considerably better compared to ones with 4 polyelectrolyte layers.

1. Introduction

The continuous search for cheap and reliable methods for controlled delivery of drugs is one of the driving forces in modern medical research. One such method is the creation of polyelectrolyte multilayers by using a layer-by-layer (LbL) deposition technique. This technique is widely used in many fields of research, such as drug delivery [1], biomedicine [2], food science [3] and the development of biosensors [4, 5]. It offers both flexibility in terms of assembly components and geometry of covered surfaces, as well as precise control of the properties of the resulting multilayers. Additionally since the LbL technique is based on the strong attraction between differently charged polyelectrolytes. It provides an easy and reliable method for assembling thin films for immobilization of different biological components. 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 [6]. One of the suitable and mostly used enzymes is β-galactosidase known also as lactase. It is enzyme belonging to glycoside hydrolase families 1, 2, 35, 42 and 59 (GH1, GH2, GH35, GH42

and GH59) [7]. These enzymes catalyze the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactoside substrates. Microbial sources of β-galactosidases are bacteria, yeasts, and fungal producers [8]. In terms of biodegradability polymer substrates offer a good basis for the creation of polyelectrolyte multilayers. One such polymer is poly-lactic acid. This material can be modified in different ways, such as corona treatment [9], chemical modification [10] and plasma treatment [11, 12], in order to improve its properties. Plasma treatment is often used for the modification of both the surface and bulk of PLA films [11]. Such modification is used to improve biocompatibility [12], as well as increase the polarity and surface roughness [11]. Corona discharge is both inexpensive and easy to control, which makes it a widely popular method for the creation of charged polymer films.
The purpose of the present paper is to investigate the immobilization of the β-galactosidase enzyme in xanthan/chitosan multilayers deposited on corona charged non lyophilized and lyophilized polylactic acid substrates.

2. Experimental details

2.1. *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 two film types (non lyophilized and lyophilized) were prepared in the following way: 1) non lyophilized films of PDLA – by dissolving 2 grams of PDLA in 100 ml chloroform. The solution was then poured in petri dishes and dried until the evaporation of the solvent; 2) lyophilized films of PDLA – by dissolving 2 grams of PDLA in 100 ml dioxan. The resulting solution was placed in a lyophilizer and dried until the evaporation of the solvent. Both substrates were dried at 35 °C for 48 h and were kept in an exicator at room temperature and 54% relative humidity (RH).The created films were cut into circular samples with a diameter of 30 mm.

2.2. Corona charging of the substrates and measurement of their surface potential

The PDLA substrates were charged in a corona discharge, in order to create a positive or negative electric charge on their surface. The charging in a corona discharge was carried out by a conventional corona triode system, consisting of a corona electrode (needle), a grounded plate electrode and a grid placed between them. The substrates were placed on the grounded plate electrode and were charged for 1 min at room conditions. Positive or negative 5 kV voltage was applied to the corona electrode. 1kV voltage 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. The electrets surface potential was measured by the vibrating electrode method with compensation [13], with anestimated error of less than 5%.

2.3. Layer-by-layer deposition of multilayers

Chitosan and xanthan gum were purchased from Sigma-Aldrich,and were used without further purification or characterization. Layer-by-layer deposition technique was used for multilayer build-up. For the LbL assembly of the multilayered films 0.1% w/v chitosan and 0.05 % w/v xanthan solutions in acetate buffer (pH 5 and ionic strength 0.1 M) were used. 1 g/L of β-galactosidase was dissolved in the xanthan solution just before the deposition process. The deposition was done by dip-coating ,with the first built-up layer always possessing an opposite electric charge to that of the substrate. For the dip-coating assembly of the multilayers a programmable slide stainer (Poly Stainer IUL, Spain) was used with the following programme: 15 min dipping step – adsorption of the first polyelectrolyte solution, 5 min washing step in acetate buffer, 15 min dipping step - adsorption of the second polyelectrolyte solution; 5 min washing in acetate buffer. The procedure was repeated until the desired even number of layers (4 or 8) was reached. The resulting multilayer structures were stored in an exicator at RH 54%.

