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Fibrous matrices facilitate pleurocidin killing of wound associated bacterial pathogens

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ABSTRACT

Conventional wound infection treatments neither actively promote wound healing nor address the growing problem of antibacterial resistance. Antimicrobial peptides (AMPs) are natural defense molecules, released from host cells, which may be rapidly bactericidal, modulate host-immune responses, and/or act as endogenous mediators for wound healing. However, their routine clinical use has hitherto been hindered due to their instability in the wound environment. Here we describe an electrospun carrier system for topical application of pleurocidin, demonstrating sufficient AMP release from matrices to kill wound-associated pathogens including *Acinetobacter baumannii* and *Pseudomonas aeruginosa.* Pleurocidin can be incorporated into polyvinyl alcohol (PVA) fiber matrices, using coaxial electrospinning, without major drug loss with a peptide content of 0.7% w/w predicted sufficient to kill most wound associated species. Pleurocidin retains its activity on release from the electrospun fiber matrix and completely inhibits growth of two strains of *A. baumannii* (AYE; ATCC 17978) and other ESKAPE pathogens. Inhibition of *P. aeruginosa* strains (PAO1; NCTC 13437) is, however, matrix weight per volume dependent, with only larger/thicker matrices maintaining complete inhibition. The resulting estimation of pleurocidin release from the matrix reveals high efficiency, facilitating a greater AMP potency. Wound matrices are often applied in parallel or sequentially with the use of standard wound care with biocides, therefore the presence and effect of biocides on pleurocidin potency was tested. It was revealed that combinations displayed additive or modestly synergistic effects depending on the biocide and pathogens which should be considered during the therapy. Taken together, we show that electrospun, pleurocidin-loaded wound matrices have potential to be investigated for wound infection treatment.

1. Introduction

Wound infections are a growing problem worldwide; their prevalence is increasing while treatment becomes complicated by antimicrobial resistance (AMR). AMR is a named priority for global public health and, in 2019, was associated with 4.95 million deaths, of which 1.27 million were directly attributable (Murray et al., 2022). Most wounds are contaminated and the risk for the development of infection can be as much as 4% even for surgical wounds (Berriós-Torres et al., 2017). Burn wound patients lose the barrier function of the skin and burn injury causes immune system dysfunction (Guo et al., 2022; Haghi et al., 2018; Javanmardi et al., 2019; Ladhani et al., 2021). Burn wounds in particular therefore have an extremely high risk for the development of infection with even small and simple wounds liable to secondary infection development and morbidity (Hermans, 2019). Major challenges for the development of treatments for wound infections are the presence of bacterial biofilm as well as the presence of extremely virulent and/or resistant wound pathogens (Malone and Schultz, 2022). *Pseudomonas aeruginosa* is the leading pathogen in invasive burn wound infection due to its virulence factors (e.g. flagellum, toxins, hemolysins, and proteolytic enzymes) (Qin et al., 2022) but burn wound infections caused by *Acinetobacter baumannii* are also a major problem in burn

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hospitals due to its high survival rate on hospital surfaces for prolonged time (Tekin et al., 2014). The prevention and treatment of wound infections caused by these difficult to eradicate bacteria is of the utmost importance, to mitigate the significant burden they cause to patients and the associated, elevated healthcare costs.

Antimicrobial peptides are part of the innate immune system of many different living organisms and are of considerable interest in this regard due to their broad mechanism of action, high potency and ability to overcome AMR. Their antibacterial mechanism of action mainly involves membrane disruption and/or genetic material damage (Miao et al., 2021). They have been widely investigated, not only because of their direct and wide antimicrobial activity but because these molecules may be involved in other processes including inflammatory regulation and angiogenesis promotion. For this reason, they are often referred as host defence peptides (Patel and Akhtar, 2017). Pleurocidin is a well-studied cationic antimicrobial peptide isolated from the Winter Flounder (*Pseudopleuronectes americanus*). It is 25 amino acid residues long and, though it can adopt α-helix conformation, it is characterised by high conformational flexibility which may underpin its relatively high antibacterial potency (Amos et al., 2016). Unlike other antimicrobial peptides, pleurocidin has shown negligible haemolytic effect on human erythrocytes (Manzo et al., 2020). Further, pleurocidin has shown other immunomodulatory characteristics, including the promotion of mast cell adhesion to fibronectin (Pundir et al., 2014), that support its development as a wound healing therapy.

