UNIVERSITY OF PLOVDIV

„PAISII HILENDARSKI“

FACULTY OF PHYSICS AND TECHNOLOGY

DEPARTMENT OF PHYSICS

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Modified multilayer structures for immobilization of bioactive molecules

Abstract

of dissertation

for the acquisition of the educational and scientific degree of “Doctor”

in the field of higher education: 4. Natural Sciences, Mathematics and Informatics,

professional field 4.1. Physical sciences

Doctoral program “Electrical, magnetic and optical properties of condensed matter”

Scientific supervisor: Assoc. Prof. Asya Viraneva PhD.

Plovdiv 2025

The dissertation contains 165 pages, in which 65 figures and 12 tables are presented. 220 literary sources were used. The PhD student works as an assistant at the Department of Physics at the Faculty of Physics and Technology of University of Plovdiv "Paisii Hilendarski".

The experimental research related to the dissertation was carried out at the Department of Physics at the Faculty of Physics and Technology of University of Plovdiv "Paisii Hilendarski" and in the laboratories of the Center for Technology at University of Plovdiv "Paisii Hilendarski".

In connection with this dissertation, 4 scientific articles have been published in indexed publications. The results have been presented at 4 international scientific forums.

The dissertation work was discussed and proposed for defense by the Department Council of the Department of Physics at the Faculty of Physics and Technology of University of Plovdiv "Paisii Hilendarski", held on 04.12.2025. The open final meeting of the scientific jury will take place on 06.03.2026 at 11 a.m. in the Conference Hall of the University of Plovdiv “Paisii Hilendarski” before a jury composed of:

Assoc. Prof. Anelia Dakova – Mollova PhD.

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Assoc. Prof. Ivan Bodurov PhD.

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The materials for the defense are provided for free access to those interested in the library of the University of Plovdiv “Paisii Hilendarski”.

Contents

Abbreviations and designations used:

PLA – poly-lactic acid
PEC – poly epsilon caprolactone
PEG – polyethylene glycol
SEM – scanning electron microscopy
DSC – differential scanning calorimetry
EDAC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
BAS – biologically active substances

I. Introduction

Modern systems for controlled release of various types of biologically active substances allow for targeted delivery of the biologically active substance to the treated areas, thus reducing not only the total amount of the substance required, but also allow the overcoming of various factors that reduce the effectiveness of the delivered substance. The first generation of technologies for controlled release were developed in the period between 1950 and 1980, and were mainly used to obtain various types of pills and patches. The second-generation methods, developed before 2010, face more and more problems caused by various physicochemical and biological factors that affect the effectiveness of the delivered biologically active substances. Some factors, such as poor water solubility, high molecular weight and difficulties in finer control of the delivered quantities, limit the development of new clinical products. Another factor influencing the development of new methods for controlled delivery is the reaction of the various biological systems that control the transfer of biologically active substances in the body. All these reasons hinder the development of modern controlled delivery systems (Y. Yun, et al. 2015).

One way to overcome the factors limiting the development of these systems is to create various multilayer structures that can be used for the controlled delivery of biologically active substances directly to the desired areas (S. Park, et al. 2018). Such multilayer systems can take the form of various micro- and nanoparticles, as well as different planar structures and fibers. This new type of materials allows for the overcoming of the shortcomings limiting the delivery of biologically active substances by incorporating the substance within the structure of the layers. In this way, the biologically active substance is isolated from external factors that can impair its effectiveness or prevent its absorption. Another advantage is the fact that the action of the substances can be delayed until it reaches the desired release zone, thus reducing side effects. By controlling the structure and number of layers, as well as the type of materials used, a sustained controlled release of certain amounts of biologically active substance can be achieved (A. Schneider, et al. 2007).

Modern controlled release systems use a wide variety of different substances, such as polymers, polyelectrolytes, and any others. Of great interest are the various biodegradable polymers (G. Kwon & D. Furgeson, 2007). Their potential ca be attributed to the fact that they do not release toxic end products during their degradation, which may allow their use for the creation of fully biodegradable alternatives to some of the more widely used medical products. Such biopolymers have already found applications as medical implants and in tissue engineering (T. Biswal, 2021), where their good biocompatible properties allow their use inside the body without unwanted side effects. These biomedical products are further improved by applying various biocompatible layers that possess antibacterial properties (B. Wang, et al. 2013; R. Pawar, et al. 2023). In recent years, more and more research has been conducted on the potential of controlled delivery systems using such biopolymers.

However, the mechanisms for controlling delivery are not yet fully understood, and the materials developed so far do not yet possess the necessary properties for the creation of commercial products. Further research is required for the development of better fully biodegradable systems for the controlled delivery of biologically active substances.

Among the biopolymers that demonstrate great potential for the development of such systems are poly-lactic acid and polycaprolactone (S Liu, et al. 2020). These two biopolymers are characterized by good biocompatibility, biodegradability and lack of toxicity, which makes them ideal candidates for the creation of various biomedical materials. Another important characteristic of biopolymers are their surface properties (C. Ha & J. Gardella, 2005), which are of great importance for their application for the creation of systems for controlled delivery of biologically active substances. However, both polymers have some disadvantages that hinder their wider application. Despite its excellent biocompatibility, poly-lactic acid is characterized by biological inertness and poor mechanical properties, while the slow degradation rate of polycaprolactone makes it more suitable for longer-term applications, such as medical implants. The disadvantages of both biopolymers can be overcome by using various modifications to improve their properties (V. Lassalle & M. Ferreira, 2007). The properties can also be improved by combining different polymers into composite materials, in which case the different components can compensate for the poor properties of each of the individual materials (V Karagkiozaki, et al. 2012). The surface properties of the biopolymers can be improved not only through various modifications (S. Nemani, et al. 2018), but also by applying a variety of biodegradable layers to their surface (Y. Yu, et al. 2020). This would allow the creation of new controlled-release systems incorporating these two materials, which would be an alternative to the non-biodegradable systems used so far.

II. GOALS AND OBJECTIVES

Based on the literature review and the systematization of scientific publications, examining multilayer structures created on modified biodegradable and biocompatible substrates based on poly-lactic acid and poly epsilon caprolactone, no sufficiently detailed studies were found regarding their application as a delivery system for various biologically active substances with controlled release.

This also justified setting the main goal of the dissertation work as a study of the possibilities for immobilization of various biologically active substances in multilayer films deposited on modified substrates of poly-lactic acid and poly epsilon caprolactone and their use as drug delivery systems.

To achieve the set goal, the following tasks were formulated and implemented:

1. Preparation of different types of polymer films to be used as substrates for the growth of polyelectrolyte multilayer structures.

1.1. Preparation of polymer films based on poly-lactic acid

1.2. Preparation of polymer films based on poly epsilon caprolactone.

1.3. Preparation of composite films based on poly-lactic acid and poly epsilon caprolactone.

2. Modification of the surface of the obtained polymer films used as substrates for the production of multilayer structures.
2.1. Surface modification by charging in a corona discharge in positive and negative corona to an initial surface potential of ± 1 kV at room conditions.
2.2. Surface modification by chemical modification of the surface using EDAC.
2.3. Surface modification by lyophilization.
2.4. Surface modification by adding polyethylene glycol.
3. Investigation of the different types of substrates used for the creation of multilayer structures.
3.1. Investigation of the time dependences of the surface potential of the obtained polymer films charged in a corona discharge.
3.2. Investigation of the morphology with scanning electron microscopy (SEM).
3.3. Investigation of the degree of crystallinity with differential scanning calorimetry (DSC).
3.4. Investigation of the contact angle and surface energy.
4. Obtaining multilayer structures by the method of deposition of polyelectrolyte multilayer films of chitosan and xanthan (casein) on all types of films used as substrates.
5. Investigation of the possibilities for immobilization of various biologically active substances (beta-galactosidase, benzydamine hydrochloride and tolfenamic acid) in the obtained polyelectrolyte multilayer films.
6. Study of the biological activity and release kinetics of the used biologically active substances from multilayer films obtained on modified substrates of polylactic acid and poly epsilon caprolactone.

III. MATERIALS AND METHODS

3.1. Materials

The following substances were used in the presented dissertation: Poly-lactic acid, poly epsilon caprolactone, chitosan, xanthan, casein, as well as the biologically active substances: beta-galactosidase, benzydamine hydrochloride, tolfenamic acid and curcumin.

