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Published in:
Materials Science and Engineering C

Link to article, DOI:
[10.1016/j.msec.2019.109789](https://doi.org/10.1016/j.msec.2019.109789)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Zdarta, J., Jankowska, K., Wyszowska, M., Kijeska-Gawroska, E., Zgoa-Grzekowiak, A., Pinelo, M., Meyer, A. S., Moszynski, D., & Jesionowski, T. (2019). Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability. *Materials Science and Engineering C*, 103, [109789]. <https://doi.org/10.1016/j.msec.2019.109789>

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Accepted Manuscript

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PII: S0928-4931(19)30317-0
DOI: <https://doi.org/10.1016/j.msec.2019.109789>
Article Number: 109789
Reference: MSC 109789
To appear in: *Materials Science & Engineering C*
Received date: 24 January 2019
Revised date: 10 May 2019
Accepted date: 23 May 2019

Please cite this article as: J. Zdarta, K. Jankowska, M. Wyszowska, et al., Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability, *Materials Science & Engineering C*, <https://doi.org/10.1016/j.msec.2019.109789>

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**Robust biodegradation of naproxen and diclofenac by laccase immobilized using
electrospun nanofibers with enhanced stability and reusability**

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Abstract

Enzymatic biodegradation of pharmaceuticals, using enzymes such as laccase, is a green solution for the removal of toxic pollutants that has attracted growing interest over recent years. Moreover, the application of immobilized biocatalysts is relevant for industrial applications, due to the improved stability and reusability of the immobilized enzymes. Thus, in the present study, laccase was immobilized by adsorption and encapsulation using poly(l-lactic acid)-co-poly(ϵ -caprolactone) (PLCL) electrospun nanofibers as a tailor-made support. The produced biocatalytic systems were applied in the biodegradation of two commonly used anti-inflammatories, naproxen and diclofenac, which are present in wastewaters at environmentally relevant concentrations. The results showed that under optimal process conditions (temperature 25 °C, pH 5 and 3 for naproxen and diclofenac respectively), even from a solution at a concentration of 1 mg L⁻¹, over 90% of both pharmaceuticals was removed by encapsulated laccase in batch mode. Both immobilized enzymes also exhibited high reusability: after five reaction cycles approximately 60% and 40% of naproxen and diclofenac were removed by encapsulated and adsorbed laccase respectively. In addition, a thorough analysis was made of the products of biodegradation of the two studied pollutants. Furthermore, toxicity study of the mixture after biodegradation of the pharmaceuticals showed that the solutions obtained after the process were approximately 65% less toxic than the initial naproxen and diclofenac solutions.

Keywords: laccase, immobilization, electrospun materials, environmental pollutants, pharmaceuticals, biodegradation

1. Introduction

Due to its high production and consumption, pharmaceutically active compounds frequently occurred in wastewater, drinking water and even ground water at concentrations usually do not exceeding nanograms or micrograms per liter [1]. Accumulation of these compounds as well as their metabolites in water compartments in unknown and uncontrolled amounts has attracted attention as they are persistent and might have negative effect on human beings and environment. For instance long-term contact with diclofenac is associated with a serious atherothrombotic and gastrointestinal issues as well as vascular risk [2]. Moreover, the adverse effect of diclofenac on environment, such as on microbial consortia and selected aquatic organisms has been previously reported [3]. On the other hand, naproxen and its metabolites also could negatively affected on human body and caused some serious

gastrointestinal and cardiovascular adverse effects and lead to an irreparable changes in a river ecosystems [4]. Due to the low bioavailability and complex structure of these compounds, their biodegradation is a serious environmental challenge. Conventional treatment plants are not able to efficiently remove and have some issues, such as generation of toxic by-products and waste stream. Thus, increasing interest was focused on a sustainable biological alternative as an eco-friendly and powerful solutions [5]. As of particular interest, enzymatic-based methods should be presented, as biocatalysts can degrade a wide range of xenobiotics under mild conditions, without toxic effluents [6].

The catalytic conversion of pharmaceutical pollutants takes place by the action of fungal oxidative enzymes, such as laccases, tyrosinases and various peroxidases [7]. Laccases, however, are the most frequently applied enzymes for biodegradation of persistent compounds due to their availability, low price, low substrate specificity and high catalytic activity. There are some literature study related to the use of free laccase for degradation on anti-inflammatories. For instance, Lonappan et al., reported that laccase produced by white rot fungi *Trametes versicolor*, degraded over 90% of diclofenac after 48 h of the process [8]. In another study Aracagök et al., showed that crude laccase from *Trametes trogii* almost completely removed diclofenac with 97% efficiency in 48 h [9]. However, practical application of free enzymes suffers from their low stability and poor reusability. Enzyme immobilization is a possible approach to overcome these limitations and at the same time increase the biodegradation efficiency and enzyme reuse [10]. In the previous study it has been reported that laccase was immobilized onto polyacrylonitrile nanofibers [11], marine sponges scaffolds [12] or biochar microparticles at various organic origin [13], and efficiently used for removal of hazardous compounds. As proper selection of the support material in enzyme immobilization is the crucial affecting properties of the biocatalysts, more and more attention is paid to evaluate immobilization protocols based on using novel and tailor-made supports.

