Glycoside hydrolase (PelA₇) immobilization prevents Pseudomonas aeruginosa biofilm formation on cellulose-based wound dressing

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Abstract

Bacterial cellulose (BC) is recognized as a wound dressing material well-suited for chronic wounds; however, it has no intrinsic antimicrobial activity. Further, the formation of biofilms can limit the effectiveness of the pre-saturation of BC with antimicrobial agents. Here, to hinder biofilm formation by P. aeruginosa, we immobilized the hydrolytic domain of PelA (a glycohydrolase involved in the synthesis of biofilm polysaccharide Pel) on the surface of BC. The immobilization of 32.35±1.05 mg PelA₇ per g BC membrane resulted in an eight-fold higher P. aeruginosa cell detachment from BC membrane, indicating reduced biofilm matrix stability. Further, 1D and 2D infrared spectroscopy analysis indicated systematic reduction of polysaccharide biofilm elements, confirming the specificity of immobilized PelA₇. Importantly, BC-PelA₇ was not cytotoxic towards L929 fibroblast cells. Thus, we conclude that PelA₇ can be used in BC wound dressings for safe and specific protection against biofilm formation by P. aeruginosa.

Keywords: bacterial cellulose, biofilm, Pseudomonas aeruginosa, PelA₇, wound dressings
1. Introduction

Natural polymers are becoming increasingly popular due to rising interest in environmentally friendly and sustainable materials. One of the most popular biopolymers is bacterial cellulose (BC), synthesized by the bacterium *Komagataeibacter xylinus*. Due to its unique properties, BC can be used in various applications e.g., paper production, textile industry, environmental protection, and in medical applications (Fijalkowski, Żywicka, Drozd, Kordas & Rakoczy, 2016; Drozd et al., 2019). Importantly, the chemical structure of BC can be modified by the addition of specific functional groups and, thus, BC can be utilized as a carrier for the immobilization of enzymes (Drozd et al., 2019; Wu, Wu & Su, 2017; Drozd, Rakoczy, Wasak, Junka & Fijałkowski, 2018) or microorganisms (Yaou et al., 2011; Nguyen, Ton & Le, 2009; Żywicka, Banach, Junka, Drozd & Fjalkowski, 2019; Żywicka et al., 2019). Further, high stability, biocompatibility, high water holding capacity, and the possibility of sterilization make BC useful as a wound dressing material. However, despite many advantages, native BC does not have any bactericidal properties, nor does it stimulate faster wound healing. For this reason, in order to increase its functionality, it is necessary to modify it, for example by introducing into its structure other polymers, such as chitosan, or antiseptics (e.g. octenidine) (Lin, Lien, Yeh, Yu & Hsu, 2013; Moritz et al., 2014).

Bacterial colonization of wound dressing materials is the major problem in wound healing therapy. The degree of colonization is the result of a combination of pathogenicity and virulence of the microorganism and the condition of the patient’s immune system. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are frequently responsible for wound infections and problems with wound healing. Importantly, both can live in the form of biofilms: communities of these microorganisms surrounded by an extracellular matrix (DeLeon et al., 2014; Bessa, Fazii, DiGiulio & Cellini, 2015). The matrix consists of various extracellular polymer substances (EPS) that maintain the three-dimensional structure of the biofilm. Additionally, the matrix is responsible for the adhesion of the biofilm to both biotic and abiotic surfaces. Finally - and perhaps most importantly - the matrix protects the bacteria against the effective action of bactericides and immune cells of the host organism (Flemming & Wingender, 2010).

A promising strategy to combat infections caused by microorganisms that form biofilms is to disrupt the integrity of the extracellular matrix structure and thus cause the bacteria to become more sensitive to antimicrobial agents. While various low-molecular substances can induce degradation of the extracellular matrix, enzymes that can hydrolyze matrix components could be an attractive method for biofilm eradication (Kaplan, 2009; Kaplan, 2019). To date, many commercially available enzymes have been tested as potential tools for biofilm eradication.

Due to the presence of proteins and polysaccharides as the main components of the biofilm matrix, amylolytic or proteolytic enzymes are most favorable (Molobela, Cloete & Beukes,
2010; Shukla & Rao, 2013; Fleming, Chahin & Rumbaugh, 2017; Hogan et al., 2017). For example, Dispersin B is a well-known enzyme used in biofilm eradication, produced by *Aggregatibacter actinomycetemcomitans*. It belongs to the glycoside hydrolase family of enzymes and cleaves poly-β-1,6-N-acetylglucosamine, the main component of the biofilm matrix of many bacterial species (Kaplan, 2009; Kaplan, Ragunath, Ramasubbu & Fine, 2003; Kaplan, Ragunath, Velliagounder, Fine & Ramasubbu, 2004; Ramasubbu, Thomas, Ragunath & Kaplan, 2005; Izano et al., 2008; Fekete et al., 2011; Little et al., 2014; Little et al., 2015; Dobrynina et al., 2015). Dispersin B increases the susceptibility of *A. actinomycetemcomitans* to cationic and anionic detergents and *Actinobacillus pleuropneumoniae* to the effective action of ampicillin (Izano et al., 2008; Izano et al., 2007). Dispersin B could also be used in combination with bactericidal nanoparticles or in an immobilized form (Tan, Ma, Liu, Yu & Han, 2015; Chen & Lee, 2018). A promising alternative approach to biofilm eradication is the use of enzymes that are elements of EPS biosynthesis pathways, including alginate lyase and the hydrolytic domains of PelA and PslG proteins (PelA<sub>h</sub> and PslG<sub>h</sub>). Alginate lyase reduces sputum viscosity in cystic fibrosis and enhances phagocytosis. Additionally, alginate lyase is an enzyme that increases the effectiveness of antibiotic therapy by disrupting the integrity of the three-dimensional structure of biofilms by hydrolyzing alginate. Thus, this enzyme could be used in combination with antibiotics such as gentamicin (Hatch & Schiller, 1998; Ghadam, Akhlaghi & Ali, 2017, Patel el al., 2019).

