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Budapest University of Technology and Economics  
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**Nanoformulating lipases for therapeutic use:**  
*A novel approach using electrospun nanofibers*

Thesis Book

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DEPARTMENT OF PHYSICAL CHEMISTRY AND MATERIALS SCIENCE

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# 1 Introduction

Enzymes are highly efficient biocatalysts that have the ability to catalyze complex chemical transformations with high efficiency and selectivity under mild, environmentally friendly conditions. They are of significant interest in the pharmaceutical industry, as beyond their utility in the synthesis of active pharmaceutical ingredients via biocatalytic processes, enzymes themselves can serve as therapeutic agents. They are employed in the treatment of various conditions, including metabolic disorders, pancreatic insufficiency, thrombosis, certain cancers, and skin diseases. Their biocompatibility and substrate specificity make them powerful tools both in drug manufacturing and in direct therapeutic interventions.

Despite their many advantages, the clinical application of enzymes is often hindered by several practical limitations. In their native form, enzymes are prone to denaturation, rapid degradation by proteases, loss of activity under physiological conditions, and poor pharmacokinetic profiles. These drawbacks limit their therapeutic effectiveness and necessitate high or repeated dosing, which may lead to increased treatment costs and reduced patient compliance. As such, there is a critical need for formulation strategies that can improve the stability, bioavailability, and controlled release of enzymes in therapeutic applications.

Nanotechnology offers a promising solution to these challenges. Through nanoformulation, enzymes can be introduced into protective environments that preserve their structure, enhance their resistance to degradation, and enable targeted or sustained delivery. Among various nanotechnological approaches, electrospinning has emerged as a particularly attractive technique for the development of enzyme-loaded nanomaterials. Electrospinning allows for the fabrication of polymeric nanofibers with an exceptionally high surface area, tunable porosity, and the capacity to immobilize sensitive biomolecules under mild conditions.

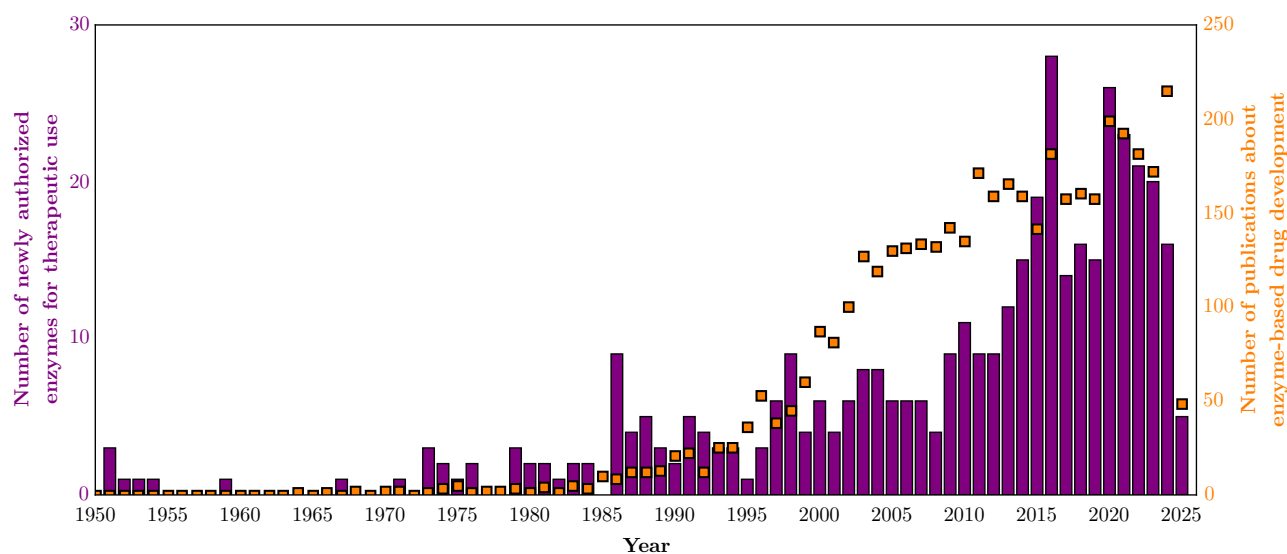
Electrospun nanofibers offer significant potential as carriers for therapeutic enzymes. Their structural properties make them suitable for topical and oral drug delivery systems, where they can act as protective matrices, release-modulating vehicles, or bioactive dressings. The ability to tailor fiber composition, morphology, and layering further expands their application scope in the field of biomedical nanotechnology.

This doctoral thesis aims to explore the use of electrospun nanofibers as innovative delivery platforms for therapeutic lipases. The research investigates how polymer properties and formulation strategies influence enzyme immobilization, stability, and catalytic performance. It also demonstrates the applicability of lipase-loaded nanofibrous formulations in two clinically relevant areas: topical treatment of dermatological conditions and oral enzyme replacement therapies. This work thoroughly investigates electrospinning's potential as a versatile and scalable nanoformulation technique for therapeutic enzymes.

## 2 Literature Background

### 2.1 Therapeutic Applications of Enzymes

Enzymes play a fundamental role in modern therapeutic strategies due to their ability to catalyze biochemical reactions with exceptional specificity and efficiency under mild physiological conditions. Their natural involvement in metabolic pathways and cellular regulation makes them highly relevant in the treatment and management of various diseases. As such, enzymes are not only indispensable in pharmaceutical manufacturing but are also directly applied as active pharmaceutical ingredients in a growing number of therapeutic formulations, as depicted in Figure 1.



**Figure 1:** Number of publications related to enzyme-based drug development and annual number of newly approved enzymes for therapy between 1950 and 2025. Publication counts were obtained from the PubMed database. The number of approved enzymes was determined based on the approval dates of the first authorized versions of enzyme-containing drugs listed in the DrugBank Online database.

The field of enzyme therapy has expanded rapidly in recent years, driven by the growing demand for more targeted and biologically compatible treatments. Enzyme-based drugs are now used across a wide spectrum of medical fields.<sup>1</sup> In oncology,<sup>2</sup> proteolytic enzymes are employed to degrade extracellular matrix components and tumor-associated proteins, aiding in tumor suppression and reducing metastatic potential. These approaches offer the potential for lower toxicity compared to conventional chemotherapeutics, especially when combined with selective targeting strategies such as protease-activatable probes or low-molecular-weight inhibitors.

In cardiovascular medicine, enzymes play a critical role in fibrinolysis.<sup>3</sup> Therapeutic agents such as serine and metalloproteases are administered to dissolve pathological blood clots in patients suffering from acute ischemic stroke, deep vein thrombosis, or pulmonary embolism.

1. Jennifer N Hennigan and Michael D Lynch, “The past, present, and future of enzyme-based therapies,” *Drug discovery today* 27, no. 1 (2022): 117–133.

2. Lennart Gremmler et al., “Proteolytic enzyme therapy in complementary oncology: a systematic review,” *Anticancer Research* 41, no. 7 (2021): 3213–3232.

3. Germana Michelle de Medeiros e SILVA et al., “Screening, production and biochemical characterization of a new fibrinolytic enzyme produced by *Streptomyces* sp.(Streptomycetaceae) isolated from Amazonian lichens,” *Acta Amazonica* 46, no. 3 (2016): 323–332.

Topical enzyme therapies have been widely adopted in dermatology and wound management.<sup>4</sup> Enzymes like papain and bromelain are used in formulations that accelerate tissue regeneration, reduce inflammation, and support the removal of necrotic tissue in chronic wounds, ulcers, and burns. Their incorporation into gels or nanofibrous dressings enhances localized efficacy and biocompatibility.

Enzyme Replacement Therapy (ERT) represents one of the most significant developments among enzyme-based treatment methods.<sup>5</sup> ERT is particularly critical in managing lysosomal storage diseases and other metabolic disorders where the absence or malfunction of a specific enzyme leads to toxic substrate accumulation and systemic symptoms. By supplementing the missing enzyme, ERT can restore metabolic balance, alleviate clinical manifestations, and improve patient outcomes. Since its first clinical application in the early 1990s, ERT has been successfully implemented for conditions such as Gaucher, Fabry, and Pompe diseases, and continues to evolve with the development of novel recombinant enzyme variants.