The materials produced by the electrospinning method are characterized by thin structures reaching a diameter in the range from 100 nm to 1 μ m, and length to even several thousand meters [14]. One of the most important features of electrospun materials is the designed geometry in a quick and relatively cheap way [15]. Although all nanofibers combine common features, the final properties of a material depend on the polymer used to obtain electrospun materials. It should be pointed that electrospun nanofibers can be also successfully used to enzyme immobilization. The important feature of materials produced by electrospinning method is porosity, biocompatibility and a large number of functional groups on the surface of

nanofibers. This is evidenced by the fact that it is possible to immobilize the protein by means of adsorption, encapsulation or covalent bonding on the electrospun material. A wide range of polymers are used to enzyme immobilization, and poly(ϵ -caprolactone) [16] and poly(vinyl alcohol) are one of them [17]. It is worth to paying attention that during the production of electrospun nanofibers it is possible to obtain composite which, having many functional groups, increase the affinity of the enzyme to the support, for example polycaprolactone/cellulose monoacetate [18] and poly(vinyl alcohol)/polyacrylamide [19].

Hence, in this study we aimed to evaluate novel immobilization protocol for laccase from *Trametes versicolor* by adsorption and encapsulation approach, using PLCL electrospun nanofibers as support material. This support was used due to its exceptional mechanical stability, numerous of functional groups facilitating stable attachment of the enzyme as well as its porosity that limit diffusional limitations and enhance activity of the biomolecules. Further, the biodegradation and conversion of diclofenac and naproxen by the biocatalytic systems obtained were investigated and the optimal process conditions for removal of both pharmaceuticals have been found during the study. Moreover, comprehensive work on characterization of degradation products and their toxicity have also been examined in details. Although degradation of pharmaceuticals by laccase has been reported, to the best of our knowledge, this is the first time, where effective removal of naproxen and diclofenac by PLCL-laccase biocatalytic systems is shown. Moreover, presented results might provide a novel and efficient technique for practical application of electrospun-based immobilized enzymes for biodegradation of hazardous pollutants from wastewaters.

2. Materials and Methods

2.1. Chemicals and reagents

Poly(l-lactic acid)-co-poly(ϵ -caprolactone) (PLCL), laccase from *Trametes versicolor* (EC 1.10.3.2, ≥ 0.5 U/mg), diclofenac sodium salt (DCF, $\geq 98\%$), naproxen (NPR, $\geq 99\%$) (see Fig. 1), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, $\geq 99\%$), Coomassie Brilliant Blue, acetate and phosphate buffers at specific pH, analytical grade chloroform and Span 80 were supplied by Sigma-Aldrich (USA). Ethanol, methanol and 85% H_3PO_4 (all laboratory grade) were supplied by Chempur (Poland). HPLC-grade methanol and LC-MS-grade acetonitrile were obtained from Merck (Germany). HPLC-grade water was produced in the laboratory using a Demiwa system (Watek, Czech Republic).

Figure 1. Structures and pK_a values of diclofenac and naproxen

2.2. Fabrication of electrospun materials

Hydrophilic PLCL nanofibers were obtained by electrospinning of an emulsion. To prepare the oil phase, 0.5 g of PLCL was dissolved in 5 mL of analytical grade chloroform to form a 10% (w/v) solution. Next, 50 μ L of the surfactant Span 80 was added dropwise. An acetate buffer at pH 5 was used as the water phase. The oil and aqueous solutions were mixed in a ratio of 1:20 (v/v), and the mixture was stirred at RT for 3 h to obtain a smooth water-in-oil emulsion. PLCL nanofibers with laccase encapsulated within the fibers were also prepared by emulsion electrospinning. The oil phase was prepared as in the case of the PLCL fibers. For the water phase, laccase was dissolved in acetate buffer at a concentration of 100 mg mL⁻¹. Next, the polymer and laccase solutions were mixed in a ratio of 1:20 (v/v) and stirred for 3 h at RT. The electrospinning of both emulsions was carried out under the same optimized conditions using a NANON-01A apparatus (MECC Co., Japan). An 18G flattened needle connected to a syringe by a PTFE tube was fixed to a moving head with a linear speed of 50 mm/sec and a working width of 100 mm. A high voltage of 25 kV was applied through the needle. The distance from the needle tip to the collector was set at 150 mm. The feed rate was fixed at 1.5 mL h⁻¹. Nanofibrous meshes were collected on an aluminum foil-covered, grounded steel plate collector and dried for 24 h in a vacuum drier (25 °C).

2.3. Immobilization of laccase by adsorption on PLCL membrane

Prior to immobilization the membrane was washed with deionized water, cut into pieces of 0.25 cm² (0.5 $\times$ 0.5 cm) and weighed. The pieces were then immersed in 5 mL of 1 mg mL⁻¹ laccase solution (acetate buffer at pH=5) for 2 h at 4 °C using a KS 4000i incubator (IKA Werke GmbH, Germany). After the process, the PLCL fibers with immobilized enzyme were separated from the mixture and washed with acetate buffer to remove unbound enzyme.

2.4. Storage stability and kinetic parameters of free and immobilized enzymes

The storage stability of free and immobilized enzyme stored at 4 °C was examined over 30 days. Briefly, ABTS was used as a substrate, to which 10 mg of free or immobilized laccase was added. Changes in the concentration of the substrate were measured spectrophotometrically ($\lambda=420$ nm), using a Jasco V-750 (Japan) spectrophotometer. For the storage stability study, the initial value of the activity of the free or immobilized enzyme was defined as 100%. The inactivation constant (k_D), and half-life ($t_{1/2}$) were evaluated based on the linear regression slope.