Alternately, *Pseudomonas aeruginosa* produces at least three exopolysaccharides as components of its biofilm matrix: alginate, Pel, and Psl. Pel is a positively charged polysaccharide, consisting of partially acetylated N-acetylglucosamine and N-acetylgalactosamine, connected by 1-4 glycosidic bonds. Pel is responsible for initiation and stabilization of the interaction between cells in the biofilm structure, and in some cases, could also play a role in cell adhesion to a surface. Pel also protects bacteria against certain aminoglycoside antibiotics. At present, the exact structure and biosynthesis process of Pel exopolysaccharide has not been fully understood. However, it is known that molecules involved in the mechanisms of synthesis and secretion of Pel are coded by an operon containing 7 genes (*pelA - G*) (Colvin, Gordon, Murakami, Borlee, Wozniak, Wong & Parsek, 2011, Franklin, Nivens, Weadge & Howell, 2011, Jennings et al., 2015, Marmont et al., 2017). The product of the first gene in the operon is the PelA molecule and bioinformatic analysis suggests that it is a 105 kD multidomain protein located in the periplasm (Franklin et al., 2011). Presumably, PelA consists of five domains and three of them have catalytic activity (Colvin, Alnabelseya, Baker, Whitney, Howell & Parsek, 2013). The first domain belongs to the glycoside hydrolase family and is particularly crucial in determining chain length and/or hydrolyzing mis-synthesized Pel molecules (Baker et al., 2016).

Thus far, the mechanisms of action of PelA<sub>h</sub> and PslG<sub>h</sub> on biofilm structure have only been partially elucidated by LeMuff et al., (2019) and Baker et a., (2015). Likewise, their use in the biofilm eradication process has been investigated only to a minor extent. One of the first
of such studies was carried out by Baker et al., (2016), demonstrating that PelA<sub>h</sub> and PslG<sub>h</sub>, even at low concentrations, could prevent the formation and remove already formed Pseudomonas spp. biofilm. More recently, Pestrak et al., (2019) showed that in P. aeruginosa-infected wounds, PelA<sub>h</sub> and PslG<sub>h</sub> enhanced antibiotic therapy effectiveness and allowed for dosage reduction. In particular, the combination of PslG<sub>h</sub> and tobramycin resulted in better penetration of the antibiotic into the biofilm and faster wound healing in an in vivo model. Further, PslG<sub>h</sub> also affected the host immune system resulting in greater complement deposition and increased neutrophil phagocytosis and reactive oxygen species production.

However, while PelA<sub>h</sub> is essential during the synthesis of Pel and for creating the biofilm structure, the possibility of using it as a therapeutic element of a wound dressing has not yet been tested. The present study explores the potential of PelA<sub>h</sub> protein immobilization to protect BC-based wound dressings from bacterial biofilm formation. Thus, the main aim of our study was the development of a novel wound dressing based on BC produced by K. xylinus, with immobilized PelA<sub>h</sub> providing protection against P. aeruginosa biofilm formation. Toward this aim, we conducted a detailed study of the properties of the developed dressing material, including the effect of immobilized PelA<sub>h</sub> protein on P. aeruginosa biofilm.

2. Materials and methods

2.1. Chemicals and reagents

All reagents and bacteria cultivation media components used in this study were purchased from Merck KGaA (Poland) and Biomaxima SA (Poland). All chemicals were at least reagent grade and used without further purification. Oligonucleotides and plasmid pET28a were purchased from Merck KGaA. Polymerase PfuPlus were purchased from Roboklon GmbH (Germany). InnuPREP Plasmid Mini Kit 2.0 (Analytic Jena AG, Germany) and ROTI®Prep PCR purification kit, were ordered from Carl Roth GmbH, (Germany). DpnI restriction enzyme and E. coli BL-21 (DE3) competent cells were purchased from New England Biolabs (Germany). E. coli TOP-10 competent cells were purchased from Thermo Fisher Scientific (Germany). Roti Garose His Beads were purchased from Carl Roth GmbH (Germany). The L929 cell line and all mammalian cell culture reagents were purchased from Merck KGaA (Poland).

2.2. Bacterial strains

Plasmid and strains used in this study were provided by the Department of Biotechnology and Enzyme Catalysis at the University of Greifswald. K. xylinus (ATCC®53524™) and P. aeruginosa PAO-1 were provided from the collection of the Department Microbiology and Biotechnology of the West Pomeranian University of Technology in Szczecin.