3.2. Methods for the creation, modification and characterization of polyelectrolyte multilayer films

3.2.1. Methods for the creation and modification of the films

3.2.1.1. Creation of nonporous polymer films

For the creation of non-porous polymer films 1 g of the chosen polymer (PLA or PEC) was dissolved in 50 ml of chloroform. The obtained solution was poured into a metal petri dishes with a diameter of 18 cm and left to dry on a level surface at room temperature and atmospheric pressure until the complete evaporation of the solvent. The resulting transparent thin film was placed in a humid desiccator until the film completely peeled off the bottom of the petri dish, after which it was stored in a dry

desiccator until its subsequent use. To obtain a non-porous film from a mixture of the two polymers, the same technology was used, with the total mass of the two polymers being 1 g for all combinations.

3.2.1.2. Creation of porous polymer films through lyophilization

To obtain porous films by lyophilization, the selected polymer or combination of polymers were dissolved in 100 ml of 1,4-dioxane and left to freeze on a level surface at a temperature of -4° C and atmospheric pressure. The concentration of the solution was determined depending on the minimum amounts of polymer from which a lyophilized film could be created. For PLA, this concentration was the same as for a non-porous film (2 g per 100 ml), while for PEC and films containing PEC the concentration was five times higher (10 g per 100 ml). After films were completely frozen, they were placed in a freeze dryer for 72 hours until the solvent was completely evaporated. The resulting films were stored in a dry desiccator until further use.

3.2.1.3. Creation of porous polymer films with added polyethylene glycol

For the creation of porous films with added polyethylene glycol to the polymer mixtures of PLA and PEC, 150% w/v polyethylene glycol was added to the polymer solutions. After complete homogenization, the solutions were poured into glass petri dishes and placed at room temperature until the solvent was completely evaporated. The created films were stored in a dry desiccator.

3.2.1.4. Modification of the surface of the created films with the corona discharge method

The samples were charged under corona discharge at room conditions using a three-electrode system consisting of a corona electrode (needle), a flat ground electrode and a grid (control electrode), placed between them.

A voltage of ±5 kV was applied to the corona electrode, and a voltage of ±1 kV with the same polarity as the corona electrode was applied to the grid. The charging time in the corona discharge was 1 minute.

3.2.1.5. Chemical modification

A chemical modification method was used in addition to corona charging for the modification of the polymer films. The modification consists in treating the PLA films with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). The chemical modification was used in parallel and in combination with corona charging, and the influence of the modification was tested both on an uncharged film and before and after corona charging.

The chemical modification of the PLA films is performed with the aim of modifying the surface to improve hydrophobicity and induce free side groups. The method used for the modification is based on hydrolysis of the polymer film surface. The chemical modification process by hydrolysis creates free –COOH groups on the surface of the polylactic acid films, with sodium hydroxide (NaOH) being used for the initial modification, followed by immersion in EDAC dissolved in methanol for 4 hours at room conditions. After modification, the films were extensively washed with methanol and allowed to dry before being used to create multilayer films.

3.2.1.6. Creation of polyelectrolyte multilayer films with the layer-by-layer deposition method

The creation of the multilayer polyelectrolyte structures, presented in this dissertation work, was carried out using an automated system for obtaining layers type Polystainer from the company IUL,S.A. with an immersion mode of 15 minutes and rinsing for 5 minutes.

The automated system operates in the immersion mode at a uniform speed and can hold the substrate motionless in the solution for a certain set time so that the coating material can attach to it. If the substrate is pre-charged and the solutions are polyelectrolytes, during the retention the surface of the sample changes its charge, because its initial charge is compensated by that of the solution and it becomes dominant. Before immersion in the second oppositely charged electrolyte solution, the sample is rinsed, during which the weakly bound molecules are removed. The sequence of polyelectrolytes is determined by the type of charge of the film, as deposition always begins with the polyelectrolyte having a charge opposite to that of the film. The described procedure creates two polyelectrolyte layers on the surface of the polymer film and is repeated until the desired number of layers is reached (4 or 8 layers).

A solution of chitosan and a solution of xanthan (or casein), dissolved in a buffer solution, were used as polyelectrolytes. Various biologically active substances were immobilized in the obtained multilayer films.

3.2.2. Methods for characterization of the created films

3.2.2.1. Vibrating electrode with compensation method

The surface potential of the electrets was measured immediately after their creation. The measurement of the surface potential was carried out by the vibrating electrode method with compensation with an error not exceeding 5%. The equipment with which the surface potential was determined included: a measuring cell, a source of constant compensating voltage, a sound frequency generator, a zero-signal indicator and a voltmeter for measuring the compensating voltage. The magnitude and sign of the compensating voltage uniquely determine the magnitude and sign of the surface potential of the electret.

As a result of the vibrations of the electrode in the electric field of the electret, the charge induced on the electrode periodically changes and an alternating current is induced in the external circuit. When an appropriate additional external voltage is applied to the vibrating electrode, its potential of the vibrating electrode becomes equal to the surface potential of the electret and the electric field in the gap becomes equal to zero, i.e. the electrode vibrates in a zero electric field and no electric current is induced in the external circuit. This voltage is called compensating, and the method is called the compensation method. The supply of compensating voltage is carried out by a constant voltage source, equipped with a regulator, by means of which the appropriate value of the compensating voltage is selected. The magnitude and polarity of the compensating voltage determine the magnitude and sign of the effective surface charge density, using the formula:

$$\sigma = \frac{\varepsilon_0 \varepsilon}{L} U_k, \qquad (1)$$

where U_k – is the compensating voltage, ε is the relative dielectric permeability of the sample, ε_0=8,85.10^{-12} F/m is the dielectric permeability of vacuum, L – is the thickness of the sample.

3.2.2.2. Differential scanning calorimetry (DSC)

Differential scanning calorimetry belongs to the group of physical and physico-chemical methods of thermal analysis, with the help of which the energy changes in the studied substance are determined. It is based on heating (or cooling) at a set constant rate of a sample of the studied substance and a thermally inert substance used as a standard, and measuring the compensating heat flow, which maintains the temperatures of the sample and the standard equal. DSC studies were conducted to determine the degree of crystallinity and compatibility of the polymers used in the construction of the composite substrates. For this purpose, the DSC 204 F1 Phoenix® device from Netzsch, Germany, was used. Its operating temperature regime is -180 ÷ 700°C, allows for a linear cooling and heating rate in the interval of 0.001 ÷ 200 °C/min and a maximum measurement range of ± 750 mW (τ sensor). The accuracy of enthalpy measurement is < 1%. The temperature and enthalpy scales are calibrated with 5 substances for the planned cooling and heating rates.

3.2.2.3. Scanning electron microscopy (SEM)

The morphology of the created polymer films was determined using a scanning electron microscope (Prisma E SEM, Thermo Scientific, Waltham, MA, USA). For this purpose, two milligrams of the investigated samples were mounted on aluminum holders and coated with carbon and gold in a Quorum Q150T Plus vacuum evaporator (Quorum Technologies, West Sussex, UK). Images were obtained using an electron acceleration detector at an accelerating voltage of 15 kV at different magnifications.

3.2.2.4. Sessile drop method

The sessile drop method was used to for the determination of the static and dynamic contact angles. Contact angle measurement is a qualitative way to assess whether a surface has hydrophobic or hydrophilic properties. It is based on the observation of intermolecular interactions between a surface and a small drop of water when the drop encounters the surface. The contact angle method is a non-invasive and rapid method for determining the wettability of various materials, including biomaterials. The method is an analytical approach widely used to determine the surface free energy of various materials.

The contact angle measurements of the studied samples were performed under standard conditions (at room temperature and normal atmospheric pressure) with two types of liquids (water and methylene iodide CH_2I_2). Small droplets of 2 µl were carefully placed on the surface of the films using a precision 10 µl micro syringe (Innovative Labor System GmbH, Germany). Six drops were placed on each sample, the contact angle was measured, the results were averaged and used to determine the hydrophobicity of each sample. Contact angles were determined by measuring the tangent of the incidence profile from images captured with a high-resolution camera. Image processing was performed using ImageJ software.