The same reaction was used to evaluate the kinetic parameters: the Michaelis–Menten constant (K_m) and the maximum rate of reaction (V_{max}). For this purpose solutions of the substrate at concentrations ranging from 1 to 100 mM were used, and the experiments were carried out under optimum assay reaction conditions. The Hanes–Woelf plot was used to calculate the apparent kinetic parameters of the free and immobilized enzyme. All measurements were made in triplicate, and the results are presented as average $\pm$ standard deviation.

2.5. Biodegradation of pharmaceuticals

The biodegradation of naproxen and diclofenac by free, adsorbed and encapsulated laccase was studied using an IKA KS 4000i incubator shaker (100 rpm). To 10 mL of a mixture of diclofenac or naproxen in buffer solution, 5 mg of free or immobilized laccase was added. The mixtures were then kept for 24 h and sampled at specified time intervals. After sampling, 0.1 mL of methanol was added prior to quantitative analysis. As naproxen is known for its resistance to enzymatic biodegradation [20] 0.1 mM of ABTS was added as a mediator agent to the solutions of that pharmaceutical.

Prior to evaluation of the efficiency of pharmaceutical biodegradation, the efficiency of adsorption of naproxen and diclofenac by the electrospun materials was examined. For this purpose, 50 mg of fibers with adsorbed and immobilized laccase were subjected to thermal deactivation (4 h, 80 °C) and placed in 10 mL of DCF and NPR solutions at concentration 1 mg L⁻¹ at pH 5 and 3 respectively. Adsorption experiments were carried out for 24 h, and the quantity of the pharmaceuticals adsorbed onto the electrospun materials was evaluated using chromatographic techniques.

The effect of pH on the biodegradation efficiency was evaluated using solutions at concentration 1 mg L⁻¹ at 25 °C over the pH range 3–7. To adjust the pH, a buffer solution with the desired pH was used. The effect of temperature on the removal efficiency was

evaluated at temperatures of 5, 25 and 50 °C using diclofenac and naproxen solutions at concentration 1 mg L⁻¹ in acetate buffer at pH 5 and 3 respectively. To assess the effect of the concentration of the pharmaceutical solution on degradation efficiency, solutions at concentrations of 0.1, 1.0 and 10.0 mg L⁻¹ were used. Experiments were carried out at a temperature of 25 °C at pH 5 (diclofenac) and pH 3 (naproxen).

The reusability of the adsorbed and encapsulated laccase was tested using diclofenac and naproxen solutions at concentration 1 mg L⁻¹ in acetate buffer at pH 5 and 3 respectively, at 25 °C. After each biodegradation cycle, the immobilized enzymes were separated from the reaction mixture, washed with acetate buffer at the desired pH and placed into a fresh pharmaceutical solution. The activity of laccase in the first biodegradation cycle was defined as 100% activity. All of the above experiments were performed in triplicate, and the results are presented as average ± standard deviation.

2.6. Analytical procedures

The morphology of the PLCL fibers before and after enzyme immobilization was evaluated based on SEM photographs (EVO40, Zeiss, Germany) after gold coating (Balzers PV205P, Switzerland). Individual fibers diameters were measured from the SEM micrographs of 100 randomly selected nanofibers using Image Analysis Software (ImageJ, National Institute of Health, USA). FTIR spectra were obtained using a Bruker Vertex 70 spectrometer (Germany) in the wavenumber range 4000–400 cm⁻¹ (resolution 0.5 cm⁻¹) using anhydrous KBr for sample preparation. The amount of immobilized enzyme (mg g⁻¹) was determined using the Bradford method, by measuring the initial enzyme dosage and the concentration of the protein in the supernatant after immobilization, and considering the mass of the support material [21]. The surface composition of the PLCL fibers before and after enzyme immobilization was analyzed by means of X-ray photoelectron spectroscopy (XPS) applying a Prevac system (ESCA, Poland) with Scienta analyzer (SES2002, Sweden) and Mg K α (h ν = 1253.7 eV) excitation radiation.

2.7. Chromatographic measurements

A chromatographic system from Dionex (USA) consisting of a P580 A LPG gradient pump, an ASI-100 autosampler, an STH 585 oven and an RF 2000 fluorescence detector was used for naproxen determination. 5 μ L samples were injected into a C18 Hypersil GOLD column (150 mm x 4.6 mm I.D.; 5 μ m) with a 2.1 mm I.D. filter cartridge (0.2 μ m) (Thermo Scientific, USA). The mobile phase consisted of 75% methanol at a flow rate of 1.5 mL min⁻¹

at 35 °C. Signal responses were measured by fluorescence detection at wavelengths set to 230 nm for excitation and 355 nm for emission.

The chromatographic system UltiMate 3000 RSLC (Dionex, USA) coupled to an API 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex, USA) was used for diclofenac determination. 5 μ L samples were injected into a KinetexEvo C18 column (150 mm x 2.1 mm I.D.; 2.6 μ m) (Phenomenex, USA) maintained at 35 °C. The mobile phase employed in the analysis consisted of 0.1% HCOOH in water and acetonitrile at a flow rate of 0.3 mL min⁻¹. The following gradient was used: 0 min 70%; 2.5 min 100%; 3.0 min 100% of acetonitrile. The LC column effluent was directed to the electrospray ionization source (Turbo Ion Spray) operating in negative ion mode. The following settings were applied for the ion source and mass spectrometer: curtain gas 10 psi, nebulizer gas 40 psi, auxiliary gas 40 psi, temperature 450 °C, ion spray voltage -4500 V, declustering potential -45 V, collision gas set to medium. The dwell time for each mass transition detected in the selected reaction monitoring mode was set to 200 ms. The quantitative transition was from 294 to 250 m/z at collision energy set to -16 V, and the confirmatory transition was from 294 to 214 m/z at collision energy set to -28 V.