Nanotechnology has significantly broadened the scope of enzyme therapy. Functional nanocarriers can protect enzymes from degradation, modulate their release kinetics, and improve cellular uptake. Both organic and inorganic nanoparticles (e.g., silica, PVA, PLA, chitosan, and carbon-based systems) have been investigated as enzyme delivery vehicles. These platforms enable new therapeutic avenues, including the oral delivery of digestive enzymes and the targeted administration of enzymes to specific organs or cell types.

## 2.2 Challenges in Enzyme-Based Therapies

Despite their clinical relevance, the therapeutic application of enzymes is limited by several critical challenges that must be addressed to take full advantage of their potential. These challenges include difficulties in achieving targeted delivery, poor stability under physiological conditions, immunogenicity, short biological half-life, and high production costs. Each of these factors poses significant barriers to the efficacy, safety, and accessibility of enzyme-based therapies.

One of the foremost obstacles is ensuring the targeted delivery of enzymes to specific tissues or cells. Due to their large size and structural complexity, enzymes often struggle to penetrate biological barriers and accumulate effectively at the intended site of action. This can diminish their therapeutic efficiency and increase the risk of off-target effects. Nanoparticle-based drug delivery systems have been developed to overcome this limitation. These systems immobilize enzymes using responsive carriers that can release their payload in reaction to specific environmental triggers – such as  $pH$  shifts or the presence of disease-specific enzymes – thereby improving site-specific activation. In parallel, protein engineering methods are being employed to enhance enzyme selectivity and improve their interactions with biological targets.<sup>6</sup>

Stability is another major concern in enzyme therapies. Enzymes are highly sensitive to changes in temperature,  $pH$ , and enzymatic degradation – particularly under harsh physiological conditions like those found in the gastrointestinal tract. Immobilization within protective matrices such as nanoparticles, or the application of protein engineering techniques such as directed evolution, can help improve enzyme resilience. These strategies aim to maintain enzyme conformation and activity, ultimately extending their shelf life and

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4. Amene Nikgoftar Fathi et al., “Use of bromelain in cutaneous wound healing in streptozocin-induced diabetic rats: an experimental model,” *Journal of Wound Care* 29, no. 9 (2020): 488–495.

5. Maryam Yari et al., “Therapeutic enzymes: applications and approaches to pharmacological improvement,” *Current Pharmaceutical Biotechnology* 18, no. 7 (2017): 531–540.

6. Miguel de la Fuente et al., “Enzyme therapy: current challenges and future perspectives,” *International Journal of Molecular Sciences* 22, no. 17 (2021): 9181.

therapeutic window.<sup>7</sup>

The immunogenicity of therapeutic enzymes also presents a considerable barrier.<sup>8</sup> Since most enzymes used in therapy are derived from non-human sources or produced recombinantly, the human immune system may recognize them as foreign, triggering the production of neutralizing antibodies. This not only reduces therapeutic efficacy but may also induce adverse immune reactions. Surface modifications like PEGylation (attachment of polyethylene glycol chains) or encapsulation within nanoparticles can mitigate immunogenicity by reducing immune system recognition and prolonging circulation time.

A short *in vivo* half-life is another common limitation, often necessitating frequent dosing to maintain effective concentrations.<sup>9</sup> Strategies such as sustained-release nanocarriers, PEGylation, or protein modifications that enhance resistance to proteolytic degradation can extend enzyme activity in the body, reducing the frequency of administration and improving patient compliance.

Finally, high production costs remain a major hurdle to the widespread adoption of enzyme therapies. The complexity of producing, purifying, and formulating active enzymes often results in high manufacturing expenses. However, progress in bioprocessing technologies – such as advanced bioreactors, optimized host strains, and refined protein expression systems – offers the potential to reduce costs and improve scalability. Protein engineering can also streamline production by increasing expression yields and simplifying downstream purification steps.

## 2.3 Nanotechnology in Enzyme Delivery

The integration of nanotechnology into enzyme-based therapies has opened new avenues for improving enzyme stability, activity, and site-specific delivery.<sup>10</sup> Nanomaterials serve as versatile carriers that protect enzymes from degradation, facilitate controlled release, and enhance therapeutic efficacy. A wide range of nanomaterials – including polymeric- and inorganic nanoparticles, as well as hybrid systems – have been explored for enzyme immobilization, each offering distinct advantages depending on the therapeutic context. These approaches not only extend the functional lifespan of enzymes but also reduce their immunogenicity and enhance bioavailability *in vivo*.

Central to this strategy is enzyme immobilization,<sup>11</sup> where enzymes are bound to or incorporated into nanostructured carriers. Immobilization methods can be classified based on the type of interaction between the enzyme and its support. Among the most common are entrapment, adsorption, covalent bonding, cross-linking, and affinity-based methods. Each technique offers unique benefits and limitations in terms of enzyme activity retention, stability, ease of production, and suitability for clinical applications.

Entrapment and encapsulation involve physically enclosing enzymes within a porous or gel-like matrix without specifically forming chemical bonds. This protects the enzyme from external stress while maintaining its native conformation. However, diffusion barriers may hinder substrate access and product release, and some enzyme leaching may occur over time.

Adsorption and electrostatic interactions rely on weak forces to bind enzymes to surfaces.

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7. Shubhrima Ghosh et al., “Stability of therapeutic enzymes: Challenges and recent advances,” *Therapeutic Enzymes: Function and Clinical Implications*, 2019, 131–150.

8. Leslie P Cousins et al., “Novel Methods for Addressing Immunogenicity of Therapeutic Enzymes,” *Biobetters: Protein Engineering to Approach the Curative*, 2015, 63–77.

9. Rahela Zaman et al., “Current strategies in extending half-lives of therapeutic proteins,” *Journal of controlled release* 301 (2019): 176–189.

10. Ambra Del Grosso et al., “Current treatment options and novel nanotechnology-driven enzyme replacement strategies for lysosomal storage disorders,” *Advanced Drug Delivery Reviews* 188 (2022): 114464.

11. Yasmin R Maghraby et al., “Enzyme immobilization technologies and industrial applications,” *ACS omega* 8, no. 6 (2023): 5184–5196.

These methods are simple and do not alter the enzyme's structure, but their effectiveness is sensitive to environmental conditions such as  $pH$ , temperature, and ionic strength, which can result in desorption and loss of activity.

Cross-linking techniques produce enzyme crystals or aggregates by covalently bonding enzyme molecules directly to one another using agents like glutaraldehyde. These systems offer high structural stability and minimal enzyme leaching, but the chemical modifications can reduce enzymatic activity and may hinder substrate accessibility.

Affinity-based immobilization leverages specific biochemical interactions between enzyme and carrier, helping to preserve enzyme activity and minimize loss. While this technique enables highly selective binding, it requires carefully engineered carrier surfaces or enzyme modifications, increasing cost and complexity.

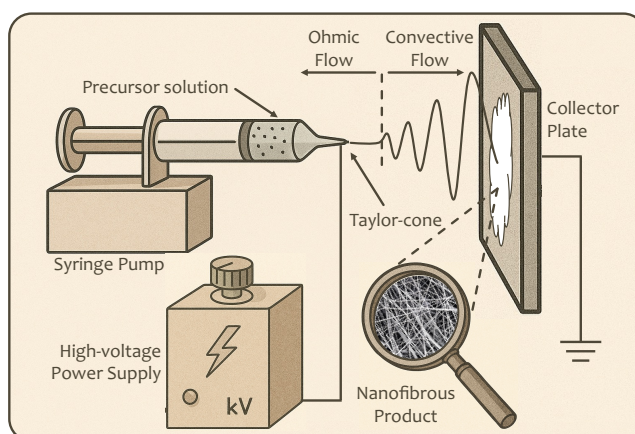
Covalent bonding, another widely used method, forms strong chemical links between functional groups on the enzyme and the carrier surface. This approach produces highly stable systems suitable for prolonged use under a range of conditions. However, covalent immobilization can induce conformational changes that negatively affect enzymatic activity, similarly to cross-linking.