Despite these properties, antimicrobial peptides also have perceived limitations which include low chemical stability and, particularly when used systemically (Lei et al., 2019; Silphaduang and Noga, 2001), host cell toxicity. The instability of antimicrobial peptides renders their delivery complicated and requires suitable technology. Indeed, because proteolytic activity is high in infected tissue, antimicrobial peptides may be degraded particularly quickly in this environment (Starr and Wimley, 2017). Therefore, increasing the stability of peptide drugs through a suitable delivery system is critical in their successful clinical application (Cesaro et al., 2023; Nasseri and Sharifi, 2022; Thapa et al., 2020). Traditional topical formulations and dosage forms, such as creams and gels, have been used, but often biofilm formation in the wound and/or the presence of wound exudate hinder their efficacy. Therefore, to overcome these issues, novel wound dressings are needed.

Electrospinning is a technique which delivers high voltage to a polymeric solution to create fibers with diameters ranging from nano- to micrometres. These fibers are then collected, forming a matrix that can be used as a wound dressing, proven to have characteristics that improve wound healing (Afsharian and Rahimnejad, 2021) and which may provide an opportunity to incorporate active drug substances (Memic et al., 2019; Preem et al., 2017), including antimicrobial drugs such as antimicrobial peptides (Dart et al., 2019). Electrospun matrices consisting of antimicrobial peptides have been developed not only for wound treatment but also other applications including food preservation (Gatti et al., 2013; Kielholz et al., 2022; Wang et al., 2015). Mostly shorter cyclic antimicrobial peptides (e.g. tyrothricin), but also LL-37 and pleurocidin have been directly electrospun from polymeric solutions via monoaxial electrospinning and/or parallel electrospinning techniques and their antimicrobial activity has been demonstrated (Gatti et al., 2013; Kielholz et al., 2022; Wang et al., 2015). Often electrospun dressings have been used as substrates on which antimicrobial peptides have been attached via adsorption from solution (Amariei et al., 2018), immobilized via coupling (Song et al., 2016) or via electrospraying of antimicrobial peptide loaded nanoparticles (He et al., 2018). However, to our knowledge no pleurocidin-loaded fiber matrix prepared by coaxial electrospinning has been reported. Core-shell (or core-sheath) fibers are usually produced by coaxial electrospinning, in which a concentric needle and two different fluids are used (Moghe and Gupta, 2008). The method allows controlling the structure of fibers, encapsulating drugs or biological agents into the polymer fibers, controlling the release of drugs from fibers, preparing fibers from materials that lack filament-forming properties or from unspinnable polymer solutions, and improving fiber quality (Yarin, 2011; Yu et al., 2011).

Pleurocidin has previously been electrospun using polyvinyl alcohol (PVA) aqueous solutions via monoaxial electrospinning for food applications, but with lower pleurocidin concentrations than might be needed to kill wound pathogens (Wang et al., 2015). Indeed, while these reported pleurocidin concentrations (0.03–0.25% w/w in solid state fibers) were able to kill less virulent *E. coli* (Wang et al., 2015), it is not known whether the antimicrobial activity of pleurocidin-loaded electrospun fiber matrices can be effective against *A. baumannii* or *P. aeruginosa* and hence suited for wound healing applications. Further, it is not known how the release of pleurocidin from electrospun matrices and the presence of such polymeric carrier systems are able to modify the antimicrobial potency and activity of antimicrobial peptides. Interestingly, the presence of other antimicrobial agents (e.g. common biocides- antiseptics used for wound treatment) may also affect the antimicrobial potency of pleurocidin. It has been shown that pleurocidin shows synergistic activity when used in combination with antibiotics through hydroxyl radical formation and membrane-active mechanism, and additionally it shows antibiofilm activity (Choi and Lee, 2012). When antibiotics and biocides are used in combination then the efficacy of both types of substances may be changed and both enhanced as well as decreased physiological effects may occur (Pietsch et al., 2021). Common biocides (e.g. silver nitrate, polyvinyl pyrrolidone iodine (povidone iodine), octenidine etc.) are used and recommended by wound associations for the cleaning of wounds (Babalska et al., 2021; Probst et al., 2022), but their presence in the wound is often neglected while the effect of novel antimicrobial peptides has been investigated.

The aim of the present study therefore, is to develop electrospun pleurocidin-loaded fiber matrices, with high pleurocidin concentrations using coaxial electrospinning, and understand their production, physicochemical properties and antibacterial activity against different pathogens relevant to wound infections. Further, we investigate whether pleurocidin activity is affected by the presence of common biocide formulations used for wound infection treatment in case pleurocidin might benefit from additive or synergistic interactions.

