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Zdarta, Jakub; Sigurdardóttir, Sigyn Björk; Jankowska, Katarzyna; Pinelo, Manuel

Published in:
Chemosphere

Link to article, DOI:
10.1016/j.chemosphere.2022.135374

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Laccase immobilization in polyelectrolyte multilayer membranes for 17α-ethynylestradiol removal: Biocatalytic approach for pharmaceuticals degradation

Jakub Zdarta a, b, *, Sigyn Björk Sigurdardóttir a, Katarzyna Jankowska a, Manuel Pinelo a

a Process and Systems Engineering Centre, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 229, 2800 Kgs Lyngby, Denmark
b Institute of Chemical Technology and Engineering, Faculty of Chemical Technology, Poznan University of Technology, 60965, Poznan, Poland

ARTICLE INFO
Handling editor: Derek Muir
Keywords:
17α-ethynylestradiol
Polyelectrolyte multilayer membranes
Layer-by-layer
Laccase
Enzyme immobilization
Biodegradation

ABSTRACT
Enzymatic membrane reactors equipped with multifunctional biocatalytic membranes are promising and sustainable alternatives for removal of micropollutants, including steroid estrogens, under mild conditions. Thus, in this study an effort was made to produce novel multifunctional biocatalytic polyelectrolyte multilayer membranes via polyelectrolyte layer-by-layer assembly with laccase enzyme immobilized between or into polyelectrolyte layers. In this study, multifunctional biocatalytic membranes are considered as systems composed of commercially available filtration membrane modified by polyelectrolytes and immobilized enzymes, which are produced for complex treatment of water pollutants. The multifunctionality of the proposed systems is related to the fact that these membranes are capable of micropollutants removal via simultaneous catalytic conversion, membrane adsorption and membrane rejection making remediation process more complex, however, also more efficient.

Briefly, cationic poly-L-lysine and polyethylenimine as well as anionic poly(sodium 4-styrenesulfonate) polyelectrolytes were deposited onto NP010 nanofiltration and UFX5 ultrafiltration membranes to produce systems for removal of 17α-ethynylestradiol. Images from scanning electron microscopy confirm effective enzyme deposition, whereas results of zeta potential measurements indicate introduction of positive charge onto the membranes. Based on preliminary results, four membranes with over 70%, activity retention produced using polyethylenimine in internal and entrapped mode, were selected for degradation tests. Systems based on UFX5
membrane allowed over 60% 17α-ethynylestradiol removal within 100 min, whereas NP010-based systems removed over 75% of estrogen within 150 min. Further, around 80% removal of 17α-ethynylestradiol was possible from the solutions at concentration up to 0.1 mg/L at pH ranging from 4 to 6 and at the pressure up to 3 bar, indicating high activity of the immobilized laccase over wide range of process conditions. Produced systems exhibited also great long-term stability followed by limited enzyme elution from the membrane. Finally, removal of over 70% and 60% of 17α-ethynylestradiol, respectively by NP010 and UFX5 systems after 8 cycles of repeated use indicate high reusability potential of the systems and suggest their practical application in removal of micropollutants, including estrogens.

1. Introduction

Estrogens are one of the most common pharmaceutical micropollutants in wastewaters, that is mainly due to their widespread use in form of oral contraceptives and their excretion by human (Wedekind, 2014). The most commonly used estrogen in birth control pills is synthetic 17α-ethynylestradiol (EE2), whose influent concentration to municipal wastewater treatment plants can reach even 40 ng/L, which in surface waters equals 20 ng/L (Tang et al., 2021). Although this estrogen has a specific role in regulation of endocrine system action, even a trace amount of EE2 in wastewaters is undesirable. As was shown, EE2 can cause a delay of sexual maturity and decrease the secondary sexual characteristics of aqueous organisms, such as freshwater fish Medaka (Oryzias latipes), three-spined stickleback (Gasterosteus aculeatus) or juvenile Atlantic salmon (Salmo salar) (Aris et al., 2014). Moreover, it was also observed that the long-term exposure of human to EE2 can reduce fertility (Laurenson et al., 2014). Therefore, the removal of this estrogen from waters is one of the most important challenges for communities.

A promising method of EE2 removal from aqueous solutions is bioconversion using enzymes, such as laccase. This oxidoreductase oxidizes phenolic compounds to usually less hazardous reaction products (Chen et al., 2019), with relatively high activities (Sun et al., 2021). Moreover, the enzymatic degradation of pharmaceutical pollutants from waters is ecofriendly and it allows to remove even trace amount of such compounds, compared to other methods commonly applied in wastewater treatment plants (Alneyadi et al., 2018). However, to use enzymes repeatedly with high catalytic activity retention, their immobilization onto stable support material is required. This kind of stabilization of enzymes is often used, for example using conventional immobilization by adsorption, covalent binding or encapsulation by applying support materials of various origin (Zucca and Sanjust, 2014; Wang et al., 2020). Among various support materials for oxidoreductase immobilization, of particular interest should be inorganic support materials, mainly due to their stability and the presence of reactive functional groups (Jesionowski et al., 2014). Further, nowadays, electrospun nanofibrous polymeric materials are considered as the most appropriate support for enzyme immobilization due to their well-developed specific surface area, inter-fiber porosity, and limited mass transfer resistance (Sathishkumar et al., 2012, 2014). Recently, however, more and more attention is paid to enzyme immobilization using layer-by-layer (LbL) assembly approach on membrane substrates, that allows to form enzyme layers with controllable density and amount of immobilized biomolecules by covalent binding and hydrogen bonds or via electrostatic interactions (Zhang et al., 2020). Moreover, the compartmentalization of enzymes within polyelectrolyte (PE) layers using the LbL approach could increase their activities due to the possibility of creating optimal conditions between specific layers of enzymes, limitation of contact with external environment and very limited blocking effect on biomolecules’ active sites caused by sticking of enzymes together. What is more, due to high-temperature tolerances of polyelectrolyte layers, they could play a role as a heat adsorber and therefore the LbL approach can reduce the negative effect of temperature on immobilized enzymes (Lee, 2016; Liu et al., 2020). It should be also noted that this type of immobilization can be effectively used for a wide variety of enzymes and supports in the form of membrane, making it possible to carry out the bioconversion processes in fed-batch or continuous reactors, which are usually used for wastewater treatment. These facts cause that this kind of highly active polyelectrolyte multilayer (PEM) biomembranes can be effectively used for degradation of various pollutants from aqueous solutions. As presented by Sarma et al. (2017), a PEM membrane with immobilized laccase was able to convert 2,4,6-trochlorophenol to dichloro-1, 4-benzoquinone with 80% efficiency. Similarly, Li et al. (2020) used a nanofiltration membrane as a support for laccase that was immobilized between poly(styrene sulfonate) and poly(allylamine hydrochloride) layers and applied for the removal of bisphenol from aqueous solution. In the case of disadvantages of LbL approach for enzyme immobilization, the fouling of the active side of polyelectrolyte multilayer biomembranes could appear. It could be especially caused by the accumulation of substrates and products of bioconversion on the active side of biosystem. Nevertheless, an area of study on enzymes immobilization using LbL assembly on membranes to form multilayer biomembranes for removal of micropollutants is still neglected and requires further research.