2.4. Enzyme activity

The enzyme, chosen for this experiment was a commercial fungal β-galactosidase (from Aspergillus niger). The influence of the substrate concentration on the initial velocity of the enzyme reaction was studied at a range 0.01 M – 1.30 M lactose. The β-galactosidase activity in the presence of lactose – 1%, 5%, and 10% and mixtures of chitosan (0,1%) and lactose (1%, 5%, and 10%) was investigated. The concentrations of the released glucose were determined by glucose oxidase method [14]. In order to determine the amount of immobilized enzyme an enzymatic assay of β – galactosidase with ONPG method was conducted. For this purpose, 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 test was repeated several times at 24 h increments to measure the remaining activity of the samples after repeated use.The activity of the enzyme was determined by measuring the absorbtion of the released o-nitrophenol (oNP) at 405 nm. One unit of enzyme activity is defined as the amount of biocatalyst required to release 1 µmol of oNP per min under the above assay conditions.

3. Results and discussion

3.1. Time Storage Influence on the Electrets Surface Potential Decay

The dependences of the normalized surface potential on time storage for positively and negatively charged PDLA non lyophilized and PDLA lyophilized electrets have been studied for 360 minutes. The surface potential was measured once of 10 minutes for the first 60 minutes,due to the rapid decay of the charge at this period. 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 non-lyophilized and lyophilized films are shown in figure 1.

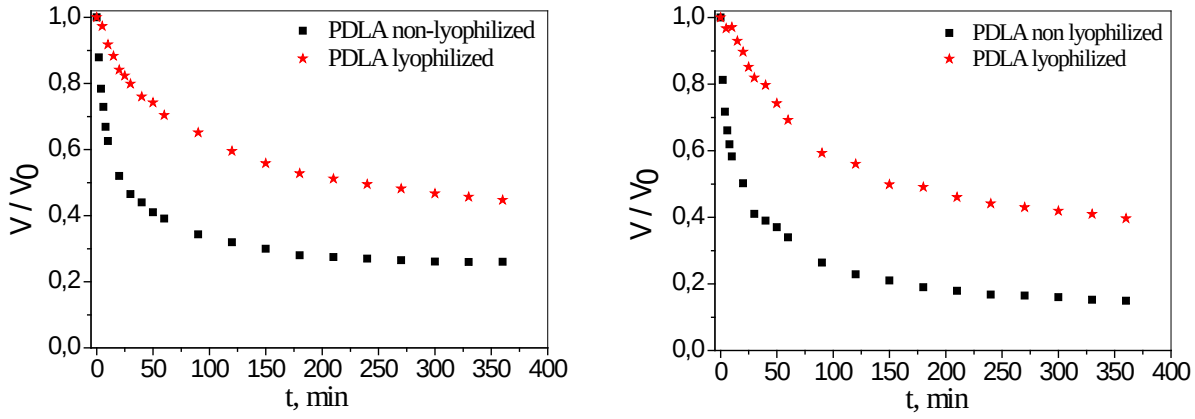

Figure 1. Normalized surface potential time dependences for PDLA non lyophilized and lyophilized electrets films at both types corona charging: a) positive and b) negative.

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

The steady state values of the normalized surface potential at a time of 360 minutes for PDLA non-lyophilized and lyophilized electret films are shown in figure 2.