## 2.4 Electrospinning as a Tool for Enzyme Nanoformulation

Electrospinning (see Figure 2) is an increasingly prominent nanomaterial fabrication technique for producing solid polymer nanofibers with high surface area-to-volume ratios and tunable structural properties, making it especially well-suited for therapeutic enzyme delivery. The process involves the use of an electric field to draw fibers from a polymer solution or melt, forming uniform nanofibrous mats.<sup>12</sup> Electrospun fibers offer several key advantages in biomedical applications, including drug delivery, wound healing, and tissue engineering.

In enzyme nanoformulation,<sup>13</sup> electrospun fibers serve as both protective matrices and efficient delivery vehicles. The encapsulation of enzymes within polymeric fibers stabilizes their structure and activity, shielding them from degradation and denaturation during storage or after administration. By tailoring the fiber composition and porosity, the release kinetics of encapsulated enzymes can be precisely controlled, enabling sustained or targeted release and reducing dosing frequency. Enzymes can also be immobilized on the surface of fibers via adsorption or covalent attachment, further expanding the range of formulation strategies.

Compared to other nanoformulation techniques, electrospun fibers are less likely to trigger immune responses and are capable of higher enzyme loading.<sup>14</sup> The process itself is versatile and compatible with a wide variety of synthetic and natural polymers, such as polyvinyl alcohol (PVA), polylactic acid (PLA),



**Figure 2:** Schematic diagram of a laboratory electrospinning device.

12. Geoffrey R Mitchell, *Electrospinning: principles, practice and possibilities* (Royal Society of Chemistry, 2015).

13. Anjum Hamid Rather et al., "Overview on immobilization of enzymes on synthetic polymeric nanofibers fabricated by electrospinning," *Biotechnology and Bioengineering* 119, no. 1 (2022): 9–33.

14. Elena Cojocaru et al., "Electrospun-fibrous-architecture-mediated non-viral gene therapy drug delivery in regenerative medicine," *Polymers* 14, no. 13 (2022): 2647.

gelatin, and chitosan. This enables the customization of fiber properties like hydrophilicity, mechanical strength, and degradation rate to meet specific therapeutic needs. Furthermore, the mild processing conditions help preserve the structural integrity of sensitive biomolecules, such as proteins and enzymes, that would be destabilized by high temperatures or harsh solvents.

Electrospun nanofibers have already shown promise in numerous preclinical studies for the oral delivery of lipases, treatment of chronic wounds, and localized drug delivery. While no enzyme-loaded electrospun therapeutic has yet reached the market, commercial devices based on electrospun matrices – such as the Spincare<sup>®</sup> wound dressing and the Rivelin<sup>®</sup> mucoadhesive patch – demonstrate the clinical viability of this technology. With continued innovation, electrospinning is poised to become a key method in the development of next-generation enzyme-based therapeutics.

## 2.5 Thesis Objectives

In this doctoral research I aim to explore the use of electrospun nanofibers as innovative carriers for therapeutic enzymes, with a particular focus on lipases. My central hypothesis is that by rationally selecting the polymer matrix, optimizing enzyme formulation parameters, and incorporating functional additives, it is possible to significantly enhance enzyme stability and catalytic performance in nanofibrous systems. The study is divided into three primary objectives, each addressing a distinct therapeutic context.

- The first objective focuses on polyvinyl alcohol as a matrix material, examining how its molecular weight, degree of hydrolysis, and concentration affect fiber formation and the catalytic activity of an entrapped model lipase from *Burkholderia cepacia*. This investigation aims to define optimal PVA characteristics for producing enzymatically active nanofibers.
- The second objective involves designing multi-layered nanofibrous face masks for topical dermatological therapy. These masks integrate two lipases (*Candida rugosa* and *Rhizomucor miehei*) and the antibiotic nadifloxacin for the potential treatment of *acne vulgaris*. The research includes structural characterization, evaluation of enzymatic activity against skin lipid analogues, and *ex vivo* skin permeability studies.
- The third objective targets oral enzyme replacement therapy by developing nanofibrous preformulations of *Porcine pancreatic* lipase. Different polymer matrices (PVA, polyvinylpyrrolidone, and polylactic acid) are tested, and enhancements in catalytic efficiency are pursued via cyclodextrin additives and substitution with alternative lipases of greater therapeutic potential.

Together, these goals aim to demonstrate the feasibility of electrospun nanofibers as a novel and versatile platform for therapeutic enzyme delivery in both topical and oral applications.

## 3 Experimental Methods

### 3.1 Production of Electrospun Nanofibers

Precursor solutions were prepared by dissolving water-soluble polymers (PVA, PVP) in water at 60 °C, or PLA in a DCM:DMF (6:1, V:V) mixture at room temperature. Electrospinning was performed using the **spincube** electrospinning device (**spinsplit**, Budapest, Hungary) under ambient conditions (23 ± 0.5 °C, ~30% RH). As the closed spinning chamber lacks internal climate control, spinning parameters were adjusted dynamically. Typical settings: 11–18 kV voltage, 10–30  $\mu\text{L min}^{-1}$  feed rate, 12–16 cm emitter-collector distance. The resulting nanofibers were collected on a flat collector plate, dried at room temperature, and stored at 4 °C.

### 3.2 Rheological Analysis

Solution viscosity was measured using a cone-plate rheometer (Physica MCR 301, Anton Paar) at 25.0 °C across shear rates of 1–631  $\text{s}^{-1}$ . Triplicate measurements were performed using 100  $\mu\text{L}$  of each sample.

### 3.3 Scanning Electron Microscopy (SEM)

Fiber morphology was examined using a JSM JEOL-5500LV SEM. Samples were gold-coated (10 mA, 180 s) and imaged in high vacuum. ImageJ software (NIH) was used to determine average fiber diameters ( $n = 100$ ).

### 3.4 Raman Microscopy

Compound distribution was mapped using a Thermo Fisher DXR Raman spectrometer with a 780 nm laser. Chemical maps were recorded over a 100 × 100  $\mu\text{m}$  area (1  $\mu\text{m}$  resolution), with 32 scans per point. Spectra were normalized to correct for intensity variations.

### 3.5 Analytical Assays for Measuring Lipase Activity

**Standard Assay:** Samples (5 mg enzyme preparation) were incubated in a mixture of TRIS buffer (650  $\mu\text{L}$ , 50 mM, pH 8.0), isopropanol (100  $\mu\text{L}$ ), and 1-octyl acetate (25  $\mu\text{L}$ ) substrate. Reactions were carried out at 37 °C for 2 hours in 4 mL screw-cap vials. Samples were analyzed using gas chromatography.

**Intestinosolvent Assay:** Identical composition as the standard assay, but using intestinosolvent buffer (650  $\mu\text{L}$ ;  $\text{KH}_2\text{PO}_4/\text{NaOH}$ , 50 mM, pH 6.8) instead of TRIS buffer to simulate intestinal conditions. Samples were analyzed using gas chromatography.

**FeSSIF Assay:** The FeSSIF reaction medium was prepared by dissolving sodium taurocholate (0.4125 g) in 500 mL of blank FeSSIF (pH 5.0; composed of NaCl,  $\text{CH}_3\text{COOH}$ , and NaOH, dissolved in deionized water and adjusted to simulate fed-state intestinal fluid conditions). A surfactant-substrate solution containing *p*-NPP (86.6 mg), Triton X100 (0.50 g), and gum arabic (0.125 g) in 2-propanol (1.477 mL) was added and sonicated for 60 minutes. For the assay, 150  $\mu\text{L}$  of lipase solution (0.1 mg  $\text{mL}^{-1}$  in blank FeSSIF) was added to 1.0 mL of FeSSIF reaction medium. The mixture was incubated at 37 °C for 1 hour in 4 mL screw-cap vials. Samples of the assay were evaluated via UV-Vis spectrophotometry.