2.3. Cloning, expression, and purification of PelA<sub>h</sub>
To obtain the target gene sequence that encodes the protein construct fragment of *pelA* (GenBank ID: AAG06452.1) from the genomic DNA of *Pseudomonas aeruginosa* PAO-1 and vector pET28a were amplified separately by polymerase chain reaction. For amplification of *pelA* gene primers: forward (5' - CTGCATATGGGCGGGCCGTCCAGCGTGGCG - 3') and reverse (5' - TTTCTCGAGTACGGTTGCACCTCGACGTCG - 3') were designed. For amplification of pET28a primers were designed as following: forward (5' - TGAGATCCGGCTGCTAACAAAGC - 3') and reverse (5' - CATATGGCTGCCGCGCGG - 3'). PCR reactions were carried by using PluPlus! DNA Polymerase (Roboclon). After confirmation of PCR products, 0.5 µl of *DpnI* was added to 50 µl of unpurified insert and vector separately and digested in 37 °C for 1 h according to the FastCloning method (Li, Wen, Shen, Lu, Huang & Chang, 2011). Digested insert and vector were purified, and gene was assembled with the pET28a (Merck KGaA) expression vector using SLiCE mix (Zhang, Werling & Edelmann, 2012) containing the following components: appropriate amount of vector and insert DNA (1:3 molar ratio), 1 µl 10x SLiCE buffer, 1 µl SLiCE extract, 1 µl ATP and MilliQ Water to a total volume of 10 µl. The SLiCE reaction mixture was incubated at 37 °C for 1 hour. 4 µl of the SLiCE reaction solution was added to 50 µL TOP10 chemically competent *E. coli* cells (Thermo Fisher Scientific). The obtained (generated) construct was confirmed by DNA sequencing using the Mix2Seq Kit (Eurofins). After confirmation, the construct was transformed to chemically competent *E. coli* BL21 (DE3) (New England Biolabs) cells. *E. coli* BL21 (DE3) cells harboring the expression vector were grown in 1 liter of LB medium containing kanamycin (50 µg/mL) at 37 °C. When OD$_{600}$ reached 0.5 – 0.6, protein expression was induced by the addition of IPTG to a final concentration of 1 mM. After induction, the cells were incubated overnight at 20 °C with shaking at 200 rpm. After overnight incubation, cells were harvested by centrifugation at 4000xg for 25 min at 4 °C and pellets were washed two times with washing buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% (v/v) glycerol and 5 mM imidazole) and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% (v/v) glycerol). The cells were lysed using sonication pulses (2x2 minutes) and then centrifuged for 30 min at 4 °C and 9000xg. Protein was purified by Nickel-based IMAC using Roti®Garose His Beads preequilibrated with washing buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol). A column with loaded protein was washed with washing buffer and expressed protein was eluted with elution buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10% (v/v) glycerol). The eluted fractions were concentrated to around 1.5 ml volume using a Vivaspin® 6 ultracentrifugation filter device (Sartorius AG) with a 10 kDa cutoff. To check the purity of the obtained protein, SDS-PAGE analysis was performed (Fig. S1).

2.4. Preparation of bacterial cellulose (BC) membranes
The culture of *K. xylinus* was carried out in 24-well plates, in Hestrin–Shramm (HS) medium containing glucose 20 g/L, yeast extract 2.0 g/L, peptone 2 g/L, citric acid 1.15 g/L, Na$_2$HPO$_4$ 2.7 g/L, and MgSO$_4$·7H$_2$O 0.06 g/L with 1% ethanol. The prepared medium was inoculated with 1% of a 1-week-old starter culture. The cultivation was carried out at 28 °C for 4 days. After this time, the formed BC membranes were collected and rinsed in dH$_2$O for 24 h. The next day, BC membranes were digested with 0.1 M NaOH at 80 °C (3x) to remove bacterial cells and residual medium components. Following digestion, BC membranes were rinsed again in dH$_2$O until the pH was stabilized and sterilized by autoclaving at 121 °C for 15 min. The prepared BC membranes were stored at 4 °C prior to further analysis.

2.5. Porosity of BC membranes
The porosity of the BC membranes was determined using the gravimetric method. Wet BC membranes were weighed using an analytical balance, dried at room temperature for 24 h, and then weighed again. Porosity was calculated using equation 1:

\[
P = \frac{W_w - W_d}{\rho_{\text{H}_2\text{O}} \cdot \left(\frac{W_w}{\rho_{\text{H}_2\text{O}}} + \frac{W_d}{\rho_{\text{PES}}}\right)} \times 100, \quad \text{eq. (1)}
\]

where: \(W_w\) is the weight of wet membrane, \(W_d\) is the weight of dry membrane, \(\rho_{\text{H}_2\text{O}}\) is the density of water at 20 °C (0.9982 g/cm$^3$), \(\rho_{\text{PES}}\) is the density of BC, 1.25 g/cm$^3$ (Lee, Blaker & Bismarck, 2009, Mozia, Grylewicz, Zgrzebnicki, Darowna & Czyżewski, 2019).

2.6. PelA$_h$ immobilization on BC membranes
A range of concentrations of PelA$_h$ enzyme, from 0.005 to 0.75 mg/ml, were prepared in SBF (Simulated Body Fluid) buffer (pH 7.4), corresponding to 5.0 to 85.0 mg/g of dry mass of carrier. Wet BC membranes with approximately equal mass were incubated in the appropriate enzyme solution in a total volume of 5 ml with mixing at 25 °C for 5 h. Every hour, a 200-µL sample of the mixture was taken and protein concentration was measured using a microplate reader with Greiner UV-star 96 well plates at 280 nm. The percentage adsorption (%) and the amount of adsorbed protein were calculated according to equation 2 and 3, respectively:

\[
\text{Yield} \left[\%\right] = \frac{C_0 - C_e}{C_0} \times 100, \quad \text{eq. (2)}
\]

\[
Q \left[\frac{\text{mg}}{\text{g}}\right] = \frac{C_0 - C_e}{W} \times V, \quad \text{eq. (3)}
\]
where: \(C_0\) is the protein concentration before adsorption, \(C_e\) is the protein concentration after adsorption, \(W\) is the dry mass of BC membranes in g, and \(V\) is the volume of the solution in cm\(^3\) (Oshima, Taguchi, Ohe & Baba, 2011).

2.7. The effect of PelAh immobilization on \(P.\ aeruginosa\) biofilm formation on BC

To check the effect of enzyme immobilization on the ability of \(P.\ aeruginosa\) to form a biofilm on BC membranes, the reference strain \(P.\ aeruginosa\) PAO-1 was grown in BHI at 37 \(^\circ\)C overnight. Following the cultivation, the culture was normalized to an OD\(_{600}\) of 0.2, diluted 1:100, and spread on BC membranes with immobilized PelAh. Finally, \(P.\ aeruginosa\) PAO-1 cells were grown at 37 \(^\circ\)C overnight.

2.8. \(Pseudomonas\ aeruginosa\) cell amount on BC membranes

The amount of \(P.\ aeruginosa\) PAO-1 released from BC membranes was assessed according to the literature (Tran et al., 2009). BC membranes with formed \(Pseudomonas\ aeruginosa\) PAO-1 biofilm were rinsed gently twice with SBF buffer (pH 7.4) to remove nonadherent bacterial cells. Then, 5 mL of SBF buffer was added to each BC membrane and the samples were vortexed three times for 1 min. The number of bacterial cells released from BC membranes was determined using a BD Accuri C6 Plus flow cytometer.