2. Materials and methods

2.1. Materials

Antimicrobial peptide, polymer, solvents. An antimicrobial peptide pleurocidin was purchased from Cambridge Research Biomedicals (Cleveland, UK) as desalted grade (crude). The peptide was amidated at the C-terminus. Biodegradable high-quality polyvinyl alcohol (PVA, KURARAY POVALTM 26–88 FA, low MW, partially saponified) as a hydrophilic polymer was obtained as a gift from Kuraray. Distilled water was applied as a solvent for electrospinning. Other solvents used for peptide purification were acetonitrile, trifluoroacetic acid (TFA) and acetic acid (10%). All solvents were purchased from Sigma-Aldrich and were of reagent grade and used as received without any further purification. All biocides: chlorhexidine digluconate (chlorhexidine, solution 20% in water), benzalkonium chloride (≥95%), silver nitrate (Bio-Reagent, >99%), silver sulfadiazine (98%), and poly(vinylpyrrolidone)-iodine complex (povidone iodine) were purchased from Sigma-Aldrich (Steinheim, Germany) except for octenidine dihydrochloride (octenidine), supplied by Schülke & Mayr (Norderstedt, Germany).

Buffers, growth media. Mueller-Hinton broth (MHB) was purchased from Thermo Fisher Scientific, Oxoid (Basingstoke, UK). All bacteria were grown in MHB culture medium. Phosphate buffered saline (1 x PBS) pH adjusted to 7.4 was used in this study.

2.2. Antimicrobial peptide purification

The crude peptide purchased was further purified using reverse

phase chromatography with a Waters SymmetryPrep C8, 7 mm, 19 × 300 mm column. A gradient of H_2O/TFA 0.1% and acetonitrile/TFA 0.1% was used for this purpose, the method that was previously validated using matrix assisted laser desorption ionization (MALDI) mass spectroscopy (Mason et al., 2006). The collected phase was then vacuum centrifuged to eliminate acetonitrile, lyophilised, resolubilised in 10% acetic acid and lyophilised again, removing the TFA counterion.

2.3. Development of electrospun matrices with pleurocidin

2.3.1. Preparation of electrospinning solutions

Hydrophilic polymer PVA was dissolved in distilled water with heating up to 80 ± 5 °C, without boiling (Palo et al., 2019). Heating was performed until the polymer was completely dissolved, the usual heating time was up to 1 h. PVA concentrations for coaxial electrospinning were 16% w/w for the inner solution (core solution) and 15% w/w for the outer solution (shell solution). Antimicrobial peptide was added into the inner solution, therefore all drug-containing formulations consisted of antimicrobial peptide within the core of the fibers. Exact electrospun matrix formulation and electrospinning method are described in Table 1.

Magnetic stirrer was always used for preparing all the electrospinning polymer solutions. In addition, pristine polymeric fiber matrices without any pleurocidin were always created and used as controls. Solution for electrospinning was obtained after 20 ± 2 h of stirring at room temperature (RT, 24 ± 2 °C). Peptide was always added into the polymer solution 20 ± 5 min prior to electrospinning and dissolution of the peptide accelerated by constant stirring.

2.3.2. Electrospinning technique

Electrospinning was performed using an ESR200RD robotized electrospinning system (NanoNC, Seoul, Republic of Korea). Two plastic syringes (12 mL) were connected to a concentric coaxial needle (p/n100–10-COAXIAL-2218, Rame-Hart Instrument Co) with inner channel size of 22 G and outer channel size of 18 G. The inner channel was loaded with a polymeric solution containing the active compound whereas the outer channel was loaded just with pristine polymer solution. Fibers were collected on a roller collector (diameter 9 cm, width 6 - 7 cm, rotating at 25 rpm) covered with aluminium foil. The solution flow rate was the same for both solutions 0.2 mL/h, the distance between the spinneret and collector was 15 cm. The voltage applied was +15 kV.

Electrospinning was performed at ambient conditions (temperature of 24 ± 2°C and relative humidity (RH) varied between 16 and 30%). Electrospun fiber matrices were stored in open zip lock bags at RT, 0% RH and vacuum desiccator (above silica gel) before further analyses.