### 3.6 Quantification of Enzymatic Activity

Enzyme activity assays were analyzed using gas chromatography (GC) or UV-Vis spectrophotometry, depending on the assay type. From the resulting chromatograms or absorbance data, the following key activity parameters were calculated: conversion ( $c$ , %), specific biocatalyst activity ( $U_B$ ,  $U\ g^{-1}$ ), specific enzyme activity ( $U_E$ ,  $U\ g^{-1}$ ), and activity yield ( $Y_A$ , %), according to the equations below.

$$c = \frac{n_P}{n_S + n_P} \times 100 \quad (1)$$

$$U_B = \frac{n_S \times c}{t \times m_B}, \quad U_E = \frac{n_S \times c}{t \times m_E} \quad (2-3)$$

$$Y_A = \frac{U_{E,immobilized}}{U_{E,native}} \times 100 \quad (4)$$

Here,  $n_P$  and  $n_S$  are product and substrate amounts ( $\mu mol$ ),  $t$  is reaction time ( $min$ ), and  $m_B$ ,  $m_E$  are biocatalyst and enzyme mass ( $g$ ), respectively.

### 3.7 Calorimetric Measurements

Calorimetric measurements were conducted using a PerkinElmer DSC 8500 differential scanning calorimeter (PerkinElmer, Waltham, MA, USA). Sample masses between 3 and 5  $mg$  were used in hermetically sealed aluminum crucibles. Nitrogen gas at a flow rate of 20  $mL\ min^{-1}$  was used as the purge gas. Samples were conditioned at  $-20\ ^\circ C$ , then heated to  $150\ ^\circ C$ , cooled to  $-20\ ^\circ C$ , and reheated to  $150\ ^\circ C$ . Heating and cooling rates were set to  $10\ ^\circ C\ min^{-1}$ .

### 3.8 Molecular Docking Simulation

PVA oligomers with varying chain lengths and hydrolysis levels (88%, 98%) were constructed in Avogadro2 and docked to *Burkholderia cepacia* lipase (*BcL*) using AutoDock Vina via DockingPie. Two receptor structures were used: apo (2LIP) and inhibitor-bound (1YS1). Ligands were docked into a grid box centered at ( $X = 15.9$ ;  $Y = 1.1$ ;  $Z = 20.0$ ), allowing both surface and active-site binding. Ten poses per ligand were generated (exhaustiveness = 8). Docking results were filtered by examining ligand orientation relative to a plane defined by Phe52, Leu293, and Phe119; only long chain polymer-mimicking poses were analyzed further. Visual inspection was done in PyMOL, and binding affinities were plotted using Seaborn.

### 3.9 Ex Vivo Skin Permeability Studies

Skin permeation was studied using Franz diffusion cells (Hanson Microette™) with heat-separated human epidermis samples (HSE) from plastic surgery patients. Electrospun mats were folded into 5-layer sheets and applied as donor phase ( $1.77\ cm^2$  area), while PBS ( $pH = 7.4$ ,  $7.0\ mL$ ) served as the acceptor phase at  $32\ ^\circ C$  over  $24\ h$ . Samples ( $0.8\ mL$ ) were taken at defined intervals and analyzed by HPLC. After the experiment, HSE and filters were extracted in methanol:water (50:50) and filtered ( $0.45\ \mu m$ ). Ethical approval: ETT-TUKEB (BMEÜ/2339-3/2022/EKU).

## 4 Results and discussion

### 4.1 Exploring the Influence of Polyvinyl Alcohol Properties on Enzyme Activity

This part of my doctoral work outlines the limitations and opportunities of electrospinning from aqueous precursor solutions of commercially available polyvinyl alcohol with different molecular weights and degrees of hydrolysis, focusing on their ability to form enzyme-loaded nanofibers. Particular attention was given to how these polymer characteristics affect the rheological properties of the precursor solutions and the morphology of the resulting fibers.

By conducting parallel rheological and electrospinning experiments, I identified an optimal viscosity range (0.1–10 *Pa s*) within which successful and continuous fiber formation could be reliably achieved. Solutions outside this range either lacked sufficient cohesion to form fibers or were too viscous to flow steadily through the electrospinning system.

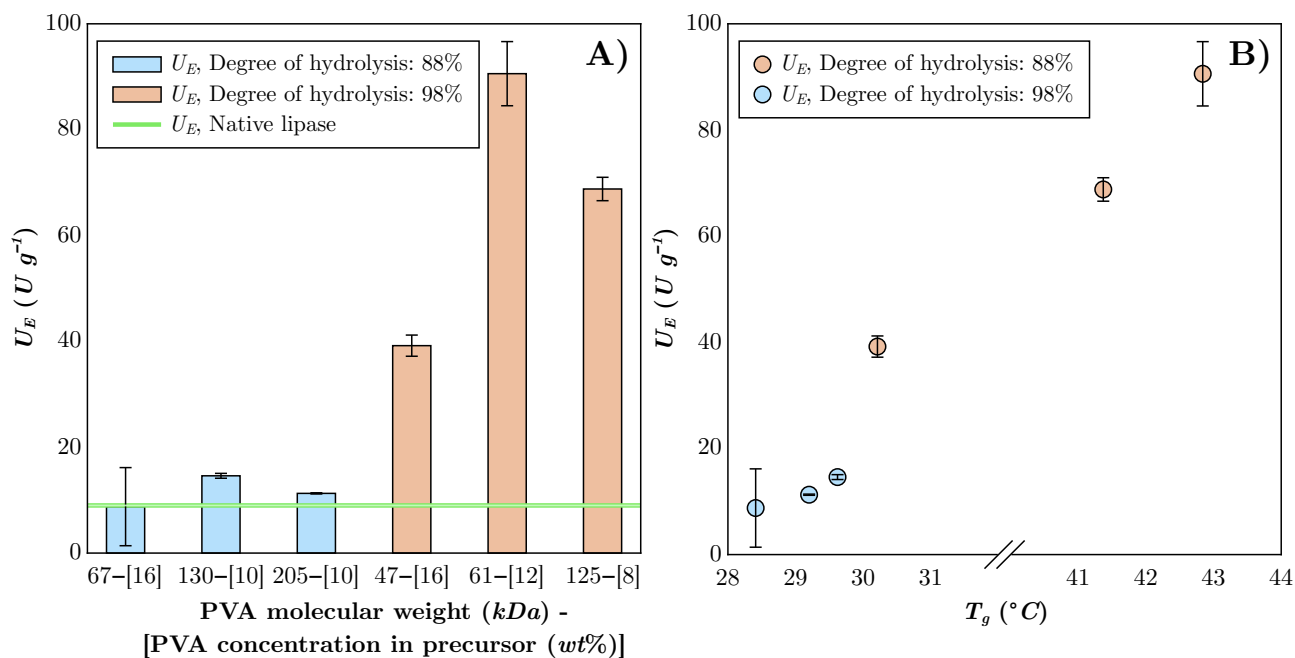
During the fiber formation experiments, several important trends emerged. As both the molecular weight and concentration of PVA increased, the resulting fibers became progressively thicker – up to a point where electrospinning became technically infeasible due to excessive viscosity and strong polymer–polymer interactions. These interactions led to flow instability and difficulty in processing the solution, even at high voltages. Beyond a critical viscosity threshold, solutions became increasingly heterogeneous and difficult to dissolve, preventing uniform fiber formation.

The inclusion of the enzyme (*BcL*) introduced additional complexity. Enzyme-containing solutions exhibited lower viscosities than enzyme-free counterparts, most likely due to specific interactions between the enzyme and the polymer chains, which altered the macromolecular structure of the solution. Consequently, the average diameter of enzyme-loaded fibers was typically smaller. This effect, combined with the stricter processing requirements, made fiber formation from enzyme-containing solutions more sensitive to formulation parameters. These observations suggest that enzyme–PVA interactions play a critical role in determining solution properties and fiber characteristics during electrospinning.