2.9. Protein Concentration Determination

Protein concentrations were assayed using the Bradford method, with bovine serum albumin as a standard (Bradford, 1976), or by measurement of absorbance at 280 nm using a molar extinction coefficient equal to 44920 M\(^{-1}\)cm\(^{-1}\), as calculated according to the amino acids sequence of PelAh (UniProt accession code Q9HZE4).

2.10. Attenuated Total Reflectance Fourier Transform Infrared Spectral Studies of BC membranes

Prior to attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, samples were dried at room temperature for 24 h. The analysis was carried out using an FTIR spectrophotometer ALPHA II (Bruker Co., Germany) with diamond ATR adapter. The spectra were collected in the range of 4000–400 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\) (32 scans). The obtained ATR-FTIR spectra were analyzed using SpectraGryph 1.2 software. The lateral order index (LOI) of dried samples BC was calculated from the ratio of band intensities at 1427 cm\(^{-1}\) and 895 cm\(^{-1}\). The total crystallinity index (TCI) was calculated from the ratio of bands intensities at 1370 cm\(^{-1}\) and 2900 cm\(^{-1}\). The allomorph \(I_{\alpha}\) was calculated according to equation 4:

\[
I_{\alpha} = 2.55 \left( \frac{A_{750}}{(A_{710} + A_{750})} \right) - 0.32 \quad \text{eq. (4)}
\]
where: $A_{750}$ is an intensity at 750 cm$^{-1}$ for $I_\alpha$, $A_{710}$ is an intensity at 710 cm$^{-1}$ for $I_\beta$ (Yamamoto, Horii & Hirai, 1996).

2.11. Scanning Electron Microscopy (SEM)

BC morphology was examined using VEGA3 (TESCAN) scanning electron microscope. For this purpose, the BC membrane samples with formed biofilm were fixed with 2% glutaraldehyde (v/v) in SBF (pH 7.4) at room temperature for 16 h. Following incubation with glutaraldehyde, samples were rinsed three times in SBF buffer (pH 7.4) for 15 min and dehydrated in ethanol-water series with increasing ethanol concentration (20%, 40%, 60%, 80%, 95% v/v) and finally two times in absolute ethanol for 15 min. The dehydrated samples were then dried at room temperature (Tran et al., 2009). Prior to SEM imaging, samples were sputtered with Au/Pd (60:40) using Q150R ES device (Quorum Technologies, Lough- ton, United Kingdom).

2.12. Cell viability studies

Potential for cytotoxicity or growth inhibitory effect of PelA$_h$ protein was screened using L929 murine fibroblast cell culture, based on ISO 10993-5 and NCI-60 methodology (Holbeck, Collins & Doroshow, 2010). Briefly, a sub-confluent T25 flask of L929 cells was trypsinized and two 96-well plates were plated: one seeded with 10000 cells per well and the second seeded with 5000 cells per well. Cells were maintained in complete growth media: Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Following 24 hours of culture, the media was aspirated and replaced with 100 µL of complete growth media containing either 1 mg/mL PelA$_h$ protein and serial, 3-fold dilutions, or elution buffer as vehicle control. Cell viability was assessed using an inverted light microscope and resazurin viability assay (Riss et al., 2004) after 24 hours of culture (plate seeded with 10000 cells per well) and 48 hours (plate seeded with 5000 cells per well).

2.13. Effect of immobilized PelA$_h$ on cytotoxicity of BC membranes

Potential cytotoxicity of BC membranes with immobilized PelA$_h$ was assessed by testing liquid extracts and performing direct-contact tests, based on ISO 10993-5. Briefly, L929 cells were plated in two plates: one 96-well plate seeded with 10000 cells per well and one 24-well plate, seeded with 50000 cells per well. Both plates were then incubated for 24 hours. In parallel, BC membranes (~2 cm$^2$ and ~3–4 mm thick) with immobilized PelA$_h$ and control, untreated BC membranes were placed individually in wells of 12-well plate, covered with 2 mL of complete growth media, and incubated for 24 hours in a CO$_2$ incubator at 37 °C. Next, the 96-well plate was aspirated, and 100 µL of media incubated with each BC
membrane was added in triplicate. As a sham control, media incubated in an empty well was used. In parallel, the 24-well plate was aspirated, and 500 µL of complete media was added to each well, followed by careful placement of each BC membrane directly on top of the cell monolayer. After a further 24 hours of culture, cell viability was assessed using an inverted light microscope and resazurin viability assay (Riss et al., 2004).

2.14. Statistical analysis
For statistical analysis of the normality of the results, the Shapiro-Wilk test was performed. Statistical analysis of the results was determined by analysis of variance ANOVA, and the statistical significance was performed by Tukey’s multiple comparison test. The level of statistical significance was p < 0.05. All statistical tests were conducted with Statistica 13 software.

3. Results and Discussion
3.1. PelA<sub>H</sub> immobilization on BC membranes - carrier properties
One of the features of <i>K. xylinus</i> is the production of high-quality cellulose, in the form of microfibrils forming a characteristic 3D structure in stationary culture. Each fibril is a linear chain composed of glucose monomers connected by β-1,4-glycosidic bonds. Further, the structure of BC consists of numerous intermolecular and intramolecular hydrogen bonds that stabilize a microfibril chain, which is stiff, linear and insoluble in most solvents (Czaja, Krystynowicz, Bielecki & Brown, 2006, Wang, Zhu & Du, 2011). Here, BC cultivation was carried out for 4 days and the molecular characteristic of obtained BC were typical of young cellulose produced by <i>K. xylinus</i> (Drozd, Rakoczy, Konopacki, Frąckowiak & Fijalkowski, 2017). After purification, the wet mass of the BC membranes was 0.96±0.02 g and dry mass was 9.92±0.28 mg. The obtained values are typical for non-modified BC membranes, where the dry mass of pellicles is approx. 1% of that of hydrated BC (Portela, Leal, Almeida & Sobral, 2019). From the point of view of potential application as a wound dressing material, the porosity and crystallinity of BC are the most important parameters, because they determine the mechanical properties, water vapor transmission, and/or water holding capacity (Li et al., 2015). The porosity of the BC membranes obtained here was 55.34±0.02%. Meanwhile, the crystallinity index values, LOI and TCI, were 2.3±0.18 and 1.6±0.04, respectively, whereas the content of the cellulose I<sub>α</sub> allomorph was 66.4±0.64. These properties of BC membranes are critical for its potential applications and affect the process of adsorbing or releasing of active agents, thereby influencing the enzyme immobilization process (Shah, Ul-Islam, Khattak & Park, 2013; Bayne, Ulijn, & Halling (2013).