3.3. Methods for determination of activity and release of immobilized biologically active substances

3.3.1. Enzymological method for determination of activity of the enzyme immobilized within the multilayer films

The enzymological method is used to determine the activity of enzymes, immobilized in the multilayer films, and is specific for each type of enzyme. For the enzyme β-galactosidase used in our studies, the ONPG method is used. It is based on the use of ortho-nitrophenyl-β-galactoside, which upon reaction with the enzyme is degraded to ortho-nitrophenol and galactose. The process occurs for a multilayer sample with immobilized enzyme, immersed in a certain amount of ONPG (0.9 ml of solution added to 1.5 ml of deionized water) for 60 minutes in a water bath at a temperature of 37 °C. At 30 and 60 minutes of the reaction, 0.8 ml of the solution is taken and the reaction is stopped with 4 ml of monomolar Na_2CO_3 solution. The reaction solution is examined on a spectrophotometer at a wavelength of 405 nm to determine the amount of ortho-nitrophenol synthesized after the reaction. The test was repeated several times every 24 hours to measure the residual activity of the samples upon reuse.

3.3.2. Release of bioactive substances from the created multilayer films

To determine the release profile of the immobilized bioactive substance, incorporated into the multilayers, the studied films were immersed in a phosphate buffer, imitating artificial saliva (pH 6.8). The bioactive substance release experiment was carried out at a temperature close to that of the human body (37±1) °C and under continuous stirring with a magnetic stirrer at 50 rpm. At certain time intervals, 3 ml samples were taken from the buffer and after removing each sample, the same amount of pure buffer was added back to the medium. Then, the resulting solutions were sonicated for 5 minutes and filtered through a ChromafilVR filter (0.45 μm). Three samples of each type were examined during the experiment. The amount of drug substance in the solutions was determined using a Metertech SP8001 spectrophotometer (Metertech Inc., Nangang, Taipei, Taiwan) at a wavelength of 306 nm according to a previously prepared calibration curve.

3.3.3. Mathematical models, used in the description of the release process

To describe the process of drug release from various modified polymer matrices, different models can be used, taking into account the solubility of the drug, the geometry of the carrier matrix and the release mechanism (N Abbasnezhad, et al. 2021; C. Corsaro, et al 2021). Several basic models are presented in this dissertation. The model for which the coefficient of determination R^2 is the highest (close to 1) is considered the most reliable.

- **First order model** – this model can be used for describing the dissolution of the drug in pharmaceutical forms, containing water soluble drugs in porous matrixes:

$$\log C = \log C_0 - \frac{Kt}{2.303}, \quad (2)$$

where C_0 is initial drug concentration, K is the release constant, expressed in в s^{-1}, and t is the release time.

- **Korsmeyer-Peppas Model**

$$\frac{M_t}{M_\infty} = Kt^n , \quad (3)$$

where M_t/M_∞ is the quantity of drug, released at the moment of time t, K is the Korsmeyer-Peppas constant and the power exponent *n* determines the diffusion mechanism.

- **Weibull model** – this model can be used for the investigation of the release from drug delivery systems of a matrix type:

$$\frac{M_t}{M_\infty} = 1 - \exp\left[-\frac{(t-T)^b}{a}\right], \quad (4)$$

where M_t/M_∞ is the amount of drug, released at the moment of time t, and T represents the delay time, measured as a result of the dissolution process. The parameter ***a*** describes the time dependency, while ***b*** describes the shape of the dissolution curve. When b = 1 the curve shape matches exactly to the shape of the exponential profile; if b has a value, larger than 1, the curve shape is sigmoidal, while at b<1 the rate of increase is faster.

IV. RESULTS AND DISCUSSION

1.1. Study of the electret properties of the created composite films

The time dependences of the normalized surface potential for all types of investigated films are presented on Figures 1-7.

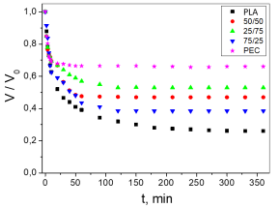


Figure 1. Time dependences of the normalized surface potential for nonporous composite films of PLA and PEC charged in a positive corona.

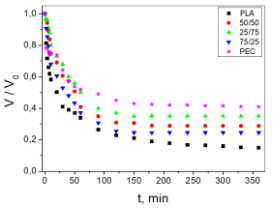


Figure 2. Time dependences of the normalized surface potential for nonporous composite films of PLA and PEC charged in a negative corona.

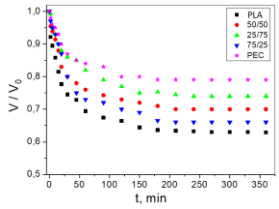


Figure 3. Time dependences of the normalized surface potential for porous composite films of PLA and PEC, prepared by lyophilization, charged in a positive corona.

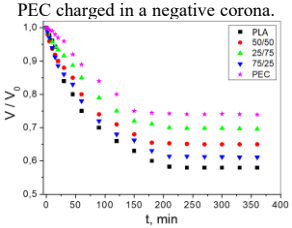


Figure 4. Time dependences of the normalized surface potential for porous composite films of PLA and PEC, prepared by lyophilization, charged in a negative corona.

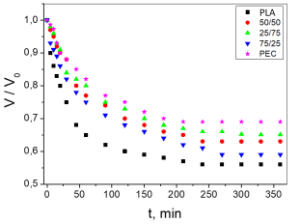


Figure 5. Time dependences of the normalized surface potential for porous composite films of PLA and PEC with incorporated polyethylene glycol, charged in a positive corona.

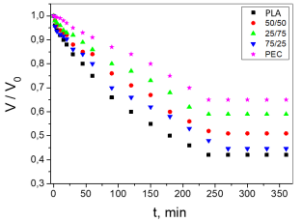


Figure 6. Time dependences of the normalized surface potential for porous composite films of PLA and PEC with incorporated polyethylene glycol, charged in a negative corona.

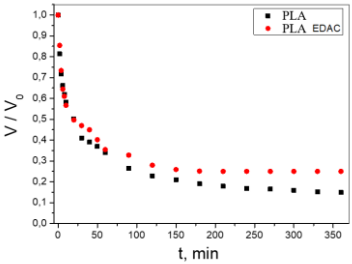


Figure 7. Time dependences of the normalized surface potential for nonporous films of PLA and PLA modified with EDAC, charged in a negative corona.

The results for the steady state values of the normalized surface potential at the 360th minutes for all samples are presented on Figures 8-10.

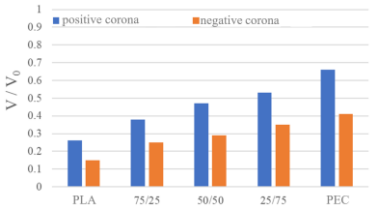


Figure 8. Steady state values of the normalized surface potential at 360 minutes for positively and negatively charged nonporous composite films.

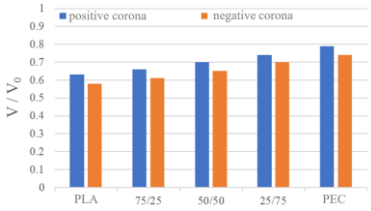


Figure 9. Steady state values of the normalized surface potential at 360 minutes for positively and negatively charged porous composite films obtained by lyophilization.

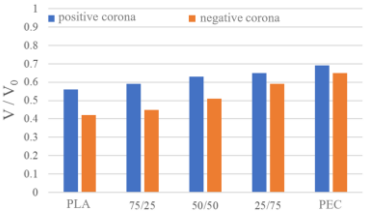


Figure 10. Steady state values of the normalized surface potential at 360 minutes for positively and negatively charged porous composite films with polyethylene glycol incorporated.

From the obtained experimental results, presented in Figures 1-10, the following conclusions can be drawn:

- For all investigated samples, the values of the normalized surface potential initially decrease exponentially, with different decay times observed for different types of films. It was found that the exponential decay occurs during the first 60 minutes for positively and negatively charged non-porous samples; during the first 120 minutes for positively and negatively charged porous lyophilized samples; and during the first 150 minutes for positively and negatively charged porous samples with PEG included. After that, the values of the surface potential decrease slightly and practically stabilize by the 360th minute.