To evaluate how the properties of PVA affect the catalytic performance of immobilized lipase, transesterification between vinyl acetate and *racemic* 2-octanol was performed in an organic solvent mixture (*n*-hexane:MTBE, 2:1, *V:V*) using *BcL* entrapped in electrospun nanofibers. Reaction progress was monitored by gas chromatography after 2, 4, and 24 *hours*.

The kinetic resolution experiments confirmed that the degree of hydrolysis, molecular weight, and concentration of PVA all had a marked effect on the biocatalytic performance of the nanofiber-immobilized enzyme. *BcL* encapsulated in PVA nanofibers exhibited significantly enhanced enzymatic activity compared to the native enzyme, as shown in Figure 3/A. Fully hydrolyzed PVA matrices consistently outperformed partially hydrolyzed ones, reaching up to tenfold activity compared to the native *BcL*.

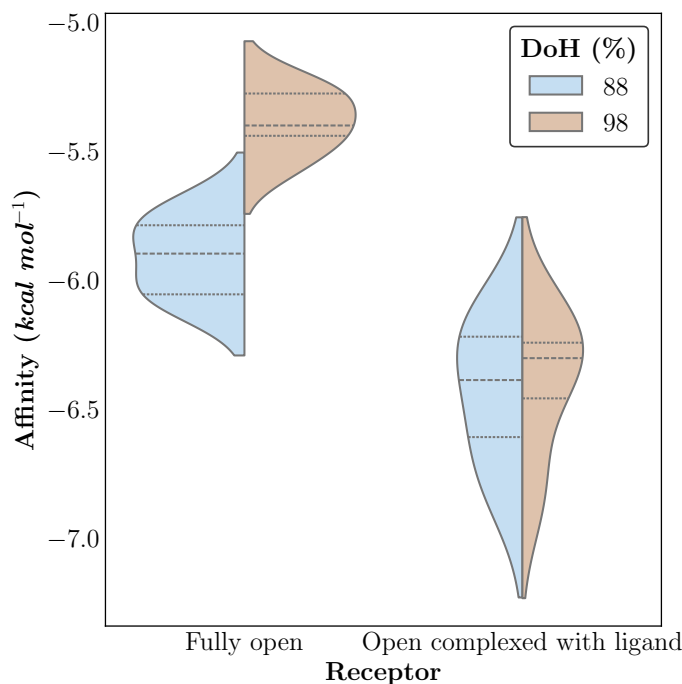
To investigate the structural basis for these improvements, differential scanning calorimetry (DSC) was used to assess the thermal properties of the nanofibers. As illustrated in Figure 3/B, enzyme-loaded fibers showed a reduction in glass transition temperature ( $T_g$ ) compared to enzyme-free ones, attributed to the plasticizing effect of the protein. Importantly, a clear correlation emerged between  $T_g$  and specific enzyme activity ( $U_E$ ): formulations with higher  $T_g$  displayed superior catalytic performance. Notably, samples with  $T_g$  above 30°C, and thus in a glassy state at the reaction temperature, achieved the highest activities. These findings support the idea that increased matrix rigidity helps maintain the active conformation of the enzyme, contributing to the enhanced activity observed after immobilization. Deviations in the thermal stability of the formulations can also suggest stabilizing interactions between the matrix polymer and the immobilized enzyme.



**Figure 3:** **A)** Specific enzyme activities ( $U_E$ ) of *BcL* entrapped in PVA nanofibers compared to the native enzyme. **B)** Correlation between glass transition temperature ( $T_g$ ) and  $U_E$ .  $T_g$  was measured by DSC;  $U_E$  was determined from transesterification reactions analyzed after 24 hours by gas chromatography.

Finally, molecular docking simulations provided further insight into the potential stabilizing role of PVA (see Figure 4). Computational models showed that PVA oligomers could interact with the active site and surface residues of *BcL* via hydrogen bonding. These interactions suggest a bioimprinting effect, in which the polymer matrix helps stabilize the enzyme's active conformation during immobilization.

These results demonstrate that by optimizing the molecular properties of PVA and processing conditions, highly active and stable nanofibrous biocatalysts can be developed. The immobilization of *BcL* in PVA nanofibers led to more than a tenfold increase in specific activity compared to the native enzyme, highlighting electrospinning as a powerful method for the nanoformulation of therapeutic enzymes. These findings establish a strong foundation for the development of enzyme-loaded nanofibers for biomedical applications, including those explored in subsequent research projects presented in this work.



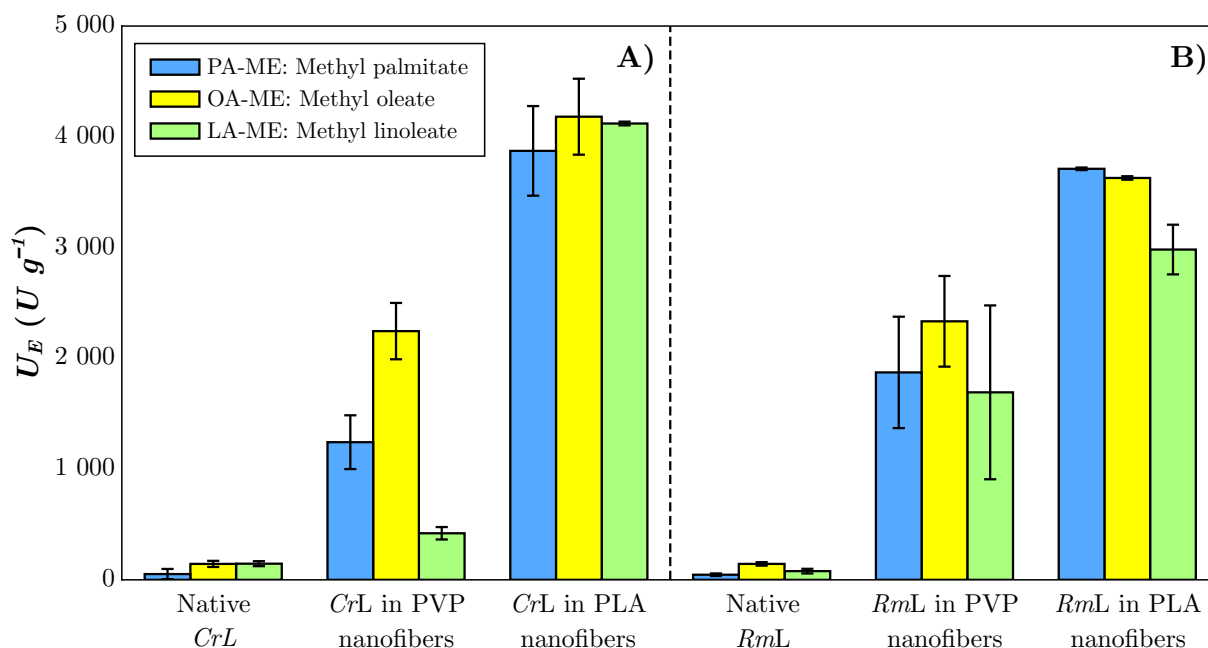
**Figure 4:** Affinities of PVA ligand poses with different degrees of hydrolysis (DoH) in molecular docking simulations with various *BcL* structures. Docking was performed using the AutoDock Vina program via the DockingPie plugin in PyMol.

## 4.2 Polymeric Nanofibers for Improved Skin Care

This part of my doctoral work focused on the development and evaluation of multilayered nanofibrous masks for topical therapeutic use. Using electrospinning, polymeric nanofibers were fabricated from polyvinylpyrrolidone (PVP) and polylactic acid (PLA), co-encapsulating two types of lipases – from *Candida rugosa* (*CrL*) and *Rhizomucor miehei* (*RmL*) – along with the antibiotic nadifloxacin. The aim was to assess the structural integrity, physicochemical properties, and biocatalytic performance of these nanofibrous formulations for potential dermatological applications.