3.2. PelA<sub>H</sub> immobilization on BC membranes – process efficiency
PelA₉ was immobilized on the BC membranes by physical adsorption, as a result of the interactions between polar groups of amino acids on the protein surface and the surface of the carrier by weak physical forces. Analysis of the charge distribution on the surface of PelA₉ at pH 7.4 showed that the enzyme molecular surface is mainly negatively charged (-7.77 [e]), in agreement with the results of Le Mauff et al., (2019) (Fig. S2), but putatively also contains positively charged patches (Fig. S3). These regions can be a responsible for facilitating the anchoring of the enzyme via electrostatic integrations to the negatively charged surface of BC membranes (Drozd et al., 2018). Importantly, according to Le Mauff et al., (2019), the active site and surrounding region of the protein surface is negatively charged, which likely enables the correct orientation of immobilized PelA₉ ensuring access to Pel exopolysaccharide secreted by bacterial cells during biofilm formation. The immobilization process was carried out in SBF buffer at pH 7.4 at 25°C for 5 h. The initial amount of PelA₉ in the adsorption medium was varied from 5.0 mg/g to 85 mg/g per dry mass of the BC carrier. The results of adsorption capacity and efficiency of immobilization are presented in Fig. 1. The adsorbed quantity increased with increasing initial protein amount and reached a maximum value of 38.5±5.01 mg per g of the dry mass of BC membrane for the initial amount of PelA₉ 85 mg/g. The yield of immobilization gradually increased up to a maximal value 23% for samples treated with an initial protein amount of 55 mg/g, with final quantity of immobilized PelA₉ 32.35±1.05 mg/g. While the yields are somewhat modest, at this stage we did not focus on process optimization, but rather testing the hypothesis that immobilized PelA₉ could disrupt biofilm formation. The effectiveness of the immobilization process depends on many factors, including the pH, temperature, and ionic strength of the adsorption medium (Jesionowski, Zdarta & Krajewska, 2014; Zdarta, Meyer, Jesionowski & Pinelo, 2018), thus optimization of conditions will require dedicated studies. Importantly, a greater amount of immobilized protein may not actually be beneficial. At the start of the adsorption process, the protein interacts mostly by hydrophobic and electrostatic interaction with the carrier and can cover the available surface. In the next stage, however, it is possible to that a second layer forms, with the protein adsorbing not on the carrier surface but on the present protein adlayer (Roach, Farrar & Perry, 2005; Hirsh et al., 2010). Thus, as a consequence of this process, the apparent biocatalyst activity can be reduced, which is why we tested different initial loading amounts (Hoarau, Badieyan & Marsh, 2017).
3.3. Biofilm formation analysis by ATR-FTIR spectroscopy

In order to assess the effect of immobilized PelA₇ protein on biofilm formation, we cultured *P. aeruginosa* PAO-1 on BC membranes impregnated with different amounts of PelA₇ protein (5.0-85 mg/g of BC membranes), as well as control samples without addition of enzyme. Representative ATR-FTIR spectra of *P. aeruginosa* PAO-1 biofilms on BC membranes are presented in Fig. 2. The spectral region between 1800 cm⁻¹ and 800 cm⁻¹ was analyzed, because it represents fingerprints of biofilms typical for *Pseudomonas sp.* (Suci, Vrany & Mittelman, 1996). The bands within this spectral region can be assigned to specific functional groups of proteins, nucleic acids, and carbohydrates. Because these are the main components of the biofilm matrix, changes in the intensity of these bands reflect the impact of immobilized PelA₇ (Pink, Smith-Palmer, Chisholm, Beveridge & Pink, 2005). The collected ATR-FTIR spectra were characterized by set of bands at the following wavenumbers: 1640 cm⁻¹, 1530 cm⁻¹, 1450 cm⁻¹, 1400 cm⁻¹, 1230 cm⁻¹, 1080 cm⁻¹, and 1040 cm⁻¹ where, depending on the quantity of immobilized PelA₇, the main changes in intensities were observed (Fig. 2a-d). The spectral bands observed at 1640 cm⁻¹ (amide I) and 1540 cm⁻¹ (amide II) were assigned to C = O stretching, C – N, -NH, and -NH₂ bending of protein and peptide amides (Tab. 1). The band at 1530 cm⁻¹ (amide II) is defined as a biomarker of biomass accumulation (Quilès et al., 2010, Wang et al., 2013). The visible band at 1450 cm⁻¹ corresponds to C – H deformation of CH₂ bending bonds, while the band at 1395 cm⁻¹ is
associated with symmetric stretching C – O of carboxylate group (COO\(^{-}\)). The band observed at 1230 cm\(^{-1}\) relates to P = O stretching of >PO\(_2\) of phosphodiesters of nucleic acids (Maquelin et al., 2002; Ojeda & Dittrich, 2012). The region between 1200 cm\(^{-1}\) and 900 cm\(^{-1}\) corresponds to C – O – H, C – C, P – O – P and P=O (stretching) and C – O – C, C – O (ring vibration), thus defining this region as the „carbohydrate region”. In this region, two dominant bands were observed at 1080 cm\(^{-1}\) and 1040 cm\(^{-1}\). The band at 1080 cm\(^{-1}\) was assigned to P = O (symmetric stretching) of >PO\(_2\) corresponding to polyphosphate products, nucleic acids, phosphodiesters, and phosphorylated proteins. The last significant band near 1040 cm\(^{-1}\) was assigned to C – O or C – C stretching and C – O – H bending vibration, typical for carbohydrate components of biofilms (Ojeda & Dittrich, 2012, Sekkal et al., 1993).