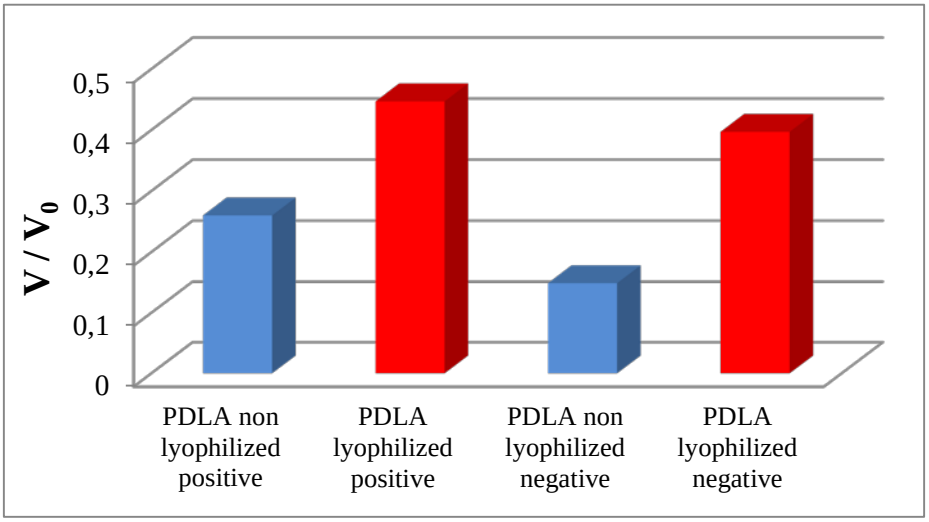

Figure 2. The steady state values of the normalized surface potential at 360 minutes for non-lyophilized and lyophilized PDLA electret films.

The experimental results presented in figure 1 and figure 2 display the following features:

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

This is probably due to the fact that in the case of a positive corona, the dominant ions are $H^+(H_2O)n$, and in the case of a negative corona - CO^{3-}. Those ions are bound in traps of various depths and their release depends on the surrounding conditions.

- ✓ For all investigated samples the normalized surface potential values are decaying exponentially for the first 60 minutes, after which the speed of charge decay decreases and is practically stable within 360 minutes.

Electrets' surface potential depends on the amount of trapped charges in the different surface states of the samples. During the initial period of time the surface potential rapidly decreases due to a release of weakly captured charges from the shallow energy states. After this initial period the surface potential stabilizes to a steady state value, caused by tightly captured charges in the deep energy traps. Similar exponential decay of the electrets charge was observed in [15].

- ✓ The steady state values of the normalized surface potential are higher for lyophilized PDLA electrets independently of the corona polarity.

The lyophilizing leads to the formation of a porous structure, thus significantly improving both the film's ability to store a charge and the stability of such stored charges for extended periods of time. This was preliminary explained in [16].

3.2. Enzyme activity of multilayer films

The enzyme activity of non-lyophilized and lyophilized PDLA multilayer films was measured at the 30^{th} and 60^{th} minute after submersion in the reactive agent, for four consecutive days. After the 60^{th} 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.

Enzyme activity of non-lyophilized positively and negatively charged PDLA multilayer films with 4 or 8 layers, measured on the 30^{th} and 60^{th} minute, are presented in figure 3 and figure 4 respectively. Figure 5 and figure 6 represent the enzyme activity of lyophilized positively and negatively charged PDLA multilayer films with 4 or 8 layers, measured on the 30^{th} and 60^{th} minute.