Scanning electron microscopy revealed that all electrospun samples exhibited a uniform fibrous morphology with minor heterogeneities. PVP nanofibers were consistently thinner than their PLA counterparts. The incorporation of enzymes slightly reduced fiber diameters, while the addition of nadifloxacin had variable effects depending on the polymer matrix. Raman microscopy confirmed the successful and homogeneous entrapment of lipases and nadifloxacin within the fibers.

Mechanical testing demonstrated that PVP fibers were more elastic than PLA fibers. However, the inclusion of enzymes and antibiotics decreased fiber rigidity in both cases. Water contact angle (CA) measurements showed that PVP nanofibers were highly hydrophilic ( $CA_{water} \approx 5^\circ$ ), dissolving rapidly in aqueous environments, whereas PLA fibers were hydrophobic ( $CA_{water} > 90^\circ$ ), indicating potential for sustained release. The presence of nadifloxacin did not significantly affect the hydrophilic-hydrophobic properties of the two matrices.



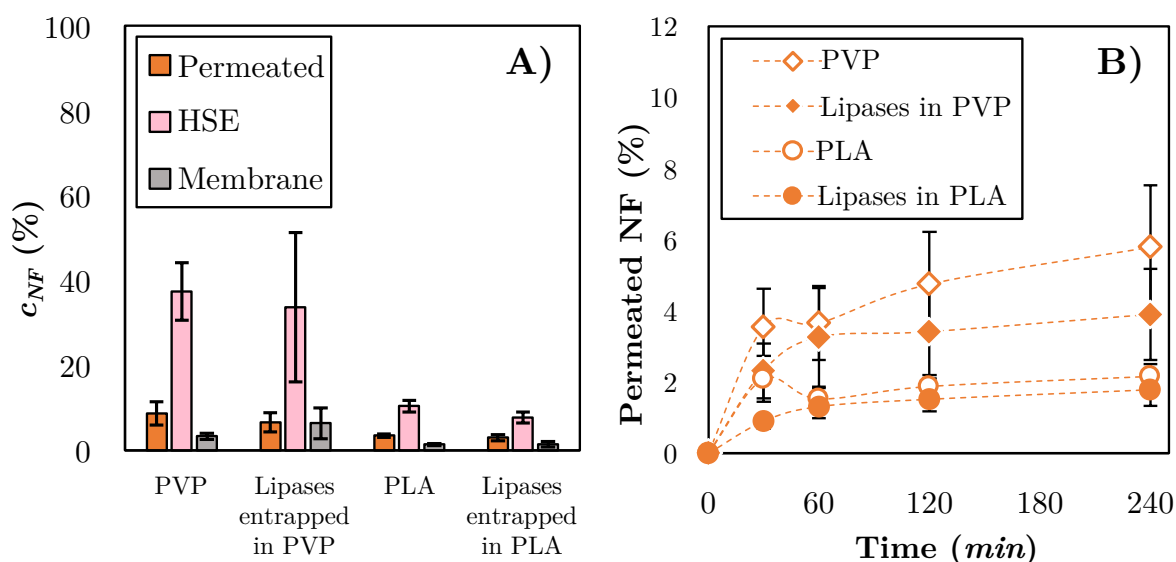
**Figure 5:** Examination of the impact of electrospinning into PVP and PLA nanofibers on the biocatalytic activity of **A)** *Candida rugosa* (*CrL*) and **B)** *Rhizomucor miehei* (*RmL*) lipases in the enzyme-catalyzed hydrolysis of methyl palmitate (PA-ME), methyl oleate (OA-ME) and methyl linoleate (LA-ME). The test reactions were conducted at  $33^\circ\text{C}$  with constant shaking. This figure presents the specific enzyme activity values, measured by gas chromatography, from samples collected after 2 *hours* of reaction time.

Lipase activity was tested in the hydrolysis of methyl esters of palmitic, oleic, and linoleic acids. Results in Figure 5 show that the immobilization in nanofibers significantly enhanced enzymatic activity compared to the native enzymes. PLA matrices provided a more stable

environment and consistently outperformed PVP in catalytic activity – despite their larger fiber diameter –, largely due to their insolubility, which preserved enzyme conformation and prevented aggregation.

The catalytic activity of the three-layer nanofibrous masks was also evaluated. We have found that the two co-formulated lipases maintained substantial activity and delivered high average performance across the full range of substrates. While the inclusion of nadifloxacin affected enzyme activity for certain substrates, this issue could be mitigated by using water-insoluble PLA nanofibers for the formulation.

Franz diffusion cell experiments confirmed the successful skin delivery of nadifloxacin across heat-separated human epidermis (HSE), as illustrated in Figure 6/A. PVP-based fibers released the drug more rapidly due to their solubility, while PLA fibers showed slower, more sustained release, as shown in Figure 6/B. The presence of enzymes slightly reduced the amount of permeated nadifloxacin, possibly due to the formation of a weakly diffusion-limiting surface film.



**Figure 6:** Analysis of nadifloxacin (NF) penetration into human heat-separated epidermis (HSE) using a topical and transdermal diffusion cell system. **A)** NF concentration in the acceptor phase (permeated), HSE, and membrane after 24 hours of incubation, and **B)** the amount of permeated NF over a 4 hour period at 33 °C. All penetration experiments were conducted using three-layered samples.

In conclusion, PLA-based multilayered nanofibrous masks demonstrated the most favorable balance between mechanical integrity, drug retention, and enzyme stabilization. These findings highlight the potential of electrospun nanofibers as customizable carriers for enzyme-based topical therapies, with particular relevance to conditions such as *acne vulgaris*, where both antimicrobial activity and lipid degradation are therapeutically desirable.

### 4.3 Electrospinning as a Nanoformulation Strategy for Digestive Enzymes

In this study, electrospinning was investigated as a novel technique for the nanoformulation of digestive enzymes, with a primary focus on lipase from *Porcine pancreas* (*PpL*). This enzyme was selected as the starting point due to its established clinical relevance and widespread use in commercial pancreatic enzyme replacement therapy (PERT) products. The aim was to develop and characterize stable nanofiber-based preformulations optimized for oral delivery, assessing the influence of polymer type and formulation parameters on the enzymatic performance.

A systematic electrospinning screening of 25 precursor solutions based on PVA, PVP, and PLA showed that fiber formation is mainly dictated by the polymer’s molecular weight and concentration, both of which are effectively reflected in the precursor’s viscosity. The optimal viscosity range for successful electrospinning was once again determined to be 0.1 – 10 *Pa s*, with best-performing solutions – PVP (360 *kDa*, 20 *wt%*), PVA (205 *kDa*, 10 *wt%*), and PLA (86 *kDa*, 8 *wt%*) – falling within a narrower range of 1.5 – 2.5 *Pa s*. These formulations consistently produced high-quality nanofibers suitable for enzyme immobilization.

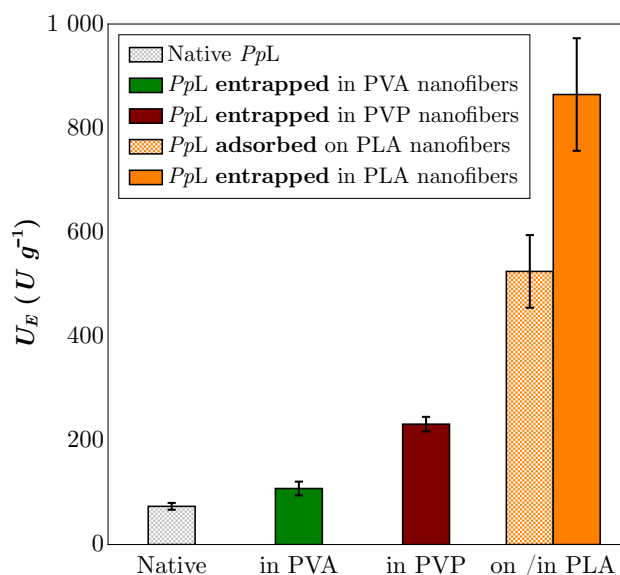
Incorporating *PpL* into the electrospun fibers led to a reduction in both viscosity and fiber diameter, likely due to protein-polymer interactions. Raman spectroscopy confirmed successful and homogeneous enzyme distribution in all formulations. SEM imaging has shown that the morphology of the nanofibers remained suitable despite the presence of enzyme, although diameter distributions were broader.