Fig. 2. The ATR – FTIR spectra of biofilms formed by \textit{P. aeruginosa} PAO-1 on BC membranes impregnated with PelA\(_h\). Spectra were normalized at 1640 cm\(^{-1}\) (amide I).
Table 1. Assignments bands for analyzed ATR-FTIR spectra *P. aeruginosa* PAO-1 biofilm formed on BC membranes.

<table>
<thead>
<tr>
<th>Wavenumber [cm(^{-1})]</th>
<th>Assignment</th>
<th>Corresponding components</th>
</tr>
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<tbody>
<tr>
<td>1640</td>
<td>N–H coupled with C–N (amide I)</td>
<td>Proteins</td>
</tr>
<tr>
<td>1530</td>
<td>N–H coupled with C–N (amide II)</td>
<td>Proteins</td>
</tr>
<tr>
<td>1450</td>
<td>C–H CH(_2)</td>
<td>Lipids</td>
</tr>
<tr>
<td>1395</td>
<td>C=O str (sym) of COO(^{-})</td>
<td>Amino acids, fatty acid chains</td>
</tr>
<tr>
<td></td>
<td>P = O str (asym) of &gt;PO(_2), C—O—C</td>
<td>Phosphodiester, Phospholipids, LPS,</td>
</tr>
<tr>
<td></td>
<td>stretching, amide III vibrations</td>
<td>nucleic acids, ribose</td>
</tr>
<tr>
<td>1230</td>
<td>P=O str (sym) of &gt;PO(_2)</td>
<td>Phosphodiester, Phospholipids, LPS,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nucleic acids</td>
</tr>
<tr>
<td>1080</td>
<td>O–H coupled with C–O</td>
<td>Polysaccharides</td>
</tr>
</tbody>
</table>

(Assignments according to Quilès et al., 2010; Wang et al., 2013; Maquelin et al., 2002; Filip & Hermann, 2001; Banyay & Sarkar, 2003)

### 3.3.1 2D-COS analysis

In order to examine the effect of the presence of immobilized PelA\(_h\) protein on the composition of *P. aeruginosa* PAO-1 biofilm formed on BC membranes, two-dimensional correlation spectroscopy was performed. The synchronous (a) and asynchronous (b) 2D correlation map of IR spectra of *P. aeruginosa* PAO-1 biofilm on BC membranes after immobilization with different initial amounts of PelA\(_h\) protein is presented in Fig 3. The synchronous spectra (Fig. 3a) show a set of auto peaks on the diagonal position, in the region between 1200 cm\(^{-1}\) and 900 cm\(^{-1}\), and two distinct auto peaks at 1530 cm\(^{-1}\) and 1395 cm\(^{-1}\). The intensity of the auto peaks indicated that the most significant differences occurred within regions characteristic for carbohydrates and proteins, whereas only minor changes in the intensity of carboxylic groups were observed. Changes in auto peak intensity were accompanied by the presence of positive and negative cross-peaks. Three positive cross-peaks between ~1040 cm\(^{-1}\) x ~1530 cm\(^{-1}\), ~1400 cm\(^{-1}\) x ~1530 cm\(^{-1}\) and ~1040 cm\(^{-1}\) x ~1395 cm\(^{-1}\) were found. Compiling these signals from 2D analysis with those from 1D spectra (Fig 2b-d),
indicates that with increasing amount of immobilized PelA$_{h}$ on BC, the band intensities decreased. However, a significant effect was observed only for BC impregnated with at least 15 mg/g of enzyme, with no significant differences between the last two tested enzyme quantities. Our results are consistent with biofilm disruption, because during undisturbed development of *Pseudomonas sp.* biofilm an increase in amide II, phospholipids, carboxyl group, and carbohydrate is expected, indicating an accumulation of biofilm components (Quilès & Humbert, 2014). Similarly, Delille, Quilès & Humbert (2007) observed a gradual decrease in the intensity of these bands for *Pseudomonas fluorescens* biofilm formed with low carbon medium as a stressor.

In addition to the positive cross-peaks, the synchronous spectra contain several negative cross-peaks between: $-1140 \text{ cm}^{-1} \times -1530 \text{ cm}^{-1}$, $-1450 \times -1530 \text{ cm}^{-1}$, $-1230 \text{ cm}^{-1} \times 1530 \text{ cm}^{-1}$, $-1038 \text{ cm}^{-1} \times -1230 \text{ cm}^{-1}$, $-980 \text{ cm}^{-1} \times -1395 \text{ cm}^{-1}$ and $-980 \times -1530 \text{ cm}^{-1}$. The negative cross-peaks indicate that changes in the amount of the specific components of the biofilm matrix are occurring in opposite directions (Noda, Dowrey, Marcott, Story & Ozaki, 2000). Specifically, here the discovered negative cross-peaks show that immobilized PelA$_{h}$ yielded a reduction of protein and peptide amides and carbohydrate components, but also an increase in the amount of lipids and nucleic acids (Table 1).

**Fig. 3.** Two-dimensional correlation spectroscopy (2D-COS): (a) synchronous and (b) asynchronous spectra of *P. aeruginosa* biofilm grown on BC membranes over the spectral range of 1800 cm$^{-1}$ to 800 cm$^{-1}$. 
3.3.2 Analysis of biofilm component changes via ratio of specific bands from ATR-FTIR spectra

To analyze the effect of the amount of immobilized PeIA₇ on the changes in the intensities of individual bands reflecting changes in biofilm structure, we calculated the average integrated areas of selected bands analyzed in 1D and 2D-COS spectra (Fig. 4).

