# Immobilization of β-galactosidase in Xanthan/Chitosan Polyelectrolyte Multilayers Deposited on Corona Charged Polylactic Acid Substrates

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**Abstract.** Polyelectrolyte multilayers (PEMs) deposited on polylactic acid (PDLA) substrates were studied. The substrates were charged in a corona discharge. Positive 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. In the present paper time dependences of the normalized surface potential of PDLA substrates were investigated. 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. In the polyelectrolyte multilayers obtained the enzyme β-galactosidase were immobilized. The enzyme activity of each multilayer configuration was measured.

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

## 1. INTRODUCTION

In the last decades, polyelectrolyte multilayer films prepared by means of layer-by-layer self-assembling have been widely used because of their fabrication easiness and great potential for application in drug delivery [1], biomedicine [2], membranes [3], food science [4] etc.

The LbL technique is based on the consecutive deposition onto solid substrate via electrostatic interaction of polyions. It is characterized by precisely defined physicochemical properties – thickness, charge, hydrophilic–hydrophobic balance [5], flexible choice of assembled components, and the ability to cover surfaces of any size and geometry.

There are several techniques for charging polymer substrates: corona discharge [6, 7], chemical modification [8, 9], plasma treatment [10] etc. The corona discharge is one of the most commonly used methods of materials treatment. 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].

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) [12]. 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 [13].

In [14] the immobilization of β-galactosidase enzyme in chitosan/xanthan and xanthan/chitosan multilayers deposited on corona charged polylactic acid substrates was investigated. It was established that in case of enzyme immobilization in a multilayer film with 4 polyelectrolyte layers the negatively charged PLA films show 30 % higher activity compared to the ones with positive charge. In case of 8 layers the efficiency of immobilization is considerably better without significant difference in activity at different corona polarity.

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

## 2. EXPERIMENT

### 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 substrates were prepared by dissolving of 2 grams PDLA in 100 ml chloroform. The solution was poured in petri dishes and dried at 35°C for 48 hours until the evaporation

of the solvent. Then the substrates were kept for 24 hours in exicator, at room temperature at relative humidity.

### 2. 2. Corona charging and surface potential measurement of the samples

The PDLA substrates were charged in a corona discharge system (Fig. 1), consisting of a corona electrode (needle), a grounded plate electrode and a grid placed between them. The charging of the samples in a corona discharge was used to achieve positive electric charge on their surface.

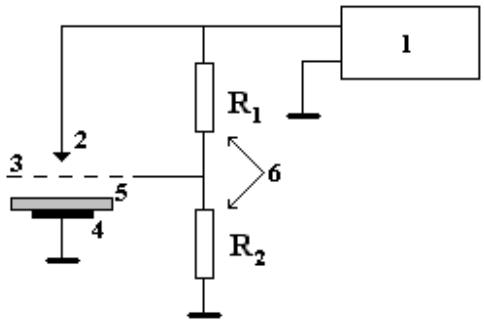


**Fig. 1** Scheme for obtaining electrets: 1. high voltage source; 2. corona electrode; 3. grid; 4. plate grounded electrode; 5. sample on a metal pad; 6. voltage divider.

Charging of the electrets was performed under relative humidity of (45% - 50%), room temperature and atmospheric pressure for 1 minute. Positive 5 kV voltage was applied to the corona electrode and 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 [15] and the estimated error was better than 5%.

### 2. 3. Polyelectrolyte multilayers deposition

Layer-by-layer deposition technique was used for multilayer build-up. Chitosan (low molecular weight) and xanthan gum were purchased from Sigma-Aldrich. They were used without further purification or characterization. For the LbL assembly 0.1% w/v chitosan and 0.05% w/v xanthan solutions in acetate buffer (pH 5 and ionic strength 0.1 M) as solvent were used. 1 g/L β-galactosidase was dissolved in the xanthan solution just before the deposition process.

The deposition was done by the dip-coating process. For dip-coating assembly a 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 solution; 5 min washing in the same acetate buffer. The procedure was repeated until reaching the desired even numbers of layers (4 or 8 xanthan/chitosan). The film was dried with hot air after the last layer deposition.

### 2. 4. Enzyme activity

β-galactosidase enzyme (from Aspergillus niger) was used in the kinetic studies. 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. 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 enzymaticaly [16]. Protein concentration was analyzed by the Bradford method [17]. 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 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 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.