Karl-Fischer titration showed low residual water content in all fibers, with PLA exhibiting the lowest ( $\sim 4\%$ ). Positron annihilation lifetime spectroscopy revealed that enzyme encapsulation reduced the free volume in the fibers, indicating successful incorporation within the polymer matrix.

As Figure 7 presents, all nanofibrous formulations exhibited enhanced catalytic activity compared to native *PpL*. Specifically, enzyme activity increased by 45% in PVA, 215% in PVP, 615% in PLA (adsorbed), and 1075% in PLA (entrapped). These improvements were attributed to favorable microenvironments formed by the matrix polymers. The PLA matrix, being water-insoluble, preserved the stabilizing interactions with the enzyme better than water-soluble matrices.

The enzymatic activity of the nanofibrous formulations was tested against several *PpL*-containing commercial drugs. Under both standard and simulated intestinal conditions, the PLA-based nanofibrous *PpL* formulation exhibited activity equal to or greater than that of commercial PERT products. This improvement is largely due to the PLA matrix’s ability to support and stabilize the enzyme in its active form, thereby amplifying catalytic efficiency even under less favorable *pH* conditions.

To avoid solvent-related toxicity, attention



**Figure 7:** Specific enzyme activity ( $U_E$ ) values of native and nanofiber-immobilized *PpL* (in PVA, PVP, and PLA) during the enzyme-catalyzed hydrolysis of 1-octyl acetate under intestinosolvent assay conditions ( $pH = 6.8$ ,  $37^\circ C$ ). Activity values were determined after 2 hours by gas chromatography.

shifted to water-soluble matrices using PVA. To enhance enzymatic activity, various cyclodextrins (CDs) were co-entrapped with lipases from *Burkholderia cepacia* (*BcL*) and *Aspergillus oryzae* (*AoL*) – two lipases that have been reported to exhibit higher therapeutic potential than *PpL* – within the electrospun nanofibers. Screening five CDs revealed that 2-hydroxypropyl- $\beta$ -cyclodextrin (HPB-CD) and  $\beta$ -cyclodextrin (B-CD) notably enhanced *BcL* activity, while HPB-CD and sulfobutylated- $\beta$ -cyclodextrin (SBB-CD) were most effective for *AoL*. Under FeSSIF conditions, these CD-templated enzymes showed significantly higher activity than native enzymes.

Electrospinning of CD-lipase pairs into PVA nanofibers yielded well-formed fibers with uniform lipase distribution, as verified by Raman mapping. Although CD-enhanced activity was somewhat diminished after electrospinning, all CD-lipase nanofibrous formulations outperformed commercial drugs in FeSSIF assays (see Table 1).

**Table 1:** Comparison of conversion rates ( $c$ , %) and specific activities ( $U_B$ ,  $U g^{-1}$ ) of various lipase formulations, including nanofibrous formulations and commercially available medicinal products, in the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) after 1 *hour* at 37 °C under standard assay conditions and in the FeSSIF assay.

Lipase	Cyclodextrin	Standard Assay		Assay in FeSSIF	
		$c$ (%)	$U_B$ ( $U g^{-1}$ )	$c$ (%)	$U_B$ ( $U g^{-1}$ )
<b>Nanofibrous formulas</b>					
<i>BcL</i>	–*	74.9 ± 4.8	137 ± 9	38.7 ± 0.5	191 ± 3
	B-CD <sup>1</sup>	98.6 ± 4.7	188 ± 9	39.6 ± 0.5	196 ± 3
	HPB-CD <sup>2</sup>	30.7 ± 0.5	56 ± 1	43.6 ± 3.5	216 ± 18
<i>AoL</i>	–*	99.1 ± 0.8	182 ± 10	22.7 ± 0.3	112 ± 1
	HPB-CD <sup>2</sup>	77.3 ± 11.1	142 ± 20	24.6 ± 4.1	122 ± 20
	SBB-CD <sup>3</sup>	96.3 ± 7.0	177 ± 13	21.0 ± 0.4	104 ± 2
<b>Marketed pharmaceutical products</b>					
Kreon <sup>®</sup> 25000		6.7 ± 1.3	12 ± 2	10.9 ± 0.1	54 ± 1
Pangrol <sup>®</sup> 25000		3.4 ± 0.3	6 ± 1	10.8 ± 0.5	54 ± 3

\* Native lipase

<sup>1</sup> B-CD:  $\beta$ -Cyclodextrin

<sup>2</sup> HPB-CD: 2-Hydroxypropyl- $\beta$ -Cyclodextrin

<sup>3</sup> SBB-CD: Sulfobutylated- $\beta$ -Cyclodextrin

This work demonstrated that electrospinning is a highly effective strategy for the nanoformulation of digestive enzymes. The use of CDs further enhanced enzyme performance, and PVA-based nanofibrous preformulations showed superior catalytic activity and handling properties. These findings highlight the potential of nanofibrous systems for advanced oral enzyme therapy applications.

## 5 Thesis statements

The key conclusions of my dissertation are concisely outlined below, with references to the relevant publications provided for each thesis statement:

1. The influence of polyvinyl alcohol's molecular weight, degree of hydrolysis, and concentration in aqueous precursor solutions on the electrospinning process was comprehensively investigated, revealing significant effects on nanofiber morphology and the biocatalytic activity of an encapsulated model lipase from *Burkholderia cepacia*. I established the parameter ranges necessary for successful electrospinning of polyvinyl alcohol nanofibers and demonstrated that optimization of these factors led to more than a tenfold increase in specific enzyme activity compared to the native lipase. [1].
2. A direct connection was established between the glass transition temperature of electrospun polyvinyl alcohol nanofibers and the specific activity of entrapped *Burkholderia cepacia* lipase. I showed that higher glass transition temperatures are associated with increased enzymatic activity. [1]
3. Innovative water-soluble (polyvinylpyrrolidone-based) and water-insoluble (polylactic acid-based) nanostructured face masks were developed using electrospinning, resulting in multi-layered formulations incorporating two lipases (*Candida rugosa* and *Rhizomucor miehei*) and the antibiotic nadifloxacin, offering a novel strategy for topical drug delivery. I demonstrated synergy between the lipases in co-hydrolyzing methyl esters of the primary fatty acids in human skin lipids – palmitic, oleic, and linoleic acids – showing enzyme activity over two orders of magnitude higher than their native forms [2].
4. Topical transdermal permeability studies have shown that the developed multi-layered nanofibrous masks, containing either nadifloxacin alone or in combination with lipases from *Candida rugosa* and *Rhizomucor miehei*, can effectively facilitate the delivery of nadifloxacin into the human epidermis, highlighting the potential of these electrospun formulations for improved topical drug delivery [2].
5. The immobilization of *Porcine pancreatic* lipase using electrospun polyvinyl alcohol, polyvinylpyrrolidone, and polylactic acid nanofibers was systematically studied using both entrapment and adsorption methods. I showed that entrapment of *Porcine pancreatic* lipase in polylactic acid nanofibers resulted in a more than ten-fold activity increase under intestinosolvent conditions compared to its native form, outperforming commercial therapeutic formulations of the same lipase. This demonstrates that these nanofibrous preformulations could serve as a foundation for developing a novel *per os* dosage form for pancreatic enzyme replacement therapy (PERT) [3].
6. The co-entrapment of lipases from *Burkholderia cepacia* and *Aspergillus oryzae* with selected cyclodextrin derivatives ( $\beta$ -cyclodextrin, 2-hydroxypropyl- $\beta$ -cyclodextrin, and sulfobutylated- $\beta$ -cyclodextrin) in polyvinyl alcohol nanofibers was shown to significantly enhance enzymatic activity, at optimal lipase:CD mass ratios. I also demonstrated that this approach promoted more homogeneous enzyme distribution within the fibers, introducing a novel strategy for enhancing nanofiber-based therapeutic enzyme formulations [4].