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

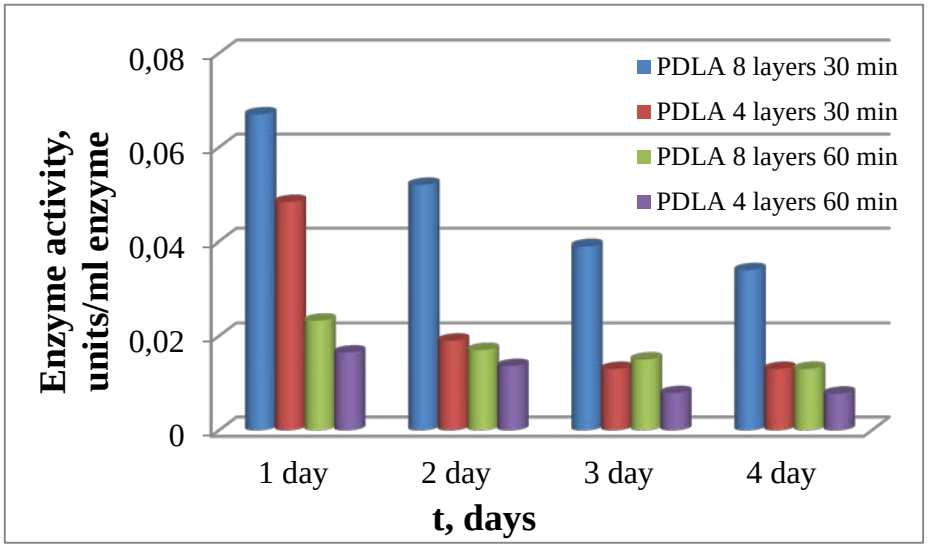

Figure 3. Enzyme activity of non lyophilized positively charged PDLA films with 4 or 8 layers measured on the 30th and 60th minute.

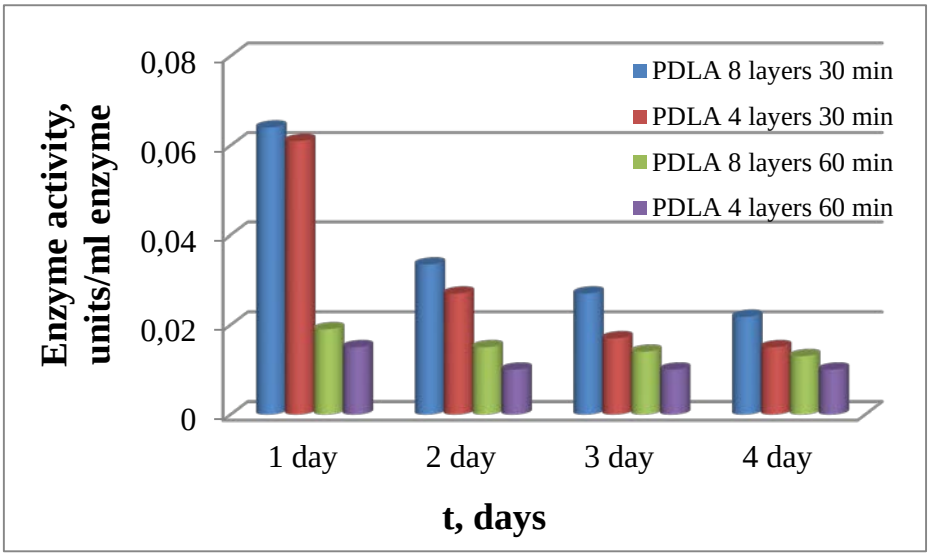

Figure 4. Enzyme activity of non lyophilized negatively charged PDLA films with 4 or 8 layers measured on the 30th and 60th minute.

The experimental results presented in Figures 3 - 6 show that:

- Enzyme immobilization in positively charged PDLA films show higher activity compared to the negatively charged ones, independently of number of polyelectrolyte layers. This can be explained by the larger amount of charge, captured in PDLA substrate, during the positive corona charging(see figure 2).
- In the case of PDLA films with 8 polyelectrolyte layers the rate of immobilization is considerably better to that of PDLA films with 4 polyelectrolyte layers, independently of corona polarity and films type. Enzyme activity of lyophilized PDLA films show lower activity compared to that of non lyophilized PDLA ones, regardless of corona polarity and number of polyelectrolyte layers.
- Lyophilized substrates are more porous, which likely results in the creation of of thinner layers of chitosan and xanthan. This, in turn, creates less suitable conditions for the capture of β-galactosidase.