![Bar charts showing changes in band ratios](image)

Fig. 4. The relative changes in the band ratio 1530 cm⁻¹:1230 cm⁻¹ (a), 1530 cm⁻¹:1450 cm⁻¹ (b), 1530 cm⁻¹:1395 cm⁻¹ (c), 1230 cm⁻¹:1040 cm⁻¹ (d) and 1230 cm⁻¹:1395 cm⁻¹ (e). Means that have no superscript in common are significantly different from each other (p < 0.05). Error bars represent standard deviation.
with increasing PelA<sub>b</sub> immobilization, indicating decreased carboxyl group content, but this effect was statistically significant only for the highest loading of PelA<sub>b</sub> (Fig. 4c). Finally, the changes in the ratio of the band at 1230 cm<sup>-1</sup> to that at 1040 cm<sup>-1</sup> indicated an increasing amount of phospholipids, LPS, and nucleic acids with decreasing polysaccharide content (Fig 4 d, e). The specific degradation of biofilm by PelA<sub>b</sub> results in the decomposition of Pel polymer that has N-acetylgalactosamine and N-acetylglucosamine as principal elements, which can also contribute to the intensity of ATR-FTIR bands specific for protein amide II (1540 cm<sup>-1</sup>, C-N) (Jennings et al., 2015; Kumirska et al., 2010). The observed decreased intensity of this band can thus be interpreted as indicating a reduction in the amount of Pel present in the biofilm matrix structure. The <i>P. aeruginosa</i> biofilm can also contain alginate that is composed form galacturonic acid rich in COO<sup>-</sup> (1395 cm<sup>-1</sup>) and reduction in band intensity can also indicate change in amount of this element of biofilm matrix (Nivens, Ohman, Williams & Franklin, 2001; Woźniak et al., 2003; Kovács, Nyerges, & Izvekov, 2008).

3.6. Influence of PelA<sub>b</sub> immobilization on <i>P. aeruginosa</i> cell release from BC.

Given the important role of biofilm matrix in protecting bacterial cells and facilitating their adhesion, we assessed the effect of the immobilized PelA<sub>b</sub> protein on the detachment of <i>P. aeruginosa</i> PAO-1 cells from biofilm, by analyzing the quantity of cells released from BC membranes. Fig. 5. shows the amount of <i>P. aeruginosa</i> PAO-1 cells recovered from the BC membranes impregnated with different initial amount of PelA<sub>b</sub> (5 – 85 mg/g of carrier) and covered with biofilm.

![Fig. 5. Amount of <i>P. aeruginosa</i> PAO-1 cells released from BC membranes. Means that have no superscript in common are significantly different from each other (p > 0.05). Error bars represent standard deviation.](image-url)
The number of cells was determined by measuring CFU of adherent cells released to SBF buffer using flow cytometry. The largest amount of *P. aeruginosa* PAO-1 cells was released from BC membranes impregnated with 85 and 55 mg of PelA<sub>H</sub> per g of carrier - the CFU was $3 \times 10^5$ and $4 \times 10^5$, respectively. No statistically significant changes were observed between 5, 15 and 30 mg/g PelA<sub>H</sub> loadings, but number of liberated cells was significantly higher than that for control samples without PelA<sub>H</sub> enzyme. These results indicate that the presence of immobilized PelA<sub>H</sub> enzyme had an influence on biofilm formation: the disrupted integrity of the biofilm matrix, resulted in easier release of adherent cells from the BC surface. This observation is in good agreement with the existing literature, including the results of Tian et al. (2014) which suggested that Pel plays a critical role in the initial bacterial cell attachment to a colonized surface. Moreover, in an earlier study Ghafoor et al. (2011) showed that *P. aeruginosa* mutants deficient in Pel production lost their ability to form stable biofilms. Likewise, glass surfaces modified with the glycohydrolase PslG<sub>H</sub> with specificity towards Psl were found to have inhibited attachment of bacterial cells (Asker, Awad, Baker, Howell & Hatton, 2018). At the same time, previous studies also indicate that the glycoside hydrolase PelA<sub>H</sub> does not influence directly bacterial cell growth (Baker et al., 2016). However, Pestrak et al. (2019) reported that the addition of PelA<sub>H</sub> can enhance the action of several antibiotics. Overall, the immobilization of PelA<sub>H</sub> on the BC surface may allow for more effective inhibition of bacterial growth using smaller doses of antibiotics, even without reducing the number of bacterial cells, by potentially destabilizing the biofilm (Tian, Xu, Hutchins, Yang & Li, 2014; Ghafoor, Hay & Rehm, 2011; Pestrak et al., 2019).

3.7. SEM analysis of biofilm morphology

Additionally, scanning electron microscopy was used to assess the morphology of the *P. aeruginosa* PAO-1 biofilm cultivated on BC membranes with the PelA<sub>H</sub> enzyme (85 mg/g mg per g of carrier) as compared to controls without immobilized PelA<sub>H</sub> (Fig. 6 a,b). The SEM images showed that after overnight incubation, the surface of control BC membranes was evenly covered with bacterial cells and appeared smooth, indicating bacterial cells surrounded by elements of biofilm matrix. In contrast, on BC membranes impregnated with PelA<sub>H</sub> the individual *P. aeruginosa* PAO-1 cells are much more distinct and visible. Snarr et al. (2017) observed the similar effect of PelA<sub>H</sub> treatment on biofilm formed by *Aspergillus fumigatus*, with a visible reduction of polysaccharide elements of the biofilm matrix associated with cell wall of hyphae. Likewise, Soler-Arango, Figoli, Muraca, Bosh & Breles-Mariño (2018) observed that using gas discharge plasma resulted in the significant reduction *P. aeruginosa* biofilm matrix and bacterial cells were more distinct on SEM images.
3.8. Cytotoxicity of native and immobilized PelAₜ

From the standpoint of possible application in wound dressings, it was important to confirm that the obtained BC membranes were not cytotoxic (Wittaya-areekul & Prahsarn, 2006; Morin & Tomaselli, 2007). To check for potential cytotoxicity of the PelAₜ protein itself, L929 murine fibroblasts were exposed to a range of enzyme concentrations (~0.004-1 mg/mL) for either 24 or 48 hours. After 24 hours, no dose response was observed (Fig. 7c). Likewise, microscopy did not reveal any changes in morphology that could indicate cytotoxicity (Fig. 7b). After 48 hours, a ~21% reduction in viability was noted for the highest amount of enzyme, 1 mg/mL (Fig. 7f); however, microscopy did not indicate cytotoxicity (Fig. 7d). Based on the observed normal morphology at 48 hours and the lack of pronounced cytotoxicity after 24 hours, the reduction in viability was ascribed to a modest
growth inhibitory effect (10-15%) of the highest enzyme dose on L929 cells, which typically have a doubling time of ~20 hours, rather than cytotoxicity.