It was found that the value of the surface potential of electrets depends on the number of trapped charges in different energy traps on the sample surface. In the initial period of time after corona charging, the surface potential decreases rapidly. This is due to the release of weakly trapped charges from lower energy states (shallow traps). The surface potential then stabilizes to a certain set value as a result of the more tightly trapped charges from higher energy states (deep traps).

- The values of the normalized surface potential for samples charged in a positive corona are higher than those for samples charged in a negative corona, regardless of the type of material and the method of modification.

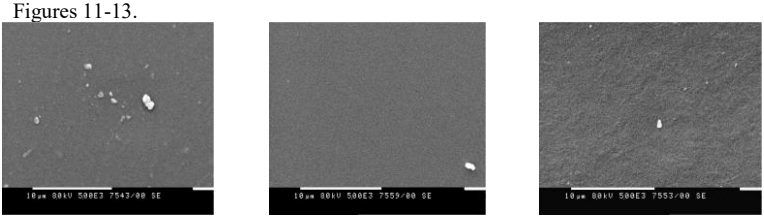
When charging in a corona discharge in air at atmospheric pressure, different types of ions are deposited on the surface of the sample. Therefore, the charging in a corona discharge depends on the polarity of the corona (J. Giacometti, et al. 1999). In the case of a positive corona, the ions that are deposited on the surface of the sample are $H_+(H_2O)_n$, and those for a negative corona - CO_3-. These ions are trapped in traps of different depths and are released from them depending on the conditions. As a result, positively and negatively charged samples differ in their properties and stability.

- The highest values of the normalized surface potential and the best electret properties are observed for PEC samples, regardless of the polarity of the corona.
- A decrease in the values of the normalized surface potential is observed with increasing PMC content in the studied samples.

The values of the normalized surface potential are highest for freeze-dried films, regardless of the type of material and the polarity of the corona. This can be explained by the morphology of the studied samples, since the largest pores were observed in freeze-dried films (T. Yovcheva et al. 2024).

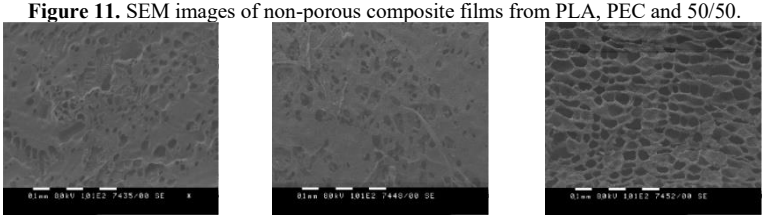
1.2. Study of the morphology of the created composite films using scanning electron microscopy (SEM)

The morphology of the resulting composite films was examined by scanning electron microscopy (SEM). The obtained images for non-porous films, porous films obtained by lyophilization and porous films with incorporated PEG are presented in Figures 11-13.



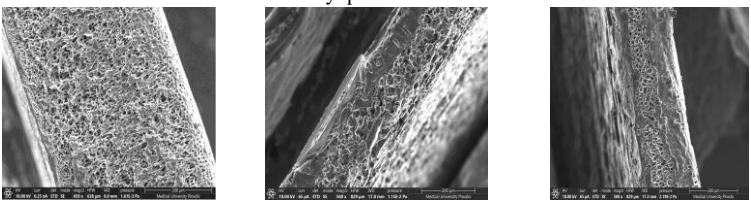
PLA 50/50 PEC

Figure 11. SEM images of non-porous composite films from PLA, PEC and 50/50.



PLA 50/50 PEC

Figure 12. SEM images of porous composite films from PLA, PEC and 50/50, created by lyophilization.



PLA 50/50 PEC

Figure 13. SEM images of nonporous composite films from PLA, PEC and 50/50 with added PEG.

The results presented in Figures 11-13 show that the non-porous composite films are characterized by a smooth homogeneous structure. It is also evident from the presented images that for both types of investigated porous films, created by lyophilization and by incorporation of PEG, a porous structure is observed, which is most likely due to the specific method used during their creation.

Due to its water-soluble nature, low molecular weight and availability in liquid form, it can be said that PEG is an excellent choice for creating microporous structures from water-insoluble polymers such as PLA and PEC and their composite blends.

With their largest pore size, lyophilized PEC films allow for greater charge capture on their surface and help retain them for extended periods of time.

1.3. Study of the degree of crystallinity with differential scanning calorimetry (DSC)

Figures 14-16 present the thermograms of the various non-porous and porous investigated films.

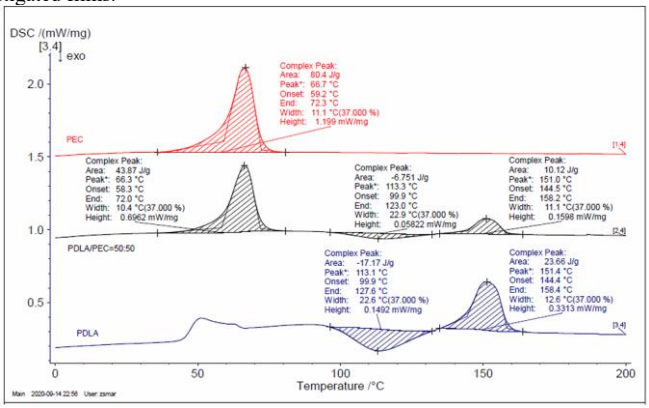


Figure 14. Thermograms for non-porous composite films from PLA, PEC and 50/50.

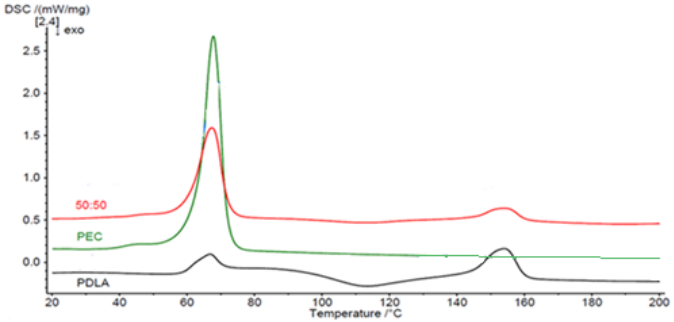


Figure 15. Thermograms for porous composite films from PLA, PEC and 50/50, created by lyophilization.

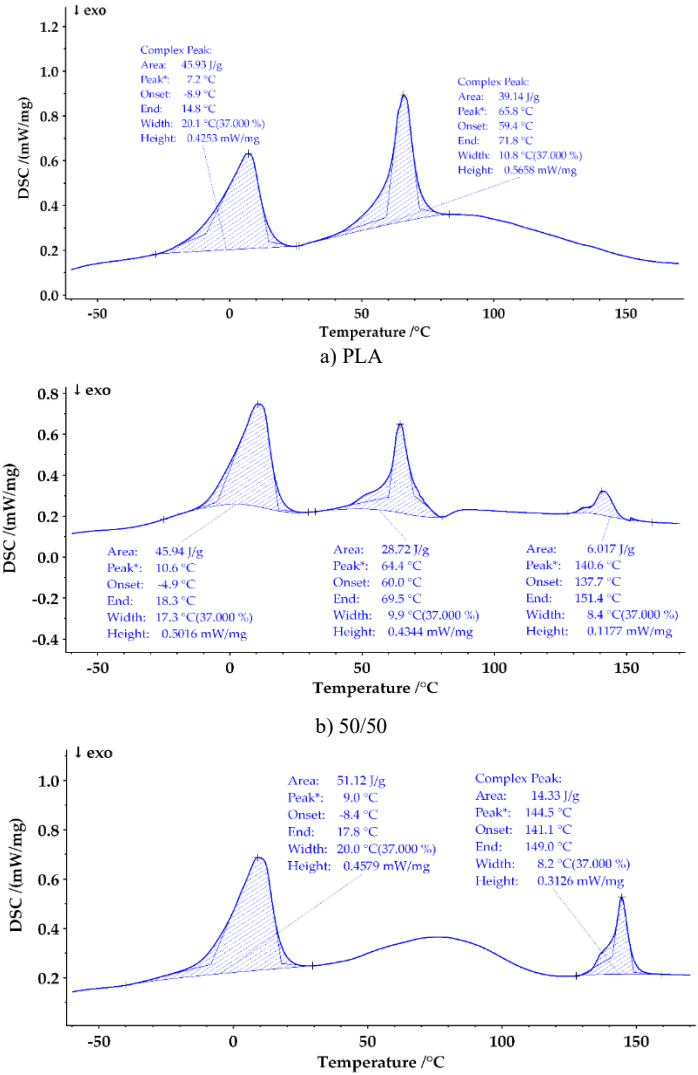


Figure 16. Thermograms for nonporous composite films from a) PLA, b) 50/50 and c) PEC with added PEG.