Therefore, in this study we have produced novel multifunctional biocatalytic PEM membranes via PE LbL assembly, using poly-γ-lysine (PLL) and polyethyleneimine (PEI) as cationic PEs and poly(sodium 4-styrenesulfonate) (PSS) as anionic PE. We proposed two methods of laccase immobilization in the PEM membranes for the fabrication of biocatalytic PEM membranes, namely with laccase immobilized between PE layers and with laccase entrapped within the cationic PE layers. We verified the applicability of the biocatalytic PEM membranes for the removal of EE2 as a micropollutant at the highest concern. Although some studies reported on use of LbL approach for laccase immobilization, to the best of our knowledge, in this manuscript we present the proof-of-concept for production and application of PEM membranes for removal of estrogens in enzymatic membranes reactor. Thorough physicochemical, morphological and catalytic characteristic of the produced membranes were performed in order to select the most suitable membranes for EE2 removal, which in the next part of the study were assessed in terms of their efficiency in removal of estrogen under various process conditions. Further, stability and reusability of the produced systems were also analyzed as crucial parameters from practical application point of view. It should be highlighted that the presented approach might open a new research area that facilitates practical application of enzymatic membrane reactors for removal of selected micropollutants in an eco-friendly and sustainable way.

2. Materials and methods

2.1. Materials

A TriSep NP010 nanofiltration membrane at molecular weight cut-off (MWCO) ~1000 Da with poly(piperazinamide) skin layer and UFX5 ultrafiltration membrane at MWCO ~5000 Da with polysulfone skin layer were delivered by Sterlitech Company (USA). Polyethyleneimine, poly(sodium 4-styrenesulfonate), sodium chloride, Bradford reagent, laccase from Trametes versicolor (EC 1.10.3.2, ≥0.5 U/mg), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 99%), 17α-ethynylestradiol (≥98%) were delivered by Sigma-Aldrich (USA). Poly-γ-lysine was obtained from Alamanda Polymers (USA). Buffer...
solution, including 50 mM acetate buffer at pH 3–5, 50 mM phosphate buffer at pH 6–8 and 50 mM Tris-HCl at pH 8 and 9 were freshly prepared. All of the reagents were of analytical grade and were used without further purification. All experiments related to water permeability tests and removal of EE2 performed within the frame of the study were conducted using Amicon 8050 stirred cell with active area of 13.4 cm² delivered from Millipore (USA) which allowed high precision at low pressure.

2.2. Preparation of biocatalytic PEM membranes

The biocatalytic PEM membranes produced in this study were prepared using PE LbL assembly on commercial nanofiltration and ultrafiltration membrane substrates, using cationic and anionic polyelectrolytes. Membranes at various characteristic and different MWCO were tested to examine how the pore size and water permeability of the substrate membrane affect final properties of the biocatalytic membranes, including their transfer limitations, enzyme activity recovery, process duration as well as retention of the micropollutants. Cationic and anionic PE with various functional groups were tested in order to identify compounds capable for binding of significant amount of the enzyme as well as providing suitable microenvironment for immobilized enzyme. Finally, two approaches were proposed for laccase immobilization (Fig. 1). In the first approach, laccase, as a negatively charged molecule at pH 5, was dissolved in acetate buffer at pH 5 and deposited onto the preceding cationic polyelectrolyte layer. In this method laccase was deposited between PE layers (internal mode membranes). In the second approach, laccase was previously dissolved in the cationic PE solution and then deposited onto the preceding anionic PE layer together with the cationic polyelectrolyte. In this mode laccase was entrapped within cationic PE layer (entrapped mode membranes). Various methods of enzyme deposition were tested, as this factor affect both, activity and stability of the immobilized laccase. It is expected, that in the first approach laccase deposited between PE layers will retain higher activity but shows lower resistance to harsh process conditions. By contrast, in the second approach enzyme is surrounded by the PE, that leads to formation of diffusional limitations and lower activity retention. However, in this approach biomolecules are less exposed to the direct contact with reaction mixture, thus are better protected against inactivation. Summarizing, various laccase immobilization approaches were presented in order to select the most suitable immobilization mode and conditions to produce highly active and stable biocatalytic PEM membranes. The membranes were produced in the Amicon 8050 cell with 150 rpm mixing during each deposition step and without pressure addition. To fabricate membranes, 8 mM solutions of PEI, PLL and PSS were obtained by dissolving a certain amount of each PE in deionized water with 0.2 M NaCl as the background ionic strength. 0.2 M NaCl solution was also used as a rinsing solution to remove unbounded PE and/or enzyme. Membrane rising consist of 5 min membrane washing, repeated twice, after each polyelectrolyte or laccase deposition step. To obtain membranes with enzyme between PE layers, 5 mg/mL laccase solution in acetate buffer at pH 5 was used, whereas to obtained membranes with enzyme entrapped, laccase was dissolved in the respective cationic PE solution at 5 mg/mL.