2.4. Electrospun fiber matrix characterization

2.4.1. Morphology

The morphology of electrospun fiber matrices was investigated using scanning electron microscopy, SEM (Zeiss EVO 15 MA, Hamburg, Germany), working with a secondary electron detector (accelerating voltage of 20–25 kV). Random areas were selected to study the diameter and surface topography of each matrix. For SEM analysis, matrices were mounted on aluminium stubs and magnetron-sputter coated with a 3-nm platinum layer in an argon atmosphere prior to microscopy. Micrographs of the top layer were analysed. Distribution of fiber diameters was manually measured by using Image J v.1.53i. software ($N = 100$).

2.4.2. Drug content

Three random 4 cm^2 pieces were cut and weighted to study the content of the drug and also evaluate its distribution across the fiber matrix. These pieces were dissolved in 10% acetic acid and studied for the peptide concentration using high performance liquid chromatography (HPLC). The stability of peptide in this solvent was separately verified. HPLC analysis was performed using modular Prominence HPLC (Shimadzu Europa GmbH, Duisburg, Germany) with solvent delivery unit LC20AD, PDA detector (wavelength at 220 nm) and Phenomenex Luna C18(2), 250 × 4.6 mm 5 μm column. For mobile phase A 0.1% (V/V) TFA in water and mobile phase B 0.1% (V/V) TFA in acetonitrile was used. The flow rate was 1.0 mL/min and injection volume 20 μL, gradient scheme used was: 0–3 min B 10%; 3–20 min B 10% -> B 80%; 20–25 min B 80%; 25–29 min B 10%. Peptide solution in water (using the theoretical peptide concentration in the fiber matrix) was used as a control.

2.5. Bacterial studies

2.5.1. Bacterial strains

Strains utilized in antibacterial activity studies of the peptide and biocides included *Acinetobacter baumannii* AYE, *A. baumannii* ATCC 17978, *Pseudomonas aeruginosa* PAO1 (reference laboratory strain, obtained originally from infected wound), *P. aeruginosa* NCTC 13437 (multi-drug resistant). These bacteria among with epidemic methicillin-resistant *Staphylococcus aureus* EMRSA-15 (NCTC 13616), *Klebsiella pneumoniae* M6, NCTC 13368 and *Escherichia coli* NCTC 12923 were selected as relevant multiresistant bacteria to study the potential antimicrobial activity of the electrospun antimicrobial peptide-loaded matrices and peptide in solution. Bacteria specified below in Table 2 were obtained through the British National Collection of Type Cultures (NCTC) or American Type Culture Collection and stored at - 80 °C.

2.5.2. Antibacterial activity assay – pleurocidin loaded electrospun fiber matrices

The antibacterial activity of antimicrobial peptide-loaded fiber matrices (PVA + H_2O + Pleurocidin) was analysed using MIC-like assay.

Table 2
Bacterial strains used in the present study together with references.

Bacterial species	Abbreviation used in study	Strain designations	References
Acinetobacter baumannii	AB AYE	AYE; ATCC BAA-1710D-5	(ATCC, 2023a)
Acinetobacter baumannii	AB 17978	5377; ATCC 17978	(ATCC, 2023b)
Pseudomonas aeruginosa	PA 13437	NCTC 13437	(NCTC, 2023a)
Pseudomonas aeruginosa	PAO1	DSM 22644, ATCC 15692, LMG 12228	(DSMZ, 2023)
Staphylococcus aureus	EMRSA-15	NCTC 13616	(Köser et al., 2012; NCTC, 2023b)
Klebsiella pneumoniae	KP M6	M6; MW228061	(Kumar et al., 2021)
Klebsiella pneumoniae	KP 13,368	NCTC 13368, ATCC 700603, CCUG 45421, LMG 20218	(NCTC, 2023c)
Escherichia coli	EC 12923	NCTC 12923; ATCC 8739. CIP 53.126, WDCM 00196	(NCTC, 2023d)

Table 1
Electrospinning method and formulation. Key: Pleu- pleurocidin.

Electrospinning method	Solvent /polymer	PVA shell solution (mg/mL)	PVA core solution (mg/mL)	Pleu concentration (mg/mL)	Pleu% w/w theoretical content
Coaxial	H_2O / PVA	149.2	162.1	2.2	0.7

0.6 cm diameter pieces of the electrospun fiber matrix were cut, weighed and inserted into the wells of a 96-well polypropylene microtiter plate previously filled with 100 μL of MHB. For investigating the relation of matrix weight and activity on *P. aeruginosa* PAO1 and *P. aeruginosa* NCTC 13437, different weights were selected in triplicates depending on the bacterial strains and their corresponding MIC values, thus for *P. aeruginosa* PAO1 fiber matrices, the weights ranged from 200 μg to 600 μg, and in case of *P. aeruginosa* NCTC 13437, the matrix weights up to 1000 μg.