## 6 Application and Prospective Utilization

The nanostructured enzyme formulations developed in this work offer several promising avenues for application in medical and pharmaceutical contexts. Electrospun nanofibrous carriers containing lipases have demonstrated particular potential in both topical and oral enzyme therapies.

The multilayered nanofiber mask prototype containing *Candida rugosa* and *Rhizomucor miehei* lipases alongside nadifloxacin exhibited outstanding biocatalytic activity and effective skin permeation. These findings suggest a future opportunity for application in dermatological products, particularly in drug-loaded face masks designed for the treatment of skin conditions such as *acne vulgaris*. The exfoliating and sebo-regulating properties of lipases may further enhance the absorption of active ingredients and can be favorably combined with other therapeutic agents.

The electrospun enzyme replacement preformulations developed for oral use demonstrated comparable or superior enzymatic activity to that of commercial pancreatic enzyme replacement products. Furthermore, nanofibrous formulations containing microbial and fungal lipases from *Burkholderia cepacia* and *Aspergillus oryzae* respectively showed greater therapeutic potential than formulations based on lipase from *Porcine pancreas*. The incorporation of functional additives such as cyclodextrins was found to further enhance enzymatic performance, offering an effective strategy to improve the therapeutic efficacy of oral enzyme formulations, especially under challenging gastrointestinal conditions.

The scalability, customizability, and compatibility of electrospun systems with other therapeutic compounds enable the design of advanced, potentially patient-specific enzyme therapies. The feasibility of industrial implementation is further supported by the fact that electrospinning is a highly automatable and GMP-compliant manufacturing technique.

In summary, the formulation strategies presented in this thesis open new directions for enzyme therapy and may contribute to the development of more effective, stable, and targeted pharmaceutical products in the future.

## 7 Publications

### Papers Used for Writing the Dissertation

- [1] **Gergő Dániel Tóth**, Zsófia Molnár, Gábor Koplányi, Benjámín Gyarmati, András Szilágyi, Gábor Katona, Alfréd Menyhárd, László Poppe, Béla Pukánszky, and Diána Balogh-Weiser. How could the physical properties of polyvinyl alcohol influence enzymatic activity? A detailed study on nanofibrous catalysts incorporating a lipase from *Burkholderia cepacia*. *ChemCatChem*, 2024. // Author's share: 90%; Quartile classification: D1; Impact Factor: 5.497
- [2] Diána Balogh-Weiser, Alexandra Molnár, **Gergő Dániel Tóth**, Gábor Koplányi, József Szemes, Balázs Decsi, Gábor Katona, Maryana Salamah, Ferenc Ender, Anita Kovács, et al. Combined nanofibrous face mask: Co-formulation of lipases and antibiotic agent by electrospinning technique. *Pharmaceutics*, 15(4):1174, 2023. // Author's share: 55%; Quartile classification: Q1; Impact Factor: 4.9
- [3] **Gergő Dániel Tóth**, Nikolett Kállai-Szabó, Miléna Lengyel, Károly Süvegh, Ferenc Ender, Gábor Katona, Adrienn Kazsoki, Romána Zelkó, István Antal, György T Balogh, et al. Nanoformulation of lipase from porcine pancreas by electrospinning as a novel alternative for enzyme-based per os therapies. *Journal of Molecular Liquids*, 389:122819,

2023. // Author's share: 100%; Quartile classification: Q1; Impact Factor: 5.3

- [4] **Gergő Dániel Tóth**, Adrienn Kazsoki, Benjámín Gyarmati, András Szilágyi, Gábor Vasvári, Gábor Katona, Lajos Sente, Romána Zelkó, László Poppe, Diána Balogh-Weiser, et al. Nanofibrous formulation of cyclodextrin stabilized lipases for efficient pancreatin replacement therapies. *Pharmaceutics*, 13(7):972, 2021. // Author's share: 100%; Quartile classification: Q1; Impact Factor: 4.9

## Other Publications

- [1] **Gergő Dániel Tóth**, Gábor Koplányi, Balázs Kenéz, and Diána Balogh-Weiser. Nanoformulation of therapeutic enzymes: A short review. *Periodica Polytechnica Chemical Engineering*, 67(4):624–635, 2023. // Author's share: 60%; Quartile classification: Q3; Impact Factor: 1.4
- [2] Gábor Koplányi, Evelin Sánta-Bell, Zsófia Molnár, **Gergő Dániel Tóth**, Muriel Józó, András Szilágyi, Ferenc Ender, Béla Pukánszky, Beáta G Vértessy, László Poppe, et al. Entrapment of phenylalanine ammonia-lyase in nanofibrous polylactic acid matrices by emulsion electrospinning. *Catalysts*, 11(10):1149, 2021. // Author's share: 20%; Quartile classification: Q2; Impact Factor: 3.8
- [3] László Nagy-Győr, Emese Farkas, Mihai Lăcătuș, **Gergő Dániel Tóth**, Dániel Incze, Gábor Hornyánszky, Viktória Bódai, Csaba Paizs, László Poppe, and Diána Balogh-Weiser. Conservation of the biocatalytic activity of whole yeast cells by supported sol-gel entrapment for efficient acyloin condensation. *Periodica Polytechnica Chemical Engineering*, 64(2):153–161, 2020. // Author's share: 20%; Quartile classification: Q3; Impact Factor: 1.4
- [4] Guoqiang Li, Waldemar Jankowski, Joanna Kujawa, Baturalp Yalcinkaya, Fatma Yalcinkaya, Diána Balogh-Weiser, **Gergő Dániel Tóth**, Ferenc Ender, Norman Sepsik and Wojciech Kujawski. Recent Advances in the Preparation and Applications in Separation Processes of Electrospun Nanofiber-Based Materials. *Journal of Environmental Chemical Engineering*, 13, 115174 2025. // Author's share: 90%; Quartile classification: Q1; Impact Factor: 7.4

## Conference Presentations

1. **G.D. Tóth**, N. Kállai, M. Lengyel, G. Katona, R. Zelkó, I. Antal, B. Gyarmati, G.T. Balogh, D. Balogh-Weiser, Formulation of Lipases into Polymer Nanofibers for Pancreatin Replacement Therapies, *Chemistry Physics and Biology of Colloids and Interfaces*, 2022. Eger, Hungary – Poster presentation
2. **Tóth G.D.**, Molnár A., Kállai N., Lengyel M., Katona G., Zelkó R., Antal I., Balogh Gy.T., Balogh-Weiser D., Nanoszálás Enzimmészitmények Fejlesztése Enzimmhelyettesítő Terápiához, *Gyógyszertechológiai és Ipari Gyógyszerészeti Konferencia*, 2022. Siófok, Hungary – Poster presentation
3. **G.D. Tóth**, New Possibilities For The Immobilization And Formulation Of Biocatalysts By Electrospinning Technique *Membrane Materials - Modification and Separation (M3-S)*, 2023. Toruń, Poland – Oral presentation

4. **G.D. Tóth**, Nanoformulating enzymes for therapeutic use: A novel approach using electrospun nanofibers, *Zechmeister Award*, 2023. Pécs, Hungary – Oral presentation
5. **G.D. Tóth**, Electrospun Nanofibers: A Novel Delivery System for Nanoformulated Therapeutic Enzymes, *Electrospun Nanofibers and Applications Workshop*, 2024. Liberec, Czech Republic – Oral presentation
6. **G.D. Tóth**, Nanofibrous Carriers For Advanced Enzyme Replacement Therapies, *Drug Delivery through the Physiological Barriers*, 2025. Budapest, Hungary – Oral presentation

## **Additional Conference Participation**

1. *SpinSpiration Symposium: Connecting Minds in Electrospinning*, 2024. Online
2. *Congressus Pharmaceuticus Hungaricus XVII. and EUFEPS Annual Meeting*, 2024. Debrecen, Hungary
3. *5<sup>th</sup> International Conference on Bio-based Polymers and Composites*, 2024. Esztergom, Hungary