Prior to PEs deposition onto the membranes surface, the membranes were immersed in 50% isopropanol for 20 min, followed by rinsing with deionized water. The pretreated membrane was then placed in an Amicon 8050 cell with the skin layer facing up. To produce biocatalytic membranes with laccase immobilized between PE layers, 10 mL of a PEI/PLL solution was deposited onto the membrane for 10 min followed by PSS deposition for 10 min from 10 mL solution. After these two steps, 10 mL of PEI/PLL solution was placed onto membrane for 10 min followed by 10 mL laccase solution deposition for 2 h. The PE/laccase deposition was repeated three times to finally obtain biocatalytic PEM membranes consisting of 8 layers. In turn, to obtain biocatalytic membranes with enzyme entrapped into PE layer at first PLL/PEI was deposited for 10 min from 10 mL solution onto pristine membrane. Next, 10 mL of PSS solution was placed for 10 min followed by deposition of laccase-PEI/PLL solution for 30 min. These two steps were then repeated three times to produce PEM membrane made of 7 layers. The membranes were then placed in deionized water and stored at 4 °C prior to further use. Before the water permeability and removal experiments, the membranes were thoroughly rinsed with deionized water.
2.3. Membranes characterisation

2.3.1. Physicochemical characterisation of the biocatalytic PEM membranes

To characterize pristine membranes and the produced biocatalytic PEM membranes, their water permeability ($I_p$), permeate flux ($J_p$) and water permeability loss were calculated under a pressure of 4 bar in the Amicon 8050 cell using deionized water according to Eq. (1) and Eq. (2), respectively.

$$L_p = \frac{J_p}{\Delta P}$$

(1)

$$J_p = \frac{V_p}{t \cdot A_m}$$

(2)

where $\Delta P$ denotes transmembrane pressure, $V_p$ denotes permeate volume after a certain time and $A_m$ denotes the effective filtration area.

To characterize changes in membranes hydrophilicity, a water contact angle of the pristine membranes and biocatalytic PEM membranes was determined. For this purpose, a Drop Shape Analysis System DSA100E (Krüss GmbH, Germany) was applied. Measurements were performed at ambient conditions by carefully dropping 1 μL of deionized water on the surface of each membrane and recording the contact angle between the water and membrane surface for 120 s. To minimize experimental error, each measurement was repeated five times on random location of each sample. Results are presented as a mean value, for which error values do not exceed 5%.

Membrane surface charge was determined using a SurPASS 3 electrokinetic analyzer (Anton Paar, Austria) in order to examine zeta potential of the tested membranes. Prior to measurements, membranes were soaked in 1 mM KCl. Next, the zeta potential measurements were performed using 1 mM KCl as a background electrolyte over the pH range from pH 4 to pH 10. The pH of the solution was adjusted by an automatic titrator. For each pH measure point, four data were recorded, and the mean values is presented. The error values for zeta potential measurements do not exceed 2%.

The morphology of the produced biocatalytic PEM membranes was determined based on scanning electron microscopy (SEM) images made on EVO40 microscope (Zeiss, Germany) 50 s after gold coating (Balzers PV205P, Switzerland).

2.3.2. Biocatalytic characterisation of the biocatalytic PEM membranes

The amount of immobilized enzyme and enzyme loading in biocatalytic PEM membranes was calculated based on spectrophotometric measurements using Jasco V-750 spectrophotometer (Japan) at 595 nm according to the Bradford method (Bradford, 1976). The total amount of the immobilized enzyme ($IM_e$) was obtained by considering the differences in the initial amount of enzyme in the immobilization solution and the amount of the enzyme in solutions after LbL deposition, and in NaCl rising residuals (Eq. (3)). To measure the absorbance, 1 mL of the enzyme solution was mixed with 1 mL of Bradford reagent and was left for 5 min.

$$IM_e = E_t \cdot V_t - E_{LbL} \cdot V_{LbL} - E_W \cdot V_W$$

(3)

where $E_t$ and $V_t$ denote initial enzyme solution concentration and its volume, $E_{LbL}$ and $E_W$ denote enzyme concentration in solutions after LbL deposition and in rising residuals, respectively, $V_{LbL}$ and $V_W$ denote volumes of solutions after LbL deposition and rising residuals.

The activity of free enzyme and laccase immobilized in biocatalytic PEM membranes was calculated based on spectrophotometric measurements using Jasco V-750 spectrophotometer (Japan) at 420 nm during continuous monitoring of the oxidation reaction of ABTS. The produced membranes were loaded into Amicon 8050 cell to which 10 mL of 5 mM ABTS solution at pH 5 was added. The process was performed in darkness (aluminum foil cover) at 25 °C with 200 rpm mixing. Reactions with free enzyme were performed at the same process conditions with respective amount of the free laccase in batch system. Membrane activity (U/mg) was calculated based on the obtained absorbance data using molar extinction coefficient of ABTS (36 1/mM-cm) and cuvette length (1 cm) and also considering membrane active area. One unit (U) of enzyme activity was defined as the amount of laccase needed to oxidize 1 mmol of ABTS per minute. The activity of free enzyme was defined as 100% relative activity. Based on this value, the activity retention of immobilized laccase was defined.

The Michaelis–Menten constant ($K_M$) and the maximum rate of reaction ($V_{max}$), of the free and immobilized laccase were calculated based on the above-mentioned model reaction under optimum assay conditions using substrate solution as a substrate under concentrations ranging from 0.01 to 1 mM. The apparent kinetic parameters were obtained using the Hanes–Woolf plot by fitting of obtained data. All of the above-mentioned experiments were performed in triplicate and the results are presented as a mean value, for which error values do not exceed 5%.

2.4. Removal study of 17α-ethynylestradiol

2.4.1. EE2 removal experiments

Prior to removal experiments, the sorption properties of the produced membranes were tested. For this reason, membranes with thermally inactivated laccase (3 h, 80 °C) were placed in an Amicon 8050 cell and 20 mL of EE2 solution at 0.1 mg/L concentration and pH 5 was added. Process was performed at 2 bars and 25 °C with mixing at 200 rpm. The process was ended when the total volume of the EE2 solution was passed through the membrane.

The biocatalytic PEM membranes selected after preliminary studies were next tested in EE2 removal experiments. In the first stage of this investigation, EE2 removal efficiency over time was examined. For this reason, PEM membranes were placed in an Amicon 8050 cell and 20 mL of EE2 solution at 0.1 mg/L concentration and pH 5 was added to the reactor vessel. The process was performed at 2 bar and 25 °C with mixing at 200 rpm. The process was ended when the total volume of the EE2 solution was passed through the membrane, thus process duration required for treatment of whole volume of the EE2 solution differ for each of the biocatalytic PEM membrane tested. During the experiment, the feed mixture was sampled every specified period and samples were subjected to GS-MS analysis.