Table 1 presents the calculated values for the degree of crystallinity for all investigated samples.

Table 1. Degree of crystallinity for all investigated samples.

Sample	Total degree of crystallinity, %		
	Non-porous	Porous lyophilized	Porous with PEG
PLA	8	6	40
50/50	50	34	56
PEC	80	59	74

The results presented in Figures 14-16 and Table 1 can be analyzed and summarized as follows:

- The melting of the composite films, regardless of the preparation method, occurs at two temperatures, which are the characteristic melting temperatures of PEC and PMK. Based on the results, it can be assumed that PLA and PEC are immiscible at the molecular level and form heterogeneous zones.
- The glass transition temperature of PEC ($T_g \approx -63$ °C) was not measured during any of the experimental processes, which may be due to the high degree of crystallinity.
- The glass transition temperature of PMK, as well as the melting temperature of both PLA and PEC, for porous lyophilized samples are close to those described by other authors.
- The glass transition temperature (T_g) of PLA for porous samples with added PEG was determined to be 50 °C, which is lower than the values reported in the literature (I. Baena, et al. 2016). This is probably due to the plasticizing effect of polyethylene glycol.

1.4. Study of the contact angle and free surface energy of the created films

Figures 17 and 18 display the values of the contact angle and the free surface energy for all types of investigated samples.

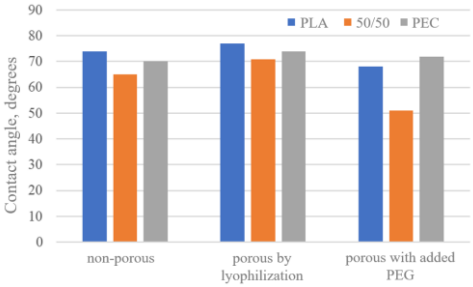


Figure 17. Values of the contact angle for all investigated composite films.

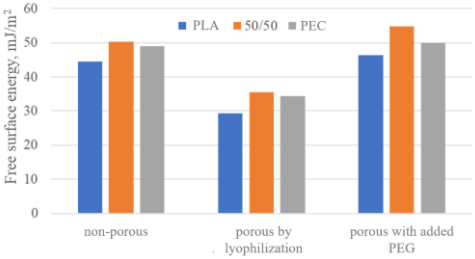


Figure 18. Values of the free surface energy for all investigated composite films.

The results presented in Figures 17 and 18 show that the free surface energy of PLA films is lower than that of PEC, regardless of the method, used during their creation. Similar results were obtained in the dissertation work of P. Wongwiwattana (2020).

It was also found that the free surface energy of PLA and PEC composites with a ratio of 50/50 increases when compared to pure PLA and PEC films.

When PEC is added to PLA in a ratio of 50/50, the free surface energy changes and reaches values of 50.30 mJ/m^2 for non-porous films, 35.45 mJ/m^2 for porous films obtained by lyophilization and 54.79 mJ/m^2 for porous films with incorporated PEG. Similar behavior in the values of free surface energy and contact angle for composite blends of PLA and PEC was observed by Alam et al. (2021).

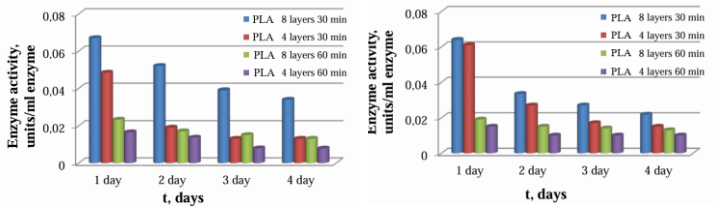
This increase in free surface energy with increasing PEC content is likely due to the increase in pore size, as shown by surface morphology measurements (see SEM images).

The free surface energy values increase significantly for porous films with PEG incorporation compared to non-porous ones, as well as for composite blends (50/50) compared to pure films of PLA and PEC. Therefore, the resulting composite blend exhibits better hydrophilic properties than the pure films (A Khandwekar, et al. 2011).

1.5. Study of the enzyme activity and release kinetics of the immobilized biologically active substances

1.5.1. Beta-galactosidase

The enzyme activity for positively and negatively charged nonporous PMC films with 4 and 8 polyelectrolyte layers is presented in Figure 19.

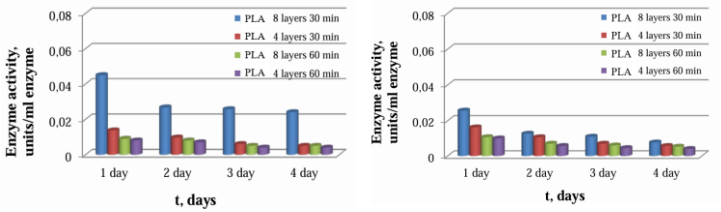


a) positively charged b) negatively charged

Figure 19. Enzyme activity of charged nonporous PLA films with 4 or 8 layers, measured at 30 and 60 minutes.

From the results, presented in Figure 19, can be seen that in the cases of immobilization of an enzyme in multilayer films with 4 polyelectrolyte layers, the negatively charged films show 30% higher activity of the enzyme beta-galactosidase compared to the positively charged ones. In the case of 8 layers, the immobilization efficiency is relatively better regardless of the type of charging. The initial activity of the films with 8 layers is about 25% higher than that of films with 4 layers. The degree of activity retention up to 48 hours is also higher and is about 40% to 50% of the initial activity. This can be explained by the type of crosslinking that occurs during the specific treatment of the gel created from two polysaccharides with different electrical charges. Additionally, it is possible that the two polysaccharides interact with each other as a result of the interaction of electrical charges, creating so-called "pockets" in which the enzyme molecules are physically positioned. This type of physical immobilization gives the enzyme spatial freedom and access of the molecules of the studied film to its active center.

Figure 20 presents the values of the enzyme activity for positively and negatively charged porous films of PMC obtained by lyophilization.



a) positively charged b) negatively charged

Figure 20. Enzyme activity of charged lyophilized PMC films with 4 or 8 layers, measured at 30 and 60 minutes.

From the results presented in Figures 19 and 20 for the enzyme activity of the multilayer structures on non-porous and porous lyophilized pure PLA films, the following conclusions can be drawn:

- In the case of immobilization of the enzyme beta-galactosidase in the multilayer structures on positively charged PLA films, higher enzyme activity is observed compared to the negatively charged ones, regardless of the number of polyelectrolyte layers (4 or 8).

This can be explained by the capture of a larger number of charges during charging in a positive corona and the obtaining of higher values of the normalized surface potential with time (Figures 1 and 2).

- When the enzyme is immobilized in multilayer films with 8 polyelectrolyte layers, the immobilization efficiency is relatively better than that for films with 4 polyelectrolyte layers, regardless of the type of film (non-porous or porous, obtained by lyophilization) and the polarity of the corona.
- The initial activity of the 8-layer films is about 25% higher than that of the 4-layer films. The degree of activity retention up to 48 hours is also higher and is about 40% to 50% of the initial activity.

During the study of the enzyme activity up to 48 hours after the initial reaction, it was found that about 30% of the initial enzyme activity was retained in both types of multilayer films. The obtained results demonstrate the prospects of this method of immobilization in multilayer films for reactions under aseptic conditions for a period of 48 hours. One possible reason for the decrease in activity may be the change in reaction conditions, which affect the access of the studied film to the enzyme contained in the inner layers of the multilayer films. Another factor that should be taken into account is the partial dissolution of the upper layers of the films placed in a buffer solution with pH 5 for a prolonged period of time.

- The enzyme activity of the multilayer structures on porous PLA films obtained by lyophilization is lower compared to non-porous ones, regardless of the polarity of the corona and the number of polyelectrolyte layers.