Identification of degradation products was performed using the above-mentioned LC-MS system. 5 μ L samples were injected into a Gemini-NX C18 column (100 mm x 2.0 mm I.D.; 3 μ m) (Phenomenex, USA) maintained at 35 °C. The mobile phase consisted of 5 mM ammonium acetate in water and methanol at flow rate 0.3 mL min⁻¹, at a gradient from 50% to 100% methanol in 4 min and then for 2 min in isocratic conditions. The electrospray ion source operated in positive and negative mode. Nitrogen was used in both the source and the mass spectrometer. The following parameters of the ESI source and mass spectrometer were used: curtain gas pressure 10 psi, nebulization gas pressure 45 psi, auxiliary gas pressure 45 psi, source temperature 450 °C, ESI voltage +/-4500 V, declustering potential -40 V. Chromatograms were collected in enhanced mass spectra mode in the range 50–900 m/z.

The linearity of the methods was tested over a wide range for all analytes. The instrumental limit of detection (LOD) and the instrumental limit of quantitation (LOQ) were calculated on the basis of the signal-to-noise (S/N) ratio, using S/N = 3 for calculation of the LOD and S/N = 10 for calculation of the LOQ. Precision and accuracy were not tested, because sample preparation included only the dilution step. Therefore, only the injection precision of the instrument is applicable in this procedure, and this was always below 1%. The values obtained for diclofenac were LOD = 0.09 (μ g L⁻¹), LOQ = 0.3 (μ g L⁻¹), linear range 0.5–1000

($\mu\text{g L}^{-1}$); the values for naproxen were LOD = 0.3 ($\mu\text{g L}^{-1}$), LOQ = 1.0 ($\mu\text{g L}^{-1}$), linear range 1–5000 ($\mu\text{g L}^{-1}$).

2.8. Toxicity tests

The acute toxicity of the untreated solutions of naproxen and diclofenac and the solutions treated with adsorbed and encapsulated laccase was evaluated at ambient temperature using *Artemia salina* as the test microorganism, according to the methodology presented by Bilal et al. [22]. Briefly, the mobility rate of 48-hour-old shrimps was measured after 48 h of incubation in untreated and enzymatically treated solutions of the pharmaceuticals. The effective concentration (EC₃₀) was then calculated. EC₃₀ is defined as the concentration of the pharmaceutical (%) at which 30% of the target population show a positive response after the exposure time. Solutions of artificial seawater and potassium permanganate were used as the negative and positive control respectively. The toxicity tests were performed in duplicate, and results are presented as average $\pm$ standard deviation.

2.9. Statistical analysis

Statistically significant differences were determined using one-way ANOVA performed in SigmaPlot 12 (Systat Software Inc., USA) using Tukey's test. Statistical significance was established at $p < 0.05$.

3. Results and discussion

3.1. Characterization of the fibers and enzyme immobilization

The PLCL electrospun materials before immobilization consisted of randomly arrayed linear and continuous fibers with diameters of around 0.5–1 μm , with a relatively uniform, folded and bead-free surface (Fig. 2a). After laccase encapsulation (Fig. 2b) the diameter of the fibers did not change significantly; however some tubercles could be observed in certain areas of the fibers and their surface was more uneven, implying effective insertion of the enzyme aggregates into the PLCL fibers. By contrast, as presented in Fig. 2c, after enzyme adsorption the total surface of the fibers became corrugated. In addition, the diameter of the fibers increased, reaching up to 2 μm , indicating that laccase was uniformly distributed on the surface of the fibers. In the study, also the average diameter of obtained fibers before and after laccase immobilization were calculated and reached 373 ± 127 nm; 469 ± 222 nm and 430 ± 143 for crude PLCL fibers, PLCL fibers with encapsulated laccase and PLCL fibers with adsorbed laccase, respectively (Fig. 2 inset).

Figure 2. SEM images of (a) crude PLCL fibers, (b) PLCL fibers with encapsulated laccase, (c) PLCL with adsorbed laccase and (d) FTIR spectra of PLCL fibers before and after laccase immobilization. Inset, the diameter distribution for each types of fibers before and after laccase immobilization.

Additionally, to investigate the functional groups present in the PLCL fibers and in the products after immobilization, FTIR spectroscopy was applied. The FTIR spectrum of the pristine fibers contains signals characteristic for the $-\text{CH}_3$ (2945 cm^{-1}), $-\text{CH}_2$ (1395 cm^{-1}) and $\text{C}-\text{O}-\text{C}$ (1185 and 1020 cm^{-1}) groups present in the structure of PLCL. A significant peak with a maximum at 1756 cm^{-1} is attributed to stretching vibrations of carbonyl groups, which are responsible for enzyme binding. As expected, when laccase was encapsulated into the electrospun fibers, peaks related to the enzyme were not observed on the FTIR spectrum. However, slight shifts of the signal maxima were observed, suggesting changes in the chemical microenvironment of the PLCL moieties after enzyme encapsulation. Comparing the FTIR spectra of pristine PLCL fibers and those with adsorbed laccase, new signals appeared at 3430 cm^{-1} and 650 cm^{-1} , corresponding to stretching vibrations of $-\text{OH}$

and -NH_2 groups and bending vibrations of C–C bonds, and thus characteristic of the enzyme structure, as shown in Fig. 2d. Moreover, compared with the spectra of PLCL and free laccase, after enzyme adsorption a shift of the signal maxima towards higher wavenumbers was observed. These indicate effective adsorption immobilization, by the formation of hydrogen bonds between carbonyl groups in the PLCL fibers and amine and hydroxyl groups in the laccase structure [23].