In the next part of the study, the effect of various process conditions (EE2 concentration, pH and pressure) on the removal efficiency of EE2 by biocatalytic PEM membranes was determined. The effect of pH on the EE2 removal efficiency was determined over the pH ranging from 3 to 9 (using buffer solution at desired pH) at a pressure of 2 bar using 20 mL EE2 solution at concentration of 0.1 mg/L. The effect of EE2 concentration on its removal rate was examined at 2 bar using 20 mL of EE2 solutions at concentrations of 0.01, 0.1, 0.5 and 1.0 at pH 5. The effect of applied pressure EE2 degradation rate was examined using 20 mL of 0.1 mg/L EE2 solution at pH 5 by applying pressure of 1, 2, 3 and 4 bar. In all removal experiments, oxygen was used as driving force, as oxygen is a laccase cosubstrate required to perform oxidation reaction. The process was ended when the total volume of the EE2 solution was passed through the membrane and the samples were subjected to GC-MS analysis to determine final EE2 concentration. The final EE2 removal rate (%) was calculated according to Eq. (4).

$$EE2 \text{ removal rate} (%) = \frac{C_0 - C_t}{C_0} \times 100\%$$

(4)

where $C_0$ and $C_t$ denote initial EE2 concentration and EE2 concentration in permeate after a specified period, respectively.

To measure the degradation efficiency of EE2, gas chromatography analysis was used. The samples were lyophilized (Alpha 1.4 LD plus,
Christ, Germany) for 96 h at the pressure of 0.36 mbar and −30 °C. The residue was then resuspended in 100 μL DME and transferred to chromatography vials. The samples were derivatized by adding 100 μL of BSTFA+1% TMCS and then heated at 65 °C for 2 h. The samples were further subjected to chromatographic analysis. Quantitative analysis of the tested compounds was carried out on a Pegasus 4D gas chromatograph (Leco, USA) with a BPX-5 column (28 m × 250 μm × 0.25 μm) with helium as the carrier gas. The sample (1 μL) was dispensed on the inlet at a temperature of 250 °C. The chromatograph was operated in programmed temperature increase mode: 80 °C for 1 min, increasing by 20 °C/min to 200 °C, then increasing by 8 °C/min to 280 °C and maintaining the final temperature for 3 min. The chromatograph was coupled to a mass spectrometer (TOF-MS), which conducted analyses of the eluent from the column using an ion source operating in positive ion mode. The temperature of the ion source was 250 °C and the energy was 70 eV. Quantitative analyses were conducted for ions of masses 73, 196, 232, 285 and 425 for EE2 based on standard solutions. Chroma TOF-GC v4.51.6.0 software was used for the data analysis.

2.4.2. Stability and reusability of the biocatalytic PEM membranes

Storage stability of the free enzyme and laccase immobilized in biocatalytic PEM membranes was determined over 20 days of storage in 50 mM acetate buffer at pH 5 at 4 °C based on model catalytic oxidation of ABTS as a substrate performed according to methodology described in Section 2.3.2. The relative activity was calculated based on spectrophotometric measurements and initial activity of immobilized laccase was determined as 100%.

Reusability of the produced PEM membranes was determined over 8 repeated cycles of EE2 degradation. Each of the removal step was carried out under optimal process conditions (temperature 25 °C, pH 5 and EE2 concentration 0.1 mg/L) according to the methodology described in Section 2.4.1. After each removal cycle, the membranes were washed with acetate buffer at pH 5 after which fresh EE2 solution was added.

During the storage stability and reusability study, the enzyme loading in the biocatalytic membranes was determined. For this reason, solutions obtained after each test were analyzed according to Bradford method (Section 2.3.2.) and enzyme concentrations was calculated. Based on these results, enzyme loading in the biocatalytic membranes was examined. All of the above-mentioned experiments were performed in triplicate and the results are presented as a mean value, for which error values do not exceed 5%.

3. Results and discussion

3.1. Physicochemical characterization of the PEM biocatalytic membranes

It is expected that during LbL coating of membrane surface and deposition of the enzyme biomolecules, changes in the structure and morphology of the membranes will occur. In Fig. 2, SEM images showing the microstructure of the pristine NP010 and UFX5 membranes and the final biocatalytic PEM membranes are presented. It can be seen that both unmodified membranes (Fig. 2(a) and (f)) are characterized by compact and uniform surfaces with limited number of microdefects. However, the surface of the NP010 nanofiltration membrane seems to be rougher and possess more irregular topography. After PE deposition and enzyme immobilization between PE layers (Fig. 2 (b, c) and (g, h)) substantial changes occurred. The surface become smoother and irregular shapes at size of 2-5 μm can be seen for each membrane. These shapes might be interpreted as laccase aggregates that were deposited onto the membrane surface as a final layer during membrane fabrication. Their presence confirms effective deposition of laccase molecules that have a direct contact with the substrate solution, as reported also earlier (Jankowska et al., 2020). By contrast, when enzyme was entrapped into PE layers, less pronounced changes in membrane morphology can be observed. The surface of membranes produced by this approach is more corrugated and more densely packed, as compared to the pristine material. However, due to enzyme entrapment within PE layers, no enzyme aggregates can be observed on the membrane surface. Presented SEM images of membranes formed in both modes indicate meaningful coverage of the pristine membranes by PE and/or laccase layers, confirming an efficient production of novel biocatalytic PEM membranes (Yurekli, 2019). Moreover, based on the SEM images, it is suggested that deposition of several PE or enzyme layers, resulted in reduced pore size and porosity of the final PEM membranes, as also reported earlier (Sarma et al., 2017).

The surface charge of NP010 and UFX5 membranes before and after PE deposition and laccase immobilization was determined via streaming potential measurements (Fig. 3). The zeta potential of the pristine NP010 and UFX5 membranes were negative across the entire pH range investigated (from pH 4–10), whereas the zeta potential generally increased upon deposition of PE and laccase, as could be expected given that the PEM films were terminated with the cationic PEs or laccase (i.e., a positively charged top-layer). Similarly for both membranes substrates, using PEI as the cationic PE resulted in a more positive surface charge than using PLL and entrapped laccase mode induced more positive surface charges than the internal laccase immobilization mode.