This is due to the fact that the porous films obtained by lyophilization have a more porous structure, which leads to the attachment of thinner layers of chitosan and xanthan. This creates conditions for weaker immobilization of the beta-galactosidase enzyme in the obtained multilayer structures and, consequently, to lower enzyme activity.

Therefore, non-porous positively charged films of PLA are most suitable for obtaining multilayer structures of chitosan and xanthan, in which the beta-galactosidase enzyme is successfully immobilized. This type of films has the best enzyme activity compared to the other considered pure films of PLA.

Polyelectrolyte layers with immobilized enzyme were also created on pure PLA substrates chemically modified with EDAC. The chemical modification was performed in order to improve the surface properties of PLA and better attachment of the polyelectrolyte layers. The results of the study of the enzymatic activity of multilayer structures on non-porous films of PLA and PMC with EDAC are presented in Figures 21 and 22.

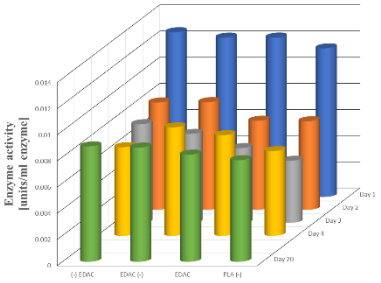


Figure 21. Enzyme activity at 30 minutes for multilayer structures obtained on all types of modified PLA substrates.

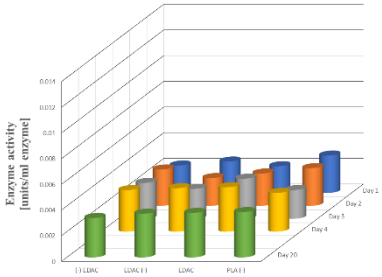


Figure 22. Enzyme activity at 60 minutes for multilayer structures obtained on all types of modified PLA substrates.

From the results presented in Figures 21 and 22 it can be concluded that:

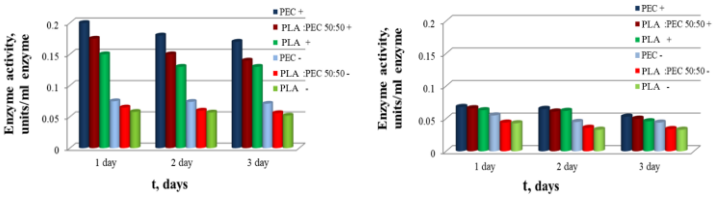
- chemical modification with EDAC of the substrate improves the degree of preservation of the enzyme activity in all considered cases, with the combination of physical and chemical modification demonstrating higher values of the enzyme activity regardless of the type of modification;
- the obtained experimental results also show that the level of activity decreases by about 40% after the first day and remains relatively stable for a long period of time (up to 20 days).

The observed initial decrease may be due to weakly captured enzyme molecules that are released from the polyelectrolyte layers after the first use of the multilayer film. The activity after the second day is due to the stably immobilized enzyme molecules captured in the structure of the layers.

The increase in enzymatic activity for the chemically modified samples can be explained by the presence of a larger number of side groups on the surface of the films, created during the hydrolysis process with sodium chloride, compared to those created during surface degradation during corona discharge treatment. These side carboxyl groups are further activated by the EDAC treatment, which changes the type of crosslinking between the polymer surface and the polysaccharides used to build the layers. Due to the fact that both chemical and physical modification increase the number of side groups on the polymer surface, their combination leads to expected results such as better attachment of the polyelectrolyte layers and a higher level of enzymatic activity.

- It was found that the combination of chemical and physical modification is suitable for improving the surface properties of pure non-porous PLA films, as well as for creating more stable multilayer films for immobilization of biologically active molecules.

To determine the influence of the addition of PEC in the structure of PLA films, three types of composite films were created with different mass ratios of the two biopolymers (PLA and PEC in the ratio 75/25, 50/50 and 25/75), on which eight of the polyelectrolyte layers with immobilized beta-galactosidase enzyme, described at the beginning of the section, were deposited. Multilayer films of pure PLA and PEC were also prepared. The results of the enzymatic activity for positively and negatively charged composite films are presented in Figure 23.



a) 30th minute b) 60th minute

Фигура 23. Enzyme activity of charged PLA and PEC composite films with 4 or 8 layers, measured at 30 and 60 minutes.

The results presented in Figure 23 show that:

- The enzyme immobilized in the multilayer structures on the positively charged films has a higher level of activity compared to the negatively charged ones, regardless of the type of film and the day of measurement.
- The enzyme activity is highest in the multilayer structures created on films of pure PEC and lowest in samples of pure PLA.
- The amount of enzyme activity is directly related to the percentage of PEC in the substrate, with films with a higher concentration of the polymer having higher levels of activity of the multilayer structures in all cases considered.
- The enzyme activity decreases on average by about 50% for all the samples studied and remains stable up to three days upon reuse, which coincides with the previously considered multilayer films on pure PLA.

The different degree of immobilization in the multilayer structures obtained on the positively and negatively charged films can be explained by the greater number of trapped charges during the positive corona charging (Figures 1 and 2). The higher surface potential, in turn, contributes to the better trapping of the polyelectrolyte layers and the greater amount of immobilized enzyme. The increase in enzyme activity with increasing PEC concentration in the composites can be explained by the higher degree of crystallinity determined by the DSC method (Table 1).

It was found that the increase in enzyme activity increases proportionally with the PEC concentration in the substrate, both in the positively and negatively charged samples.

The obtained experimental results show that the stabilization of enzyme activity confirms the retention of the enzyme in the polyelectrolyte layers, which allows the reuse of the created films. The films thus created can find potential applications in various fields requiring the repeated use of the same film for extended periods of time.

The results also show that the inclusion of another biopolymer in PLA films can also be used to increase the level of immobilization of biologically active substances in polyelectrolyte layers deposited on the surface of such biopolymer composites.

1.5.2. Curcumin

The amount of curcumin incorporated into the polyelectrolyte multilayer films at different pH values and ionic strength of the solutions, for both positively and negatively charged films, is presented in Table 2.

Table 2. Amount of curcumin incorporated into polyelectrolyte multilayer films

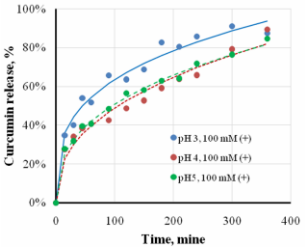
Type of sample	*Curcumin amount, μg*	
	positively charged sample	*negatively charged sample*
pH 3, 100 mM	0,391	0,862
pH 4, 100 mM	1,481	1,239
pH 5, 10 mM	2,234	1,868
pH 5, 100 mM	0,983	0,537
pH 5, 1000 mM	0,692	0,850

The results presented in Table 2 show that the highest amount of curcumin is incorporated into polyelectrolyte multilayer films formed at pH 5 and ionic strength 10 mM. The second highest amount is at pH 4 and ionic strength 100 mM. Decreasing the pH level to 3 and increasing the ionic strength of the solution above 100 mM at lower acidity levels leads to a lower amount of curcumin incorporated into the multilayer structures. These results are related to the different structure of the polyelectrolyte multilayer films constructed under the different conditions.

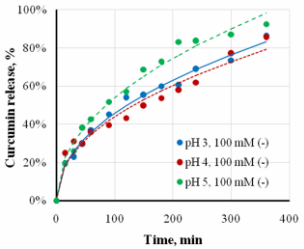
At pH 3, strong electrostatic interactions result in ordered dense polyelectrolyte layers with very few pores. Since curcumin is electroneutral, its incorporation occurs entirely by diffusion. Due to the lack of sufficient “empty” space in the layers, the amount of curcumin incorporated is significantly lower.

The increase in the porosity of the structures, which is realized at higher ionic strengths, is unfavorable for the incorporation of curcumin. Since the latter is a low-molecular compound with relatively small dimensions, it is likely that with larger pores in the layers the diffusion process of the biologically active substance from the polyelectrolyte multilayer film to the solution prevails. Therefore, it turns out that the most optimal conditions for the incorporation of curcumin into polyelectrolyte multilayer films are at low ionic strength and pH 4 and 5.