The structure of the PLCL crude nanofibers and immobilization of laccase were followed using the surface analysis by X-ray photoelectron spectroscopy. The surface composition of PLCL fibers before immobilization, after laccase encapsulation and after enzyme adsorption was analysed. It has been found that on the surface of PLCL nanofibers before laccase immobilization mainly C–H, C–OH and carboxylic groups (COOH) occur. Furthermore, the elemental analysis of PLCL fibers and material after laccase encapsulation gives identical result. The surface of these materials is composed of about 77% of carbon atoms and about 22% of oxygen atoms. In case of the material after enzyme adsorption apart of the above mentioned elements a distinctive though low (about 0.5 at%) signal of nitrogen was detected. Since immobilization of enzyme takes place on a surface of PLCL fibers only a presence of nitrogen atoms originating from peptide bonds is expected. Its low intensity is justified by a relatively high information depth for organic material and the fact that PLCL molecules dominates in the region of analysis. Therefore the presence of nitrogen is considered as a confirmation of successful immobilization of laccase. In the material after laccase encapsulation the outer material (PLCL fibers) screens the XPS signal from enzyme molecules inside capsules.

The high-resolution XPS N 1s spectrum from material after enzyme adsorption indicates that nitrogen atoms are in chemical environment characteristic for peptide bonds at 400.4 eV [24]. The XPS C 1s spectra of all investigated samples are shown in Fig. 3 The spectrum of PLCL fibers is presented as a grey fill and is virtually identical with a spectrum coming from encapsulated laccase (dotted line). A spectrum originating from adsorbed laccase differs in the region about 289 eV which is characteristic for carboxyl functional groups. The intensity of the peak in these region is lower than that from other samples. It indicates that part of the carboxylic groups from PLCL fibers is involved with the bonding of immobilized enzyme.

Figure 3. XPS C 1s spectra of PLCL nanofibers, PLCL nanofibers with encapsulated laccase and PLCL nanofibers with adsorbed laccase.

3.2. Characterization of products after laccase immobilization

According to the data in Table 1, the amount of encapsulated laccase (340 mg g^{-1}) was approximately 25% higher than the quantity of adsorbed laccase (292 mg g^{-1}). This is directly related to the fact that before encapsulation the laccase was dispersed in the solution used for electrospinning, and thus the total amount of the enzyme was incorporated into the fibers. Meanwhile, during adsorption immobilization, some enzyme losses were observed, resulting in lower laccase loading.

Table 1. Laccase loading and kinetic parameters of free and immobilized laccases. Standard deviation do not exceed 5%.

Laccase	Laccase loading (mg g^{-1} fibers)	K_m (mM)	V_{max} (mM/s)
free laccase	-	0.051	0.039
adsorbed laccase	292	0.143	0.033
encapsulated laccase	340	0.215	0.024

In line with expectations, the Michaelis–Menten constant (K_m) of adsorbed laccase was three times higher (0.143 mM) and K_m of encapsulated laccase was over four times higher (0.215 mM), than that of the free enzyme (0.051 mM), indicating lower substrate affinity to the enzyme after immobilization. Whereas, the maximum reaction rate (V_{max}) of both immobilized laccases was lower than that of the free enzyme. The differences in K_m and V_{max}

between the free and immobilized enzymes might be explained by an increase in diffusional limitations and lower accessibility of the enzyme's active sites after immobilization, caused by the spatial barriers of the matrix [25]. The effect of the diffusion limitations on K_m is particularly pronounced for the encapsulated enzyme, as the PLCL surrounds the enzyme molecules and reduces their availability to the substrates. Nevertheless, in our study both immobilized laccases have a V_{max} value up to 75% of that of the free laccase, indicating that they can still be considered efficient catalytic systems for the removal of pharmaceuticals. The retention of such good catalytic properties, particularly in the case of encapsulated laccase, was an unexpected finding. It might be explained by the highly porous structure of the electrospun nanofibers used that partially reduces diffusional limitations, as has been reported previously [26].

3.3. Storage stability of free and PLCL-immobilized laccase

Irrespective of the immobilization protocol used, the storage stability of both PLCL-immobilized enzymes was significantly enhanced compared with the free laccase (Fig. 4). Accordingly, encapsulated and adsorbed laccase retained respectively over 90% and around 70% of their initial activity after 30 days of storage at 4 °C. These values correspond to the inactivation constant (k_D) of 0.004 and 0.014 1/day and enzyme half-life ($t_{1/2}$) of 173.3 and 49.5 days for encapsulated and adsorbed laccase, respectively. By contrast, the free enzyme retained 60% of its initial activity after 10 days, and only around 30% after 30 days. Also noticed k_D and $t_{1/2}$ of free enzyme were significantly lower, as compared to immobilized biocatalysts and reached 0.0397 1/day and 17.5 days, indicating lower stability of the free enzyme. These results might be explained by the protection of the enzyme molecules against conformational changes by the PLCL electrospun supports. Thus, better stabilization of the three-dimensional structure of the enzyme is provided. The higher activity retention in the case of the encapsulated laccase is also related to the formation of a unique core-shell structure after laccase immobilization into PLCL fibers, which protects the enzyme molecules against deactivation caused by harsh reaction conditions [27].