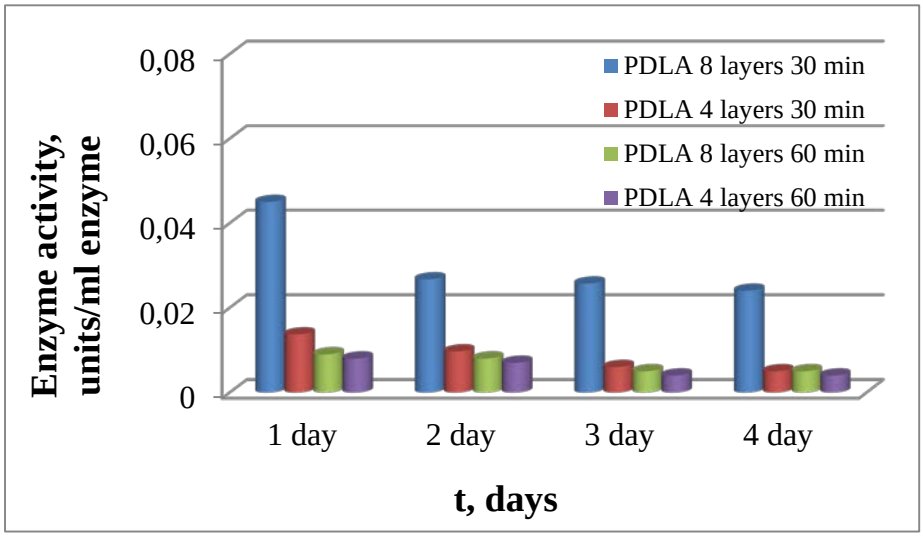

Figure 5. Enzyme activity of lyophilized positively charged PDLA films with 4 or 8 layers measured on the 30th and 60th minute.

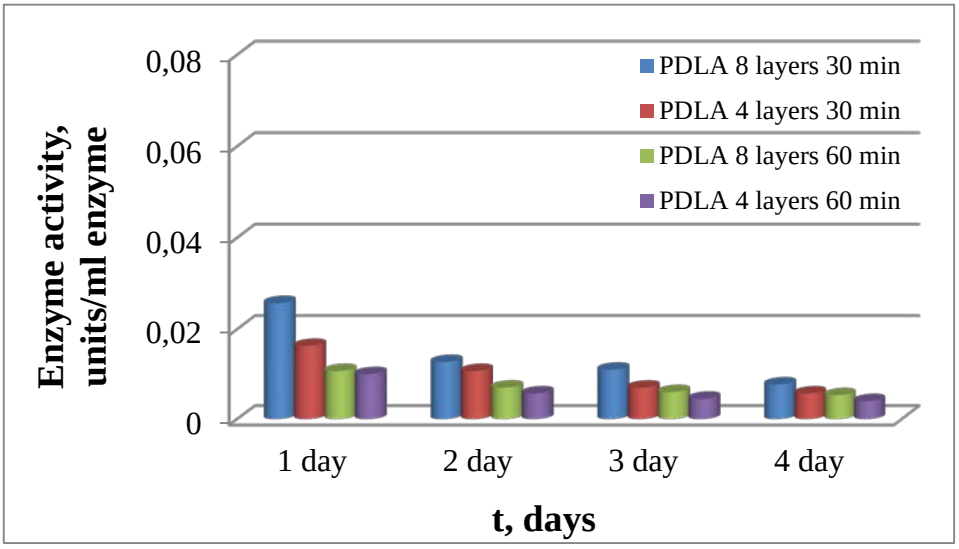

Figure 6. Enzyme activity of lyophilized negatively charged PDLA films with 4 or 8 layers measured on the 30th and 60th minute.

Therefore, non-lyophilized positively charged PDLA substrates are more suitable for building multilayer films with successfully included β-galactosidase. This results in an increase of enzyme activity.

4. Conclusion

.The creation of multilayer structures of the natural polymers xanthan and chitosan, with the use of the layer-by-layer deposition method, on preliminary charged lyophilized and non-lyophilized PDLA substrates was successfully carried out. Immobilization of β-galactosidase in the created polyelectrolyte multilayers was also achieved. The experimental results show that, in case of enzyme immobilization in multilayer films with 8 layers, the efficiency of immobilization is considerably better, when compared

to ones with 4 polyelectrolyte layers, independent of corona polarity. Overall enzyme activity is highest in PEMs on non-lyophilized positively charged PDLA substrates. Because the immobilization technique, described in this paper, is still in the initial stages of development, its full potential cannot be determined with certainty. Some possible applications can be the creation of improved packaging materials, affordable biodegradable sensors and a number of medical applications – such as new types of bandages.

Acknowledgments

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