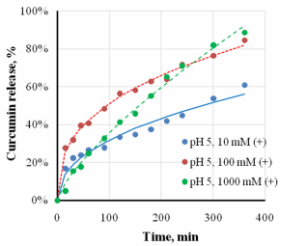
The dependences of the released amount of curcumin on the release time for multilayer films obtained at different pH and ionic strength values are presented in Figure 24.



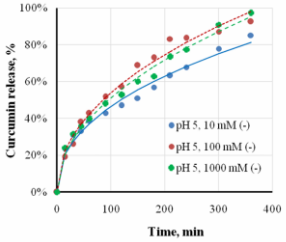
a) positively charged samples at different pH levels



b) negatively charged samples at different pH levels



c) positively charged samples at different ionic strengths



d) negatively charged samples at different ionic strengths

Figure 24. Curcumin release from multilayer films at different values of pH and ionic strength.

From the results presented in Figure 24, can be seen that the curcumin release rate is relatively stable under all investigated conditions, with up to 40% of the substance being released during the first hour, and by the sixth hour the release reaches 90%. The lowest release rate is observed at an ionic strength of 10 millimoles, which may be due to the increased density of the layer structure. Increasing the structure leads to an increase in the release rate, suggesting a looser structure. Changes in pH levels also affect the rate, with the slowest release observed at pH 4. For positively charged supports, a decrease in the release rate of the immobilized substance with an increase in pH is observed, while for negatively charged supports the opposite trend is observed. Overall, it can be concluded from the results that changing the pH and ionic strength of polyelectrolyte solutions can be used to control the release parameters of biologically active substances from multilayer polyelectrolyte films.

1.5.3. Benzydamine hydrochloride

The effect of the number of polyelectrolyte layers on the release rate and the amount of immobilized benzydamine hydrochloride was investigated. The results for the amount of immobilized substance are presented in Table 3.

Table 3. Amount of immobilized benzydamine hydrochloride in multilayer films obtained on non-porous composite positively and negatively charged substrates of PLA and PEC.

Amount of immobilized benzydamine hydrochloride, µg

Type of film	*Positive charge*		*Negative charge*	
	4 layers	**8 layers**	**4 layers**	**8 layers**
PLA	457.6572	593.6974	1004.33	1320.204
PLA/PEC 50/50	396.1382	483.9637	536.8884	625.7096
PEC	944.0957	1023.681	2004.398	2476.466

From the results presented in Table 3, the following conclusions can be drawn:

- The amount of immobilized benzydamine hydrochloride is higher in films with 8 polyelectrolyte layers.

- The layers built on negatively charged films immobilized a larger amount of biologically active substance. This may be due to the higher initial surface potential for the negatively charged samples.
- The highest amount of immobilized substance was measured for polyelectrolyte layers deposited on PEC films.

The graphs representing the release rate of benzydamine hydrochloride for 4 and 8 layers are shown in Figure 25.

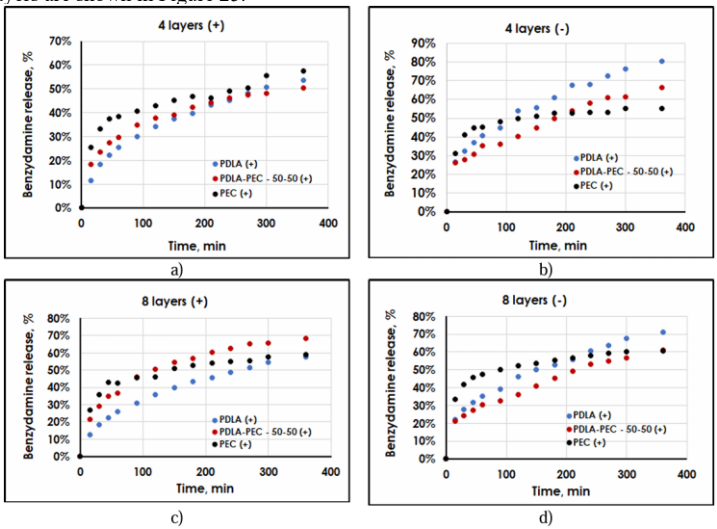


Figure 25. Release of benzydamine hydrochloride from 4 and 8 layered multilayer structures on positively and negatively charged PLA, PEC and 50/50 substrates.

From the graphs can be determined that the release of the biologically active substance during the first hour is about 30 to 50%, and by the sixth hour between 60 and 80% of the substance is released for all the samples studied. These results demonstrate the lack of a sudden release (burst effect) of the immobilized substance, which means that the benzydamine hydrochloride is firmly attached to the polyelectrolyte layers. The higher release rate in the negatively charged samples may be due to the fact that in them the last deposited polyelectrolyte layer is casein, which reduces the diffusion time of the layers and allows for faster release. The lowest release rate for the multilayer structures on positively charged films is observed in the pure PLA films, and for the negatively charged ones in the PLA and PEC composites (50/50).

The results obtained show that the release profile of benzydamine hydrochloride can be controlled by changing the composition and charge of the polymer film supports.

The influence of the porosity of pure and composite polymer films of PLA and PEC used as supports on the release rate and the amount of immobilized benzydamine hydrochloride was investigated. Porous composite films of PLA and PEC obtained by

lyophilization and porous films with the inclusion of PEG 400 were used. The release results are presented in Figures 26-28 and in Table 4.

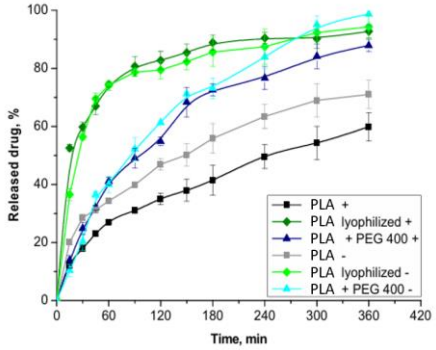


Figure 26. Release profile of benzydamine hydrochloride from multilayer films deposited on positively and negatively charged nonporous, lyophilized porous, and PEG-incorporated porous PLA substrates.

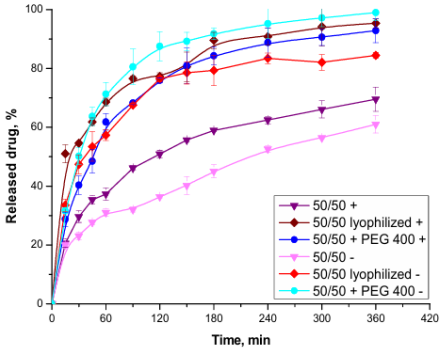


Figure 27. Release profile of benzydamine hydrochloride from multilayer films deposited on positively and negatively charged nonporous, lyophilized porous, and PEG-incorporated porous PLA and PEC composite (50/50) substrates.

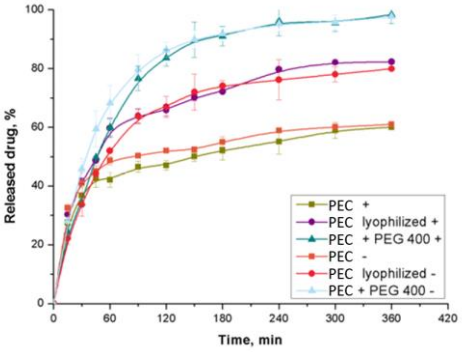


Figure 28. Release profile of benzydamine hydrochloride from multilayer films deposited on positively and negatively charged nonporous, lyophilized porous, and PEG-incorporated porous PEC substrates.