Figure 4. Storage stability of laccase from *Trametes versicolor*, free, encapsulated into PLCL fibres and adsorbed onto PLCL fibres.

3.4. Biodegradation of pharmaceuticals

It is known that the yield of enzymatic reactions is strongly affected by the process conditions. For practical applications it is important to evaluate the effect of the reaction conditions and determine the most suitable parameters for diclofenac and naproxen degradation. For this reason, the degradation experiments were carried out under various pH and temperature conditions. Additionally, in view of the well-developed porous structure of the electrospun fibers, the sorption capacity of the produced materials with deactivated enzyme was evaluated. In the latter case, less than 10% of both pharmaceuticals were removed from the solution by adsorption. It was thus concluded that the remediation of anti-inflammatories is related mainly to the catalytic action of the immobilized laccase. Moreover, to evaluate the effect of immobilization on the catalytic properties of the laccase, biodegradation processes were carried out using the free enzyme. Under optimal conditions (pH 5, temperature 25 °C) the removal of naproxen and diclofenac by water-soluble laccase reached about 95%. However, under conditions deviating from the optimum, degradation efficiencies were significantly lower than those noticed for the immobilized laccase, falling below 50% for both pharmaceuticals. Moreover, the reusability of the free biocatalyst is extremely limited.

3.4.1. Effect of pH

As Figure 5 shows, the highest degradation of diclofenac, around 90%, was observed when encapsulated laccase was used in acidic conditions (pH 3 and 5). Degradation by adsorbed laccase in these conditions was lower, at around 60%. This may be explained by the fact that the adsorbed laccase is more exposed to deactivation by the process conditions, as well as

some enzyme leakage occurred, resulting in decreased biodegradation efficiency. The optimal pH range of 3 to 5 for diclofenac removal may also be related to its pK_a value (4.15). In this pH range the ratio of undissociated and dissociated molecules of diclofenac are equal; this creates the most suitable environment for the action of laccase [28]. Surprisingly, the highest rate of degradation of naproxen by the immobilized enzyme was observed at pH 3; it reached almost 100% and around 90% for encapsulated and adsorbed laccase respectively. However, even at pH 5, PLCL-laccase systems were able to degrade over 80% of the pollutant. The shift of the optimum pH and significantly higher rates of biodegradation by the adsorbed enzyme, compared with the case of diclofenac, are probably related to the presence of the mediator (ABTS in this study) in the reaction mixture, which slightly altered the microenvironment around the active sites of the enzyme and suppressed the laccase deactivation process [29]. The presence of the mediator is required to achieve satisfactory rates of reaction, as it is known that naproxen is insufficiently oxidized by the laccase and is resistant to enzymatic degradation [20]. The significant drop in the rates of degradation of both anti-inflammatory drugs at pH 7 is related to the formation of hydroxide ions, which can bind to the T2/T3 copper ions in the laccase structure. This causes the internal transfer of electrons from T1 to T2/T3 to be blocked, so that the reduction potential of oxygen is decreased and in consequence the reaction efficiency is reduced [30].

Figure 5. Effect of solution pH on efficiencies of removal of: (a) diclofenac and (b) naproxen by encapsulated and adsorbed laccase. Degradation conditions: temperature 25 °C, pharmaceutical concentration 1.0 mg L⁻¹.

Nevertheless, it should be emphasized that both immobilized laccases exhibited higher removal rates at strongly acidic (pH 3) and even neutral pH, compared with the free enzyme (data not presented), indicating the lower sensitivity of the bound enzymes to changes in pH.

This is probably due to the rigidification of the enzyme structure after immobilization and its better protection against conformational changes and chemical deactivation [31].

3.4.2. Effect of temperature

As it can be seen in Fig. 6, degradation of naproxen and diclofenac increased with an increase in temperature, reaching a maximum at 25 °C (92% and 99% respectively for encapsulated laccase, 62% and 87% for adsorbed laccase), and decreasing slightly at 50 °C. On the one hand, the lower degradation rates of both pharmaceuticals observed at 5 °C are related to inhibition of the enzyme and its insufficient activation. On the other hand, the high removal efficiencies observed even at 50 °C indicate that the negative effect of high temperature on the catalytic properties of the immobilized enzyme was strongly reduced. This might be related to the stabilization of the laccase structure after immobilization that prevent against conformational changes, as well as to the protective effect of the PLCL electrospun fibers. High degradation efficiencies at higher temperatures are also related to the better solubility and mobility of the pharmaceuticals at elevated temperatures, as well as reduced diffusional limitations of the substrate molecules [32]. However, it should be noted that the temperature of real effluents containing pharmaceuticals is usually in the range 15–35 °C [33]; thus, the high degradation efficiencies observed at 25 and 50 °C indicate that the resistance of the enzyme to thermal deactivation was improved after immobilization and suggesting the possible practical application of the produced biocatalytic systems for wastewater treatment.

Figure 6. Effect of process temperature on the efficiency of removal of: (a) diclofenac and (b) naproxen by encapsulated and adsorbed laccase. Degradation conditions: pharmaceutical concentration 1.0 mg L⁻¹, pH 5 and 3 respectively for diclofenac and naproxen.