Considering the different polyelectrolytes, PEI and PLL, the higher positive charge induced by PEI was presumably due to the branched structure of PEI, as opposed to the linear structure of PLL. The electrostatic properties of the two polyelectrolytes are similar, with the IEP of PEI reported as 10.6 (Ufer et al., 2008) compared to 10.5 for PLL (Morga et al., 2015), and are therefore not expected to be the main factor controlling the PE deposition. The branched PE structure could however promote thicker layers and more shielding of the underlying negatively charged membrane surface.

The two different immobilization strategies, internal and entrapped modes, also resulted in considerable difference in surface charge. In the internal mode, laccase (with two IEPs, IEP-1 around pH 3 and IEP-2 around 4.6–6.8 (Jankowska et al., 2019) was used instead of the anionic PE and deposited between and on top of the cationic PE layers from buffer at pH 5. At pH 5, laccase is mostly negatively charged and can interact with the positive charges of PEI or PLL. In the entrapped mode on the other hand, laccase was deposited together with PEI or PLL between and on top of layers of PSS. The surface charge was generally more positive in entrapped mode than in internal mode, since in entrapped mode, the laccase was entrapped inside the positive PEI or PLL top-layer, whereas in internal mode, laccase was deposited directly on the surface and hence its negative charges were exposed, inducing negative surface charge.

Interestingly, although the entrapped immobilization mode included additional layers of PSS (strong anionic PE) (Sigurjardottir et al., 2020), this mode generally resulted in a more positive surface charge than the internal mode. By including PSS in the whole PEM film, the overall amount of charges in the system could be increased, resulting in increased deposition and again, more shielding of the negatively charged surface of the membrane substrate. Similar results were observed by Ji et al. (2020), who prepared PEM membranes using polydopamine (PDA), poly(acrylic acid) (PAA) and poly-(diallyldimethylammoniumchloride) (PDADMAC). They observed a higher positive surface charge when depositing PDADMAC on a highly negatively charged PDA/PAA surface, as opposed to a less negatively charged PDA surface. The increase in absolute surface charge (negative or positive) promotes further deposition of additional polyelectrolytes to the surface, with increased charge density and more shielding of the membrane surface below.

Beside examination of changes in membrane morphological and electrokinetic properties upon Lbl modification, from membrane technology point of view it is also crucial to determine changes in membrane hydrophobicity and water permeability as these parameters directly affect process duration and its costs. Lbl assembly usually reduces water permeability and flux due to the deposition of additional layers onto the
Fig. 2. SEM images of (a) pristine NP010 nanofiltration membrane and (f) pristine UFX5 ultrafiltration membrane as well as produced PEM biocatalytic membranes: (b) NP010-PEI-intLAC, (c) NP010-PLL-intLAC, (d) NP010-PEI-entLAC, (e) NP010-PLL-entLAC, (g) UFX5-PEI-intLAC, (h) UFX5-PLL-intLAC, (i) UFX5-PEI-entLAC, (j) UFX5-PLL-entLAC.
membrane surface and into membrane pores, leading to even 80% reduction in water permeability, as was reported for a nanofiltration membrane modified by 6 bilayers of PEs (Dizge et al., 2018). In this study, for membranes produced with PEI and PLL, respectively, around 50% and 60% drop of water permeability was observed (Table 1) due to coating of the membrane surface and membrane pores by PEs and enzyme, which enhances mass transfer limitations and causes a decrease in membrane pore size (Cheng et al., 2018; Dizge et al., 2018; Yabuki, 2011). Although the NP010 membrane is more hydrophilic, the decrease of $L_p$ of this membrane is more pronounced compared to the UFX5 membrane. This observation suggests that partitioning into the pores of this membrane indicates lower resistance to transport through the membrane, as compared to NP010 (Zwolinski et al., 1949). Nevertheless, the decrease in the membrane flux confirms successful PEs and enzyme deposition onto the membrane matrix and production of biocatalytic membranes as reported also in previous findings (DuChanois et al., 2019; Datta et al., 2008).

Moreover, membrane modification by PEs gives a possibility to control membrane hydrophobicity by selection of suitable PEs (Petriła et al., 2021). To quantify changes in the hydrophilicity of the pristine membranes and produced PEM membranes, the water contact angle was determined and compared (Table 1). Pristine NP010 and UFX5 membranes are characterized by water contact angle of 59° and 77°, indicating higher hydrophilicity of the poly(piperazinamide) skin layer of the NP010 membrane. After PEs and laccase deposition, the contact angle of all membranes dropped by around 10–20% indicating slightly higher hydrophilicity of the produced membranes. An increase of membrane hydrophilicity was expected and facilitates membrane flux and reduces membrane fouling (Qasim et al., 2019).

### 3.2. Biocatalytic characterization of the PEM biocatalytic membranes

Besides physicochemical and morphological analysis of the produced PEM membranes, it is crucial to provide thorough characterization of the produced systems in terms of their catalytic and kinetic properties. This characterization is to verify potential applicability of the formed systems towards estrogen removal, as the selected membranes will act as a biocatalytic unit in enzymatic membrane reactors. From Table 2 it is clear that the highest amount of immobilized laccase was observed for UFX5-PEI-intLAC (93 mg), whereas the lowest amount of laccase (43 mg) was deposited in NP010-PLL-entLAC. In general, higher amount of immobilized laccase was observed for UFX5-based membranes produced with laccase deposited between PE layers. This might be due to direct laccase immobilization from buffer solution in internal mode membranes and lack of competition between PEs and enzymes molecules for membranes sorption active sites that occurred in ent-membranes (Malinin et al., 2011). Further, around 20% higher amount of laccase was immobilized in membranes formed using PEI, irrespectively of the membrane used and immobilization mode applied. The difference indicates significantly higher number of amine groups in the PEI structure, that are responsible for enzyme binding (Virgen-Ortíz et al., 2017) as well and higher strength of hydrophilic interactions between PEI and laccase molecules (Orrego et al., 2020). Obviously, results on enzyme loading followed the same trends as results on amount of immobilized laccase, as membrane reactors with the same working area was used. The presented data on enzyme loading should be considered as relatively high and produced relatively high membrane activity, that also corresponds to the trend that higher in enzyme loading, higher is membrane activity. Among produced UFX5-based membranes, the highest membrane activity reached 59.7 U/cm² for...
UFX5-PEI-intLAC whereas for NP010-based membranes it was 48.7 U/cm² indicating high catalytic retention by the produced membranes. By contrast, in another study, laccase immobilized in an LbL poly(allyl amine hydrochloride) nanofiltration membrane cross-linked by glutaraldehyde showed less than 0.3 U/cm² activity (Li et al., 2020). From Table 2 it is also clear that higher enzyme loading generated higher activity retention. Around 80% activity recovery was observed for UFX5 membranes produced using PEI, whereas for NP010 membranes with PEI, the activity recovery was around 70%. Further, from data in Table 2 it is also clear that higher activity retention was observed for internal mode membranes (laccase between PE layers). This is directly related to the approach of PEM membrane fabrication and is related to the fact that in this mode enzyme biomolecules were deposited from buffer solution and biomolecules are less strictly surrounded by the PE. This generates lower changes in enzyme structure and produces lower diffusional limitations leading to the higher activity retention. The obtained values should be considered as high and suggest possible practical application of these systems in real processes. This data shows also that laccase is well deposited between/within PEI layers, which generate a suitable microenvironment for the immobilized enzyme, mainly due to creation of stable hydrophilic interactions and limited interference in the enzyme structure (Li et al., 2021). Moreover, the high catalytic activity retention indicates that the methods proposed in this investigation for the production of biocatalytic membranes limited enzyme inactivation due to immobilization and produced systems in which the active site of the enzyme is not sterically blocked. Recently published studies on formation of biocatalytic membranes with immobilized laccase showed that production approach and used pristine filtration membrane affect activity retention of the produced systems. For instance, glutaraldehyde functionalized polyamide 6,6 membranes with immobilized laccase showed less than 10% activity recovery (Silva et al., 2007), whereas laccase deposited onto nanofiltration membrane covered by polydopamine showed around 60% activity retention (Cao et al., 2016).