Table 4. Summary of results for the release of benzydamine hydrochloride from non-porous, porous, lyophilized, and porous PEG-incorporated composite films of PLA and PEC

Sample Composition	Corona Polarity	Modification	Encapsulated Benzydamine, μg/4 cm^2	Released Drug After 6 h, %	Major Transport Mechanism
PDLA	Positive	None	59.3 ± 1.6	59 ± 5	Fickian diffusion
PDLA	Positive	Lyophilized	588 ± 20	92 ± 3	Fickian diffusion
PDLA	Positive	PEG 400	98 ± 6	87 ± 2	Fick diffusion and swelling controlled transport
PDLA	Negative	None	132.0 ± 2.4	71 ± 5	Fickian diffusion
PDLA	Negative	Lyophilized	168 ± 13	94 ± 4	Fick diffusion and swelling controlled transport
PDLA	Negative	PEG 400	50 ± 5	98 ± 5	Complex release mechanism
50/50	Positive	None	48.3 ± 1.7	69 ± 4	Fickian diffusion
50/50	Positive	Lyophilized	499 ± 60	95 ± 2	Fickian diffusion
50/50	Positive	PEG 400	264 ± 18	92 ± 4	Fick diffusion and swelling controlled transport
50/50	Negative	None	62.5 ± 2.7	61 ± 3	Fickian diffusion
50/50	Negative	Lyophilized	313 ± 25	84 ± 1	Complex release mechanism
50/50	Negative	PEG 400	183 ± 15	98 ± 3	Complex release mechanism
PCL	Positive	None	102.3 ± 6.8	60 ± 1	Fickian diffusion
PCL	Positive	Lyophilized	2526 ± 50	82 ± 1	Fickian diffusion
PCL	Positive	PEG 400	361 ± 38	98 ± 3	Fick diffusion and swelling controlled transport
PCL	Negative	None	247.6 ± 6.6	60 ± 1	Fickian diffusion
PCL	Negative	Lyophilized	684 ± 34	79 ± 1	Complex release mechanism
PCL	Negative	PEG 400	350 ± 41	97 ± 2	Complex release mechanism

From the results presented in Figures 26-28 and Table 4, can be determined that the film structure plays a major role and can be used to control the immobilization and release of biologically active substances from polyelectrolyte multilayer structures deposited on the surface of various modified composite films.

1.5.4. Tolfenamic acid

The kinetics of tolfenamic acid release from polyelectrolyte multilayer films deposited on porous positively and negatively charged substrates of PLA and PEC obtained by lyophilization are presented in Figure 29, and those with incorporated PEG – in Figure 30.

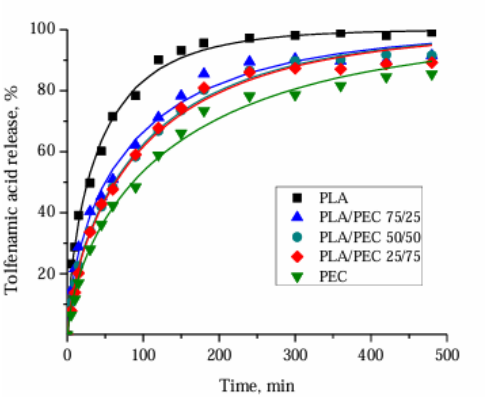


Figure 30. Release of tolfenamic acid from polyelectrolyte multilayer films deposited on positively charged porous composite substrates of PLA and PEC prepared by lyophilization.

The results obtained show that:

- The drug release process occurs in a single phase and no pronounced initial burst effect is observed. This type of process is characteristic of the release of non-water-soluble substances, such as tolfenalic acid (M. Józó, et al. 2021).
- The total amount of tolfenalic acid released is within 40 to 70% during the first hour and between 85 and 100% after the first 8 hours. Therefore, it can be concluded that complete release of the drug substance is achieved within 8 hours.

V. CONCLUSIONS

The following conclusions can be drawn from the presented experimental results:

1. The applied electric field, as a result of charging under corona discharge, contributes to the better attachment of the polyelectrolyte layers and immobilization of a larger amount of biologically active substance.
2. Surface modifications, such as corona discharge treatment and chemical modification of the polymer films, used as substrates on which the multilayer structures are built, improve their surface properties and help control the release process of the immobilized substances.
3. The creation of pores by lyophilization or by adding polyethylene glycol to the structure of the polymer films used as substrates for the multilayer structures increases the amount of immobilized substance and slows its release.
4. Combining the two studied polymers in composite films, which are used as supports for multilayer structures, leads to changes in the degree of immobilization and the release rate of biologically active substances, depending on the ratio of polymers in the composites.
5. Increasing the number of polyelectrolyte layers leads to an increase in the amount of immobilized substance.
6. Immobilization of the enzyme beta-galactosidase in polyelectrolyte multilayer films retains its activity for long periods of time when reused.
7. The release properties depend not only on the properties and structure of the substrate, but also on the type of immobilized substance.
8. Varying the properties of the polymer supports and polyelectrolyte layers allows controlling the release properties of the immobilized biologically active substances.

VI. CONTRIBUTIONS

- For the first time, the influence of the electric field in which the lyophilized and chemically modified composite substrates of poly-lactic acid and poly epsilon caprolactone are modified on the kinetics of release of various biologically active substances from the multilayer structures built on these substrates, was investigated and established.
- For the first time, the influence of various types of modification of polymer films on the immobilization of biologically active substances in polyelectrolyte multilayer structures built on their surface was studied.
- For the first time, multilayer structures were created on corona-charged porous composite substrates based on PLA and PEC and the influence of the resulting porous structure on the immobilization and controlled release of various biologically active substances was established.
- For the first time, multilayer polyelectrolyte layers of chitosan, xanthan or casein were created on the surface of modified polymer films of polylactic acid and polyepsilon caprolactone for immobilization of biologically active substances.
- For the first time, the enzymatic activity of beta-galactosidase and the release of benzydamine hydrochloride, tolfenamic acid and curcumin from fully biodegradable polyelectrolyte multilayer composite polymer structures were studied
- The potential of the studied polyelectrolyte multilayer structures, built on polylactic acid and polyepsilon caprolactone substrates, for the creation of systems for immobilization and controlled release of various biologically active substances was confirmed.

VII. BIBLIOGRAPHY

The complete literature review and the list of cited literature sources can be found in the dissertation.

VIII. APENDICES

List of scientific publications related to the dissertation:

1. Viraneva, A., Marudova, M., Milenkova, S., Grigorov, A., & Yovcheva, T. (2024). Investigation of Polyelectrolyte Multilayers Deposited on Biodegradable Corona-Charged Substrates Used as Drug Delivery Systems. *Coatings*, *14*(1), 85. Q1 – 25 т.
2. A Grigorov, A., Yovcheva, T., Iliev, I., Vlaeva, I., & Viraneva, A. (2022). Impact of physical and chemical modification on the immobilization of β-galactosidase in poly-lactic acid multilayer structures. *Bulg. Chem. Commun, 54*, 47-52. Q4 – 12 т.
3. Viraneva, A., Marudova, M., Grigorov, A., & Yovcheva, T. (2024). BENZYDAMINE HYDROCHLORIDE IMMOBILIZATION IN MULTILAYER STRUCTURES BASED ON LYOPHILIZED COMPOSITE POLYLACTIC ACID/POLY (E-CAPROLACTONE) SUBSTRATES. Journal of Chemical Technology and Metallurgy, 59(4), 823-830. Q3 – 15 т.
4. Viraneva, A., Marudova, M., Grigorov, A., Milenkova, S., & Yovcheva, T. (2025). Multilayered Polyelectrolyte Structures Deposited on Corona-Charged Substrate Blends as Potential Drug Delivery Systems. *Coatings*, *15*(2), 240. Q1 – 25т.

Participation in scientific conferences:

- 9th International conference of FMNS (FMNS-2021), June 2021, Blagoevgrad, Bulgaria.
- 10th Jubilee International Conference of FMNS (FMNS-2023), June 2023, Blagoevgrad, Bulgaria.
- 10th International School on Condensed Matter Physics (ISCMP), September 2022, Varna, Bulgaria .
- 23rd International School on Condensed Matter Physics (ISCMP), 25-30 August, 2024, Varna, Bulgaria.

Participation in scientific projects:

- BG05M20P001-1.002-0005, Personalized Innovative Medicine Competence Center (PERIMED), operational program "Science and education for smart growth" 2014-2020.
- D24-FTF-009 Digital Sustainable Ecosystems - Technological Solutions and Social Models for Ecosystem Sustainability (DUEkoS) BG-RRP-2.004-0001-C01, 2024-2026.
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- Project “Green Technologies GT-1/2020", Scientific Found of University of Plovdiv "Paisii Hilendarski", Bulgaria 2020-2022.
- MUPD23-FTF 014/25.04.2023, Department of Scientific Research at the University of Plovdiv "Paisii Hilendarski", 2023-2024.