3.4.3. Effect of pharmaceutical solutions concentration

After determining the optimal pH and temperature conditions for the remediation processes (temperature 25 °C, pH 5 and 3 for diclofenac and naproxen respectively), we also examined how the concentration of the anti-inflammatory drugs affected the efficiency of their remediation (Fig. 7). Although the concentration of both compounds in municipal and pharmaceutical wastewaters is usually at levels of $\mu\text{g L}^{-1}$ [34] we decided to test higher concentrations to determine whether the produced biocatalytic systems offer good potential for the bioconversion of hazardous pollutants from wastewaters. As expected, the higher is the concentration of the pharmaceutical, the lower is the degradation efficiency. This might be explained by two main factors: (i) insufficient amount of the immobilized enzyme and (ii) inhibition of the laccase active sites by substrates molecules, observed at higher concentrations of pharmaceutical. Similarly as in the previous experiments, in case of both analyzed compounds, encapsulated laccase is characterized by significantly higher biodegradation ability. However, less significant drop of the removal rates was observed during degradation of diclofenac (80% of diclofenac removal from 10 mg L^{-1} solution), compared to naproxen conversion, that is directly related to the presence of the mediator (ABTS) in the reaction mixture, which generates radical species. These radical species enhance biodegradation rate by the increasing of the laccase redox potential and reduce steric hindrances [35].

Figure 7. Effect of initial concentration of (a) diclofenac and (b) naproxen solution on the efficiency of their removal by encapsulated and adsorbed laccase. Degradation conditions: temperature 25 °C, pH 5 and 3 respectively for diclofenac and naproxen.

3.5. Operational stability of the immobilized enzymes

Figure 8. Reusability of the encapsulated and adsorbed laccase for degradation of (a) diclofenac and (b) naproxen over five removal cycles. Degradation conditions: temperature 25 °C, pharmaceutical concentration 1.0 mg L⁻¹, pH 5 and 3 respectively for diclofenac and naproxen.

Reusability is the main advantage of immobilized enzymes, and is particularly important for practical applications. Hence, the operational stability of the encapsulated and adsorbed laccase was determined over five successive cycles of diclofenac and naproxen degradation (Fig. 8). The degradation of diclofenac and naproxen by adsorbed laccase followed the same trend, reaching approximately 20% and 40% respectively in the fifth reaction cycle. The sharp drop in the removal efficiency of the adsorbed enzyme is caused by laccase leakage (about 50% of the enzyme). As expected, encapsulated laccase demonstrated significantly higher reusability, enabling the removal of over 60% of diclofenac and 70% of naproxen in the fifth cycle. This is directly related to the effect of the core-shell structure of the PLCL nanofibers, which protects enzyme molecules against conformational changes caused by the external environment. Nevertheless, the activity of both immobilized laccases may also decline due to thermal and chemical denaturation and deactivation and due to partial inhibition of the biomolecules by the biodegradation products, as previously mentioned.

3.6. Products of biodegradation and reaction pathways

During the study, the degradation products of diclofenac and naproxen were identified by analyzing the mixture obtained after the process that resulted in the highest efficiency of degradation. Characteristic ions of diclofenac were found at a retention time of 2.6 min, namely the pseudomolecular ion (at $m/z = 296$) due to its incomplete conversion, and its

daughter ions formed after elimination of the water molecule (at $m/z = 278$), the carbon monoxide molecule (at $m/z = 250$) and the chlorine radical (at $m/z = 215$). Two degradation products were identified at retention times of 3.6 and 4.0 min (Fig. 9a). The first was formed after elimination of the water molecule from diclofenac, and the pseudomolecular ion of this compound (found at $m/z = 278$) has the same structure as the first fragmentation ion of diclofenac. Further fragmentation ions were not found due to the low concentration of this degradation product and the rapid formation of the second bioconversion product, whose structure was confirmed by the presence of the pseudomolecular ion (at $m/z = 284$) and two abundant daughter ions formed after elimination of the hydrogen peroxide molecule (at $m/z = 250$) and the chlorine radical (at $m/z = 215$). Surprisingly, results show that hydroxy- and dihydroxydiclofenac (products of enzymatic conversion of diclofenac) were not detected, indicating the transformation of diclofenac into non-detected metabolites, as presented also earlier [8,36].

In the second test, apart from the naproxen peak at retention time 2.2 min, a number of other compounds were found and identified (Fig. 9b). The degradation process of naproxen led to the elimination of the formic acid molecule from the carboxylic group of naproxen. As a result three different degradation products were found: two formed immediately and one after oxidation of the carbon atom adjacent to the aromatic ring. For the first two compounds detected at retention times 2.1 and 3.3 min, only the pseudomolecular ion at $m/z = 185$ was found, due to their relatively stable nature under soft electrospray ionization. The first of these compounds had a similar retention time to naproxen and the same m/z value as the daughter ion of naproxen. It was identified as a degradation product because during the test the pseudomolecular ion of naproxen (at $m/z = 231$) completely disappeared. The initially determined ratio of the ions at $m/z = 231$ to those at $m/z = 185$, equal to 3:4, is characteristic of naproxen in the given LC-MS system and the conditions of the analytical method. The disappearance of the first peak with simultaneous increase in the second is evidence of the formation of a new product found at a similar retention time. Unfortunately, due to lack of further fragmentation, the compounds eluted at retention times 2.1 and 3.3 min could not be distinguished from each other and assigned reliably to peaks. The third degradation product was found at retention time 2.6 min. The pseudomolecular ion of this compound found at $m/z = 201$ was decomposed by the loss of the ketene molecule, leading to an ion at $m/z = 159$. Thus, 1-(6-metoxynaphtalen-2-yl)ethanone was determined to be the main degradation product formed in the presence of laccase in our study. Moreover, as the test with naproxen was performed with the ABTS mediator, this compound was also present in the