In studies on immobilized enzymes, it is important to determine kinetic parameters of the immobilized catalysts in order to determine changes in enzyme-substrate affinity (Michaelis-Menten constant, \( K_M \)) and maximum reaction rate (\( V_{\text{max}} \)) between free and immobilized enzyme. The \( K_M \) of free laccase was found to be 0.057 mM, whereas \( V_{\text{max}} \) was determined as 0.046 mM/s. For all tested membranes, higher \( K_M \) values were observed, indicating lower substrate affinity by the immobilized laccase as compared to its free counterpart, which suggests that the LbL approach negatively affects laccase interactions with substrate molecules, as reported also earlier (Habimana et al., 2021). Although the \( K_M \) value increased for all tested membranes, less pronounced increase was observed for the UFX5-based systems and for membranes with laccase deposited between PE layers. For instance, UFX5-PEI-intLAC exhibited \( K_M \) of 0.063 mM that was around 10% higher than for the free enzyme. This difference is because the UFX5 membrane has bigger MWCO, which facilitates substrate flow, and likewise, the active sites of laccase deposited between PE layers, generate direct enzyme-substrate contact. Simultaneously, around 30%–50% lower values of maximum reaction rate were observed for produced systems with immobilized laccase. This drop in \( V_{\text{max}} \) values can be attributed to the slight constitutive rearrangements in the laccase molecules and creation of diffusional limitations in substrate and product transport due to formation of additional layers around the enzyme that decrease the possibility of substrate and laccase binding (Birhanh et al., 2022).

It could be summarized that all produced biocatalytic PEM membranes are characterized by good catalytic properties and might be considered as efficient biocatalytic tools. Nevertheless, based on the data presented in Table 1 (membrane flux) and Table 2 (catalytic properties), four membranes were selected for further study on catalytic degradation of EE2, namely NP010-PEI-intLAC, NP010-PEI-entLAC, UFX5-PEI-intLAC and UFX5-PEI-entLAC, as these systems are characterized by the highest catalytic properties and water permeability values, suggesting high conversion efficiencies in a reasonable time.

### 3.3. Removal study of 17α-ethynylestradiol

#### 3.3.1. 17α-ethynylestradiol removal

The process duration required to achieve high removal rate is one of the most important parameters in degradation of micropollutants. Prior to the experiment, in order to determine sorption capacity of the PEM membranes, adsorption tests were performed, which showed that removal of EE2 by adsorption did not exceed 20%, making enzymatic conversion a main driving force of the removal process. Moreover, for comparison, a test with free enzyme was performed, which resulted in 94% removal efficiency of EE2 after 180 min. It was also reported that produced biocatalytic PEM membranes are characterized by various water permeability and hydrophilicity, thus it is expected that also duration of removal experiments and their efficiency will be different for each system. That is why curves presented at Fig. 4 end at different times. The removal rate of EE2 by the biocatalytic membranes was slightly lower and reached a maximum of 91% for NP010-PEI-intLAC systems.

![Fig. 4. Time course for the removal of EE2 at the pressure of 2 bar from water solutions using various PEM biocatalytic membranes produced. Results are presented as a mean value, for which error values do not exceed 5%](image-url)
after 150 min of the degradation at the pressure of 2 bar (Fig. 4). In turn, the removal of EE2 by UFX5-PEI-intLAC reached 86% in just 60 min, which is directly related to the bigger MWCO of the UFX5 ultrafiltration membrane and over three-times higher water permeability of this membrane compared to the NP010 membrane. By contrast, due to the enzyme immobilization within PE layers, with subsequent lower enzyme accessibility for the EE2 molecules, lower micropollutants removal rate and longer process duration were observed for membranes with entrapped laccase. UFX5-PEI-entLAC membrane was capable for removal of 69% of EE2 after 105 min, whereas for NP010-PEI-entLAC removal rate attained 77%, however, 180 min of the process was needed. It should also be noted that PEM membranes for removal experiment were selected based on their catalytic properties - mainly their catalytic activity retention (Table 2). Interestingly, data on activity retention and EE2 removal rate are not entirely consistent. For instance, higher activity retention was obtained for UFX5-based membranes, whereas higher removal rate was noticed for NP010 membrane indicating that not only enzyme activity determined high removal rate. Also other factors, such as membrane MWCO, membrane material and flux during the process should be considered as important factors influencing the total removal rate (Cao et al., 2018; Luo et al., 2020). Moreover, it should be highlighted that the highest removal rate of EE2 was noticed after 150 min of the process and the maximal process time required to treat the whole volume of EE2 solution was 180 min, which was relatively short, as compared to other studies.