## 3. RESULTS AND DISCUSSION

### 3. 1. Influence of Time Storage 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 charged PDLA films were investigated for 360 minutes. The surface potential was measured once of 10 minutes except for the first mi-

nutes when it was measured more often 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 charged PDLA films are presented in Fig. 2.

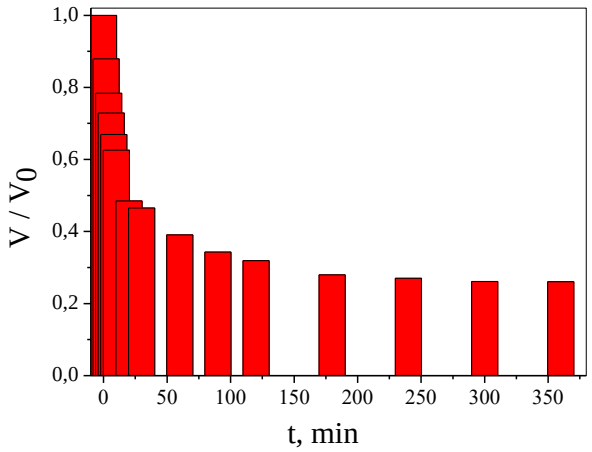


**Fig. 2** Time dependences of the normalized surface potential for positively charged PDLA electret films.

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 that the values of the normalized surface potential are initially decaying exponentially for the first 60 minutes and then are slowly decreasing and are practically stabilized to the 360 minute.

It was established that the electret surface potential depends on the amount of trapped charges in different surface states of the samples. In the initial period of time after corona charging, the surface potential rapidly decreases. Probably 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 of the electrets charge was observed in [18].

### 3. 2. Effect of type of PEMs on immobilization and enzyme activity

The enzyme activity of multilayer films obtained was measured on 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 positively charged PDLA multilayer films with 4 or 8 layers measured on the 30$^{th}$ and 60$^{th}$ minute are presented in Fig. 3 and Fig. 4 respectively.

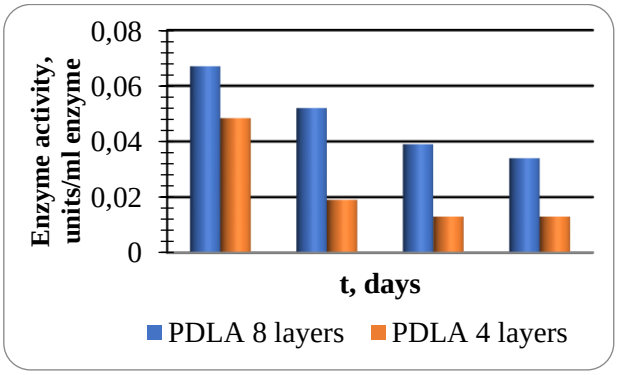


**Fig.3.** Enzyme activity (in units/ml enzyme) of positively charged PDLA films with 4 or 8 layers measured on the 30$^{th}$ minute.

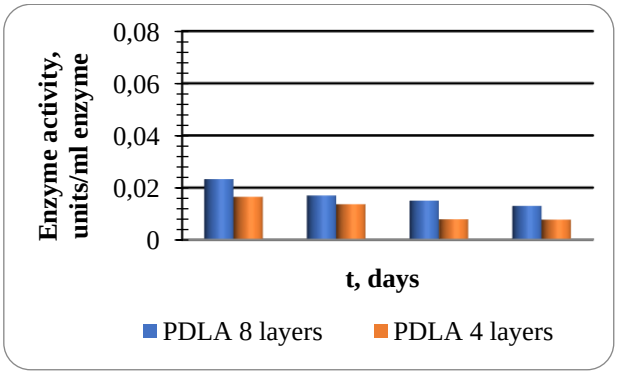


**Fig.4.** Enzyme activity (in units/ml enzyme) of positively charged PDLA films with 4 or 8 layers measured on the 60$^{th}$ minute.

Each point in the figure is a mean value from 8 samples. The calculated standard deviation was better than 5 % from the mean value with confidence level 95 %.

The results obtained 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 independently of minute of measured (30$^{th}$ or 60$^{th}$ minute).