chromatogram. More interestingly, there were two ions found which may be attributed to conjugation products of the naproxen molecule with the ABTS molecule and a large part of that molecule. These two ions were found at retention times 1.4 min (at $m/z = 715$) and 1.8 min (at $m/z = 458$) respectively. These conjugation products of naproxen and ABTS mediator have not previously been reported in literature. In the course of the degradation, only the first can potentially be transformed back to ABTS. The formation of the second results in an inevitable loss of ABTS and a potentially lower rate of degradation of naproxen due to the lower concentration of the mediator.

(b)

Figure 9. Extracted ion chromatogram after 6 h of biodegradation by encapsulated laccase of (a) diclofenac and (b) naproxen. The structures of ions found at retention times (a) 2.6, 3.6 and 4.0 min after diclofenac degradation and (b) 1.4, 1.8, 2.1, 2.6 and 3.3 min after naproxen degradation are given below the chromatogram.

3.7. Toxicity study

One of the most important objectives in research related to the removal of pollutants is to reduce the toxicity of the effluent after treatment. Thus, ecotoxicity studies against *A. salina* were carried out, to evaluate changes in the toxicity of the untreated and enzymatically treated solutions of both pharmaceuticals. For this purpose, mixtures produced after 24 h of biodegradation under optimal process conditions, were examined. The results are presented in terms of EC_{30} (%), the concentration of the pollutant at which 30% of the microorganisms showed a positive response after the exposure time (Fig. 10).

The untreated diclofenac and naproxen solutions have EC_{30} values of around 25% and 20%, indicating the high toxicity of both pharmaceutical solutions. After enzymatic treatment by both biocatalytic systems, the toxicity was substantially reduced, with EC_{30} ranging from 60% to around 85%. However, the use of encapsulated laccase led to higher EC_{30} values (79.6% and 84.7% for diclofenac and naproxen respectively) than in the case of the adsorbed enzyme. This suggests the lower toxicity of the effluent, and is consistent with the results

obtained for biodegradation efficiency, which showed the removal rate to be higher when the encapsulated biocatalyst was used. The decrease in the toxicity of the tested solutions may be explained by the elimination of both anti-inflammatories from the mixture by way of enzymatic conversion [37]. It has been shown that both native laccase and laccase-containing fungal strains may be used for the removal of hazardous pharmaceuticals from wastewaters; however, monitoring of the toxicity of the effluents is key to determining the practical application of the evaluated methods, as intermediates and/or final products might be even more toxic than the initial compounds [38]. Nevertheless, the higher values of EC_{30} obtained suggest that the products of biodegradation are in fact less toxic than the initial solutions of the tested pharmaceuticals. Thus, our findings show that the use of laccase immobilized on electrospun PLCL nanofibers is a very promising choice for the effective biodegradation and detoxification of naproxen and diclofenac from water solutions, and may lead to practical applications.

Figure 10. Toxicity study of untreated solutions of naproxen and diclofenac and solutions treated with immobilized laccase, using the *A. salina* test. $EC_{30} \geq 100\%$ = nontoxic.

4. Conclusions

The main obstacles to the widespread implementation of laccase-based systems for biodegradation of hazardous pollutants, including various pharmaceutical compounds, are the cost of the enzyme and its poor reusability. Thus, in the present study, we have demonstrated a proof-of-concept for the use of PLCL electrospun fibers as a support material for the adsorption and encapsulation of laccase, an enzyme of enormous environmental potential, and an application of the obtained biocatalytic systems in the removal of naproxen and diclofenac from water solutions. After 24 h of the process, encapsulated laccase biodegraded over 90% of naproxen and diclofenac under optimal process conditions, namely a temperature of 25 °C

and pH of 5 and 3 respectively, from a solution at an environmentally relevant concentration (1 mg L^{-1}). The efficiency of removal of both compounds by the adsorbed enzyme was lower, mainly due to deactivation of the enzyme and its elution from the support. The obtained biocatalytic systems exhibited excellent operational stability: even after five consecutive degradation cycles the encapsulated laccase removed over 60% of the pollutants, which is of particular interest for practical applications. Furthermore, the storage stability of the immobilized laccase was significantly better than that of the free enzyme. Finally, we identified products of the enzymatic conversion of both anti-inflammatories, and showed them to be substantially less toxic than the parent compound. It should be added that the metabolites formed might be easily removed from water solutions using simple physical processes, such as precipitation. In summary, the presented results illustrate the excellent potential of PLCL-immobilized laccase for the removal of pharmaceuticals from aqueous solutions, and indicate the possibility of its practical application in real wastewater treatment on an industrial scale. However, further studies in this area are still needed.

Acknowledgements: This research was funded by Ministry of Science and Higher Education (Poland) as financial subsidy to PUT.

Declarations of interest: none.

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Graphical abstract

Highlights:

- Use of electrospun PLCL nanofibers as effective support for laccase immobilization.
- Application of PLCL-laccase systems for removal of diclofenac and naproxen.
- Evaluation of optimal biodegradation conditions for the highest removal efficiency.
- Improvement of storage stability and reusability of immobilized laccase.
- Decrease in the toxicity of the solutions after biodegradation.