For instance, in our previous study, over 5 h was required to achieve 89% removal efficiency of tetracycline by laccase immobilized in nanofiltration membrane in reverse mode (Zdarta et al., 2022). Further, laccase incorporated into biocatalytic nanofiltration membrane made using LbL approach exhibited 80% removal of bisphenol A after 24 h of degradation (Li et al., 2020). Finally, Nguyen et al. (2015) showed that 72 h was needed to attain around 90% removal of 17β-estradiol and EE2 by laccase immobilized onto ultrafiltration hollow fiber membrane. Although laccase immobilized in the produced membranes showed slightly lower removal rate compared to free laccase, this disadvantage might be overcome by higher stability and reusability of the immobilized laccase due to enzyme structure stabilization and its stable binding to the support, as reported also by Xu et al. (2018).

Besides the process duration, also other parameters such as pH and temperature might significantly affect enzymatic removal of micropollutants. Moreover, it is known that EE2 occurs in wastewater at various concentration, depending on the wastewater source. Thus, removal of EE2 by selected PEM membranes was examined over a wide pH and temperature range and from solutions at concentrations exceeding environmental relevant amount of this micropollutant to investigate the degradation potential of the produced systems. Prior to removal experiments using PEM membranes, also EE2 degradation by free laccase was tested. The obtained data (not presented in the manuscript) showed that 94% EE2 removal was reached only at pH 5 from solutions up to 0.1 mg/L. Change of the pH and increase in EE2 concentration lead to significant drop of the removal rate implying low stability of the laccase. From Fig. 5(a) it is clear that the higher the EE2 concentration, the lower is its removal rate, irrespectively of the initial membrane used and enzyme deposition within membrane structure. All tested membranes allowed over 90% removal of EE2 from 0.01 mg/L solution, whereas around 20–30% lower removal rate was observed for 0.5 mg/L solution indicating that catalytic potential of the produced system is getting exploited with increasing EE2 concentration. Moreover, the decrease in removal rate might be related to partial laccase inhibition at higher estrogen concentration as well as blocking of membrane pores that produces limitations in transport of substrate and products, as observed also in previous study on laccase immobilization using membranes (Ahmad et al., 2021). Nevertheless, removal of over 60% of EE2 even from 0.5 mg/L solution clearly shows that produced membranes should be considered as effective for removal of even highly concentrated estrogens.

On the other hand, Fig. 5(b) suggests that a compromise has to be found between process duration and process efficiency with respect to the pressure applied. As expected, the higher the pressure applied, the shorter the process duration. At the pressure of 3 bar and 4 bar, the degradation time was reduced by around 10% and 15% respectively, as compared to the process performed at 2 bar (data not presented). Note however, that EE2 removal dropped up to 15% at higher pressure applied, mostly due to increased flux of the substrates and limited enzyme-estrogen contact and binding time. It was also reported, that at higher pressure enzyme might undergo partial inactivation leading to its lower activity (Hou et al., 2014). At the pressure of 2 bar, the removal rate of EE2 reached over 85% for membranes with laccase deposited between PE layers, which was only around 5% less than at 1 bar, thus 2 bar of oxygen pressure was selected as the most suitable pressure and applied in other experiments.

Fig. 5(c) shows that removal rate of EE2 increased with increasing the pH from 3 to 5, where it reached its maximum and then started to decrease by further increasing the pH. This trend is observed for all tested membranes and is related to the catalytic properties of the
laccase. *Trametes versicolor* laccase shows highest activity at slight acidic pH (from 3 to 5) (Zdarta et al., 2020), whereas at basic pH the single electron oxidation of EE2 at laccase T1 copper atom is interrupted as well as the intramolecular electron transfer between T1 and T2/T3 copper sites due to hydroxylation of the copper ions (Sun et al., 2021). Nevertheless, use of free enzymes resulted in around 40% drop of EE2 removal in conditions different from the optimal (pH 5) indicating significantly improved pH tolerance of laccase upon immobilization in both modes. It should be highlighted that over 50% of the estrogen was removed by all PEM membranes at pH ranging from 3 to 6, whereas over 80% process efficiency was attained at pH from 4 to 6 for membranes with laccase immobilized between PE layers. This stability increase might be explained mainly by rigidization and stabilization of the enzyme structure upon immobilization, as well as by protective effects exerted by the membrane and PEs on the biomolecules against chemical inactivation at harsh pH (Zhang et al., 2021). Further, at all tested process conditions, higher removal rates were observed for membranes with laccase between PE layers, probably due to the fact that enzymes in these membranes are better exposed to direct contact with substrate molecules. On the other hand, access to the active sites of laccase entrapped within PE layers is confined, generating diffusional limitations in transport of reaction ingredients, which is in agreement with the results on kinetic parameters and was suggested also in our previous study (Zdarta et al., 2022). Although the number of studies on estrogens removal by membrane-immobilized laccase are limited, other published results on laccase immobilization using various types of membranes also suggest careful selection of process conditions in order to achieve high removal rate. For instance, laccase immobilized into polyvinylidene fluoride microfiltration membrane showed around 90% removal of bisphenol A at a constant operation flux of 50 L/m²·h, however, increase of the flux lead to enzyme elution and lower removal rate (Fan et al., 2017). Further, over 70% removal rate of tetracycline was reached by laccase immobilized into modified ceramic membranes. However, it was observed that, beside substrate flow-rate and membrane MWCO, also micropollutant concentration and changes in solution pH significantly affected the removal rate of antibiotic (de Cazes et al., 2015).

3.3.2. Stability study of biocatalytic PEM membranes

Improvement of storage stability and reusability of the enzymes upon binding to solid support is one of the biggest advantages of the immobilization process. Further, long-term stability and the possibility to reuse the biocatalysts are key features encouraging large-scale applications of the produced system in wastewater treatment. From Fig. 6 (a) it is clear that all produced biocatalytic membranes showed improved storage stability during storage at 4°C over 20 days, as compared to free enzyme. All tested membranes retained over 75% of initial activity after 20 days, which is over 30% higher than free laccase that showed 41% relative activity after the same storage time. However, biocatalytic PEM membranes with laccase entrapped within PE layers, irrespectively of the membrane used, showed around 10% higher relative activity. This fact is directly related to the enzyme deposition inside PE layers that protect the biomolecules against harsh process conditions and reduce enzyme leaching over time (Liao et al., 2013). These findings are confirmed by the results of enzyme leaching tests (Fig. 6(b)) that showed that after 20 days around 15% of the laccase was leached from the membranes with entrapped enzymes, compared to 35% for the membranes with laccase between PE layers. Note however, that due to bigger MWCO of the UFX5 membrane, more pronounced enzyme leaching, around 5% higher than for the NP010 membrane was observed for systems built on this membrane. Similar observation were made by Li et al. (2020), who immobilized laccase onto nanofiltration PEM membrane made of poly(allylamine hydrochloride) and poly(styrenesulfonic acid) sodium salt and observed around 7% enzyme leaching from the membrane after 10 days.