Fig. 7. Representative micrographs of L929 cells exposed to (a) vehicle control or (b) 1 mg/mL PelA₆ protein for 24 hours; (c) vehicle control or (d) 1 mg/mL PelA₆ protein for 48 hours and cell viability normalized to vehicle control of L929 cells exposed to range of recombinant enzyme for (e) 24 hours and (f) 48 hours. Each point represents a technical replicate, n = 5.
Next, potential cytotoxicity of BC membranes with immobilized PelA \textsubscript{h} was assessed, based on ISO10993-5. We tested the effect of both liquid extracts prepared in complete growth media, as well as BC membranes placed in direct contact with L929 cells. After 24-hour incubation, there was no cytotoxic effect of extracts of BC membranes with immobilized PelA \textsubscript{h} or control membranes with no enzyme (Fig. 8e).

![Image of micrographs and bar graphs](image)

**Fig. 8.** Representative micrographs of L929 cells after 24 hours of incubation in direct contact with cellulose discs with the immobilized enzyme (top row) or without (bottom row). Micrographs on the left were taken with disc remaining in well, while those on the right are of the same well, but after careful removal of the disc and viability of L929 cells (normalized to sham) after 24 hours of: (e) incubation with liquid extracts (complete media, 24 hours, 37 °C) of cellulose discs with PelA \textsubscript{h} protein or without (Control) or (f) incubation in direct contact with cellulose discs with PelA \textsubscript{h} protein or without (Control).

More importantly, there was also no decrease in the viability of L929 cells incubated in direct contact with either type of BC membranes (Fig. 8f). Further, microscopy revealed robust...
cell proliferation beneath the tested membranes and removal of the BC membranes did not appear to cause damage to the monolayer (Fig. 8a-d), indicating low adhesion.

Overall, our results are in good agreement with previous data obtained for IMR-90 human lung fibroblast cells treated with by PelAₜ₉ protein (Baker et al., 2016). In another study, Snarr et al., 2017 also confirmed a lack of toxicity of PelAₜ₉ using IMR-90 human lung fibroblast cells and lung cancer (line A549) cells. In addition, PelAₜ₉ protein has also been shown to be nontoxic to human red blood cells and did not have any impact on neutrophil function (Pestrak et al., 2019). Collectively all these results indicate that neither native nor immobilized PelAₜ₉ affects the morphology nor viability of mammalian cells. Thus, these promising in vitro results motivate further studies of these materials as wound dressings in animal models, in order to assess risk of sensitization and irritation.

4. Conclusions
Bacterial biofilms are responsible for almost 80% of microbial infections in humans. Bacterial cells within biofilm reveal from 10 to 1000 times the highest resistance to antibiotics than planktonic form of these bacteria (Khatoon, McTiernan, Suuronen, Mah & Alarcon, 2018). One of the main challenges in the treatment of chronic wounds is the protection against biofilm-mediated infection. In case of BC-based wound dressings, due to the structure of BC it is possible to carry out several modifications aimed at obtaining bactericidal properties, by introduction of specific functional group or by the addition of another biopolymer (Lin et al 2013., Figueiredo, Figueiredo, Silva, Timmons, Almeida, Silvestre & Freire, 2015). Alternately, one can rely on adsorbing an active substance to be delivered to the wound, such as quaternary ammonium salts or octenidine (Moritz et al., 2014, Żywicka, Fijalkowski, Junka, Grzesiak & El Fray, 2018). However, antibiotics and antiseptic agents are not always able to fully inhibit microbial growth, particularly if a biofilm is formed. Further, the phenomenon of drug resistance forces us to look for new safe and effective methods for the treatment microbial infections, particularly those caused by bacteria that form biofilms. Here we explored a novel approach towards addressing this problem in chronic wound infection. We developed an effective method of BC dressing protection by immobilization of the hydrolytic domain of the PelAₜ₉ protein on BC membranes. We showed that immobilized PelAₜ₉ resulted in destabilization of biofilm formed by Pseudomonas aeruginosa. Importantly, no cytotoxic effect was detected indicating the safe application of PelAₜ₉ in novel BC-based dressing materials. Collectively, our results suggest the potential of the obtained materials in the development of „intelligent” wound dressings that can mitigate the risk of P. aeruginosa infection during chronic wound therapy. Further, the developed method can be potentially improved by combining a set of specific glycohydrolases, including those specific for other elements of biofilm matrix. This could yield a synergistic effect, allowing for even more
effective biofilm eradication and as well as decreasing the required dosage of any additional antimicrobial chemotherapeutic.

**Author contributions**

Magdalena Szymańska: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Investigation, Visualization Jolanta Karakulska: Writing - Review & Editing Peter Sobolewski: Investigation, Writing - Review & Editing Urszula Kowalska: Investigation Bartłomiej Grygorcewicz: Investigation, Writing - Review & Editing Dominique Böttcher: Resources, Writing - Review & Editing Uwe T. Bornscheuer: Resources, Writing - Review & Editing Radosław Drozd: Conceptualization, Methodology, Formal analysis, Project administration, Investigation, Writing - Original Draft, Visualization, Supervision.

**Conflicts of Interest:**

The authors declare no conflicts of interest.

5. References