The initial enzyme activity of the 8 layer PDLA films is around 25% higher than that of the 4 layer films. The degree of storage of the activity to 48 hour is also higher of 8 layer PDLA films.

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 enzyme activity investigation up to 48 hours was established that after the first reaction around 20 % of the initial activity of the enzyme is retained in both types of multilayer films. The results obtained demonstrate the prospect of this immobilization method in multilayer films for reactions in aseptic conditions during of 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 reason can be 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.

## 4. CONCLUSION

Xanthan/chitosan polyelectrolyte multilayer films deposited on PDLA substrates, previously charged in a corona discharge, were investigated. In multilayer films obtained the enzyme β-galactosidase were immobilized. The enzyme activity of multilayer films obtained was measured on the $30^{th}$ and $60^{th}$ minute after submersion in the reactive agent for four consecutive days. 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. The initial enzyme activity of the 8 layer PDLA films is around 25% higher than that of the 4 layer films.

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## REFERENCES

1. V. Gunjkar, S. Patwekar, and S. Dhage, *WJPPS*, **4(6)**, 216 (2015).
2. R. Costa, and J. Mano, *Chem Soc Rev*., **43**, 3453-3479 (2014).
3. E. Pasco, H. Shi, I. Xagoraraki, S. Hashsham, K. Parent, M. Bruening, and V. Tarabara, *J. Membrane Sci.*, **469**, 140-150 (2014).
4. E. Poverenov, S. Danino, B. Horev, R. Granit, Y. Vinokur, and V. Rodov, *Food and Bioprocess Tech.*, **7(5)**, 1424-1432 (2014).
5. Eds. G. Decher, and J. B. Schlenoff, *Multilayer Thin Films*, Wiley VCH, Weinheim (2002).
6. T. Yovcheva, M. Marudova, A. Viraneva, S. Sotirov, S. Rusev, I. Bodurov, B. Pilicheva, Y. Uzunova, G. Exner, Ts. Grancharova, and I. Vlaeva, *AIP Conf. Proc.*, **1722**, Art. No. 220026, 1-4 (2016).
7. M. Marudova, I. Bodurov, S. Sotirov, Y. Uzunova, B. Pilicheva, I. Avramova, A. Viraneva, I. Vlaeva, G. Exner, and T. Yovcheva, *Bulgarian Chem. Comm.*, **48(C)**, 468-474 (2016).
8. A. Delcorte, P. Bertrand, E. Wischerhoff, and A. Laschewsky, *Adsorption of polyelectrolyte multilayers on polymer surfaces*, Langmuir, **13**, 5125-5136 (1997).
9. Z. Wang, P. Hauser, J. Laine, and O. Rojas, *J. Adhesion Sci. Technol.*, **25**, 643-660 (2011).
10. T. Tamai, M. Watanabe, and K. Mitamura, *Colloid Polym. Sci.*, **293**, 1349-1356 (2015).
11. S. Datta, L. Rene Christena, and Y. Rani Sriramulu Rajaram, *Biotech.*, **3**, 1-9 (2013).
12. B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat, *Nucleic Acid Research.*, **37**, 233–238 (2008).
13. B. Rodriguez-Colinas, L. Fernandez-Arrojo, M. De Abreu, P. Urrutia, M. Fernandez-Lobato, A. O. Ballesteros, and F. J. Plou, *Advances in Enzyme Biotech.* (ed. P. Shukla, B. I. Pletschke), New Delhi, Springer, 23–39 (2013).
14. A. P. Viraneva, I. P. Bodurov, A. V. Grigorov, T. A. Yovcheva, T. A. Vasileva, V. P. Bivolarski, I. N. Iliev, *Bulgarian Chemical Communications*, **52(A)**, 165-170, (2020).

15. M. Knoll, F. Ollendorff, and R. Rompe, *Gasentladungstabelen*, Springer-Verlag, Berlin, 84 (1935).
16. V. Bivolarski, T. Vasileva, B. Dzhambazov, A. Momchilova, J.-M. Chobert, I. Ivanova, and I. Iliev, *Biotechnol. Biotec. Eq.*, **27**, No 3, 3811–3820, (2013).
17. M. M. Bradford, *Anal. Biochem.*, **72**, No 1–2, 248–254, (1976).
18. G. M. Sessler, *Electrets: Recent developments* Journal of Electrostatics, **51-52**, 137-145 (2001)