![Fig. 6.](image)

(a) Storage stability and (b) enzyme loading over 20 days of storage time as well as (c) removal efficiency of EE2 and (d) enzyme loading over 8 cycles of repeated use of PEM biocatalytic membranes produced. Results are presented as a mean value, for which error values do not exceed 5%.
In addition, the results of reusability tests confirm the great potential of the produced membrane for repeated use in removal of EE2. PEM biocatalytic membranes made on NP010 nanofiltration membranes were capable for removal of around 70% of EE2, whereas systems produced using UF55 ultrafiltration membrane removed around 55% of micropollutants after 8 repeated degradation cycles. By contrast, in another study, urine immobilized onto sulfonated polysulfone ultrafiltration membrane modified by PEI and alginate showed less than 60% activity retention after 5 repeated cycles of model reaction (Yürekli, 2019). The high reusability potential of the produced membranes is mainly due to stabilization of the enzyme structure and preservation of its high catalytic activity. Although membranes made by enzyme entrapment initially showed lower removal rate of estrogen, the NP010-PEI-emtLAC and UF55-PEI-emtLAC membranes showed higher removal rate of EE2 as compared to membranes with laccase immobilized between PE layers after 4 and 6 degradation cycles, respectively. These results indicate that the enzyme is better protected when immobilized within the PE layer, than between PE layers and further suggests that the PEs provide a suitable microenvironment for the immobilized enzyme (del Castillo et al., 2019). On the other hand, the slight continuous drop in degradation properties of the tested membranes might be ascribed to enzyme inactivation and its inhibition by the substrates and products of the process, as well as formation of membrane fouling over repeated use that limits the access of substrates to the enzyme active sites (Sarma et al., 2017). Moreover, the results of enzyme loading over repeated use (Fig. 6(d)) showed around 10% and 20% elution of the laccase from entrapped and internal mode membranes, respectively suggesting that partial enzyme elution is also responsible for decrease of EE2 removal rate over repeated use. Nevertheless, based on the obtained data it may be concluded that the biocatalytic PEM membranes proposed herein, based on immobilized laccase, exhibit great stability and reusability indicating promising economic feasibility and applications in removal of micropollutants.

4. Conclusions

In this study, novel multifunctional biocatalytic PEM membranes were produced via LbL assembly from cationic and anionic PEs with laccase enzyme immobilized in internal and entrapped mode. NP010 nanofiltration and UF55 ultrafiltration membranes were found to be suitable as support layers for productions of PEM systems for removal of EE2 from water solutions. Although in this study EE2 was used as a model microenvironment to demonstrate the potential of the produced systems in remediation processes, the main idea of the study is to produce systems capable for treatment of real wastewater containing a huge variety of micropollutants. Due to the multifunctional character of the produced PEM membranes and possibility to remove compounds by simultaneous adsorption, membrane rejection and biocatalytic conversion, these systems might be applied for conversion of a wide range of pollutants. By proper selection of membrane MWCO as well as selection of type and number of polyelectrolyte layers, both sorption properties and membrane rejection could be controlled that enhances removal of various pollutants containing various functional groups and differ by the structure and properties. Moreover, in the presented study laccase was used as an enzyme capable for EE2 conversion. Laccase enzyme is known by its low specificity and ability to convert wide range of organic micropollutants, facilitating multipurpose application of the produced systems for complex treatment of wastewater.

In the study membranes based on branched PEI polyelectrolyte showed the highest enzyme loading and over 70% activity retention, making these systems promising for application in biodgradation study. The removal study performed showed that systems produced on UF55 membrane were capable for removal of over 60% of EE2 in less than 100 min. In comparison, up to 180 min was required to perform degradation using NP010-based systems, however, about 15% higher removal rate was achieved. Moreover, it was possible to remove over 70% of the targeted micropollutant over a wide range of pH (from 3 to 6) and pressure (from 1 to 3 bar) even from solutions at environmentally relevant concentration of 0.1 mg/L. Finally, long-term stability and great reusability potential (removal of over 60% of EE2 after 8 repeated cycles) indicate high application potential of the proposed systems in removal processes of not only estrogens, but also other micropollutants. Although nanofiltration or reverse osmosis membranes could also be considered as an efficient platforms for removal of micropollutants, from the point of view of practical application, the simultaneous membrane rejection and bioconversion seems to be more efficient way of micropollutants removal rather than membrane rejection and enzymatic bioconversion, separately. The advantage of the PEM biomembranes over pristine membranes is also the fact that the second disposal problem, which occurs after application of membranes removal processes, can be significantly decreased due to fact the initial compounds can be converted into less toxic products in eco-friendly way. Nevertheless, further studies in this research area are still of highest importance, focusing on application of enzymatic membrane reactors for removal of various micropollutants from real wastewater. To support real wastewater treatment, PEM biomembranes produced in this study should be transferred from lab-scale into pilot- and large-scale. From one perspective moving of the presented solutions into industrial scale might be partially challenging due to relatively high enzyme costs and requirements for the proper bioreactor. On the other hand, significant improvement of enzyme stability, possibility of the multiple use of the PEM biomembranes as well as high removal efficiencies of the estrogens supported by the omnipresence and low-cost of the commercially membranes facilitate transfer of the developed membranes into larger scale. However, detailed studies on economic feasibility of this process as well as advanced studies on effect of real wastewater matrix constituents and implementation of continuous bioreactors are required.

Credit author statement

Jakub Zdarta: Conceptualization, Methodology, Investigation, Manuscript writing, Formal analysis, Project supervision. Sigyn Bjork Sigurðardóttir: Investigation, Zeta potential analysis, Data Formal analysis, Manuscript writing. Katarzyna Jankowska: Conceptualization, Methodology, Investigation, Manuscript writing. Manuel Pinelo: Project supervision, Verification of the final version of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by the National Science Centre, Poland under the research Grant number 2019/35/D/ST8/02087.

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