The theoretical concentration of antimicrobial peptide pleurocidin in each well was calculated based on each independent matrix weight and this information was used for the preparation of positive controls. Non-loaded control electrospun matrices and filter paper (Whatman, grade 1) were used as negative controls. 100 μL of bacteria were added at a starting concentration of 10^5 CFU/mL, from a diluted overnight culture. Plates were incubated for 20 h at 37°C in static conditions. Matrices and controls were carefully removed from each well before using the plate reader. Bacterial growth was visualised against a black surface. Although the matrix is hydrophilic, the loss of medium after removal was negligible and no major changes between samples nor controls were found. The drug incorporated within fiber matrices did not affect the loss of medium after matrix removal compared to the non-loaded matrices nor controls.

2.5.3. Antibacterial activity assay – pleurocidin and biocides in solution

The antibacterial activity of pleurocidin and biocides was assessed through a modified two-fold broth microdilution assay with modal minimal inhibitory concentrations (MICs) generated from at least three biological replicate experiments. The method broadly followed EUCAST methodology, with the exception that in this case non-cationic-adjusted MHB and polypropylene plates were used, as recommended for cationic antimicrobial peptides (Wiegand et al., 2008). All peptide and biocides stock solutions, except for silver sulphadiazine, were prepared using Milli-Q water as solvent. Silver sulphadiazine was dissolved in DMSO: MHB (1:100) for solubility enhancement. Peptide and biocides were diluted in a two-fold dilution in media down on a 96-well polypropylene microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany) to a final volume of 100 μL. The same volume of bacterial dispersion was then added, at a starting concentration of 10^5 CFU/mL, from a diluted overnight culture. Plates were incubated for 20 h at 37°C in static conditions. The OD_{600} was determined using a Clariostar plate reader (BMG Labtech). The MIC was defined as the lowest concentration where OD_{600} was below a value of 0.1 after subtracting the background absorbance and there was no visible growth.

2.5.4. Checkerboard assay

Phenotypic effects (synergy, antagonism, additive and indifferent) between pleurocidin and biocides in solution were measured using standard microdilution checkerboard assay. The two-fold dilution series of pleurocidin and one biocide were prepared in MHB in each 96-well polypropylene microtiter plate to a final volume of 100 μL. The biocide was first diluted across the columns leaving the last one free from biocide (peptide MIC column). Then, the pleurocidin was diluted down the plate, leaving the last row free from pleurocidin (biocide MIC row). Afterwards, 100 μL of bacterial dispersion, at a starting concentration of 10^5 CFU/mL, from a diluted overnight culture were added in each well. Preparing these two-fold dilutions in the same microtiter plate results in the second compound added (pleurocidin) decreasing its concentration by half with each dilution. However, the first compound (biocide) is not always reduced by half with each dilution. The biocide was first diluted across columns and then, during the pleurocidin dilution process, it got reduced again across rows. In the first row, where pleurocidin solution was added, the biocide was diluted by half. However, when pleurocidin was diluted through following rows, the starting solution contained in row A consists of pleurocidin but also biocide. This biocide is transferred to the following row, increasing its final concentration, and so this occurs across the plate. This deviation from a two-fold dilution series has been considered, and real concentrations for biocides were calculated and incorporated in the analysis (schematics shown in Fig. 5**A and** 5**B**). The growth or absence of growth was concreted using the same definition as the MIC. The fractional inhibitory concentration (FIC) was calculated with the formula (Eq. (1)):

$$FIC = \frac{MIC\ A_{\ A+B}}{MIC\ A} + \frac{MIC\ B_{\ A+B}}{MIC\ B} \quad (1)$$

where A corresponds to the compound A alone, B corresponds to the compound B alone and $A + B$ corresponds to both compounds in combination.

FIC presented was calculated using three independent experiments and presented together with an arithmetic mean. FIC $\leq$ to 0.5 were considered strongly synergistic, and consistent with a recent interpretation of FIC, which stressed the importance of measuring the MIC in the same microarray plate, values of 0.5 to <1 were considered to indicate weak synergism (Fratini et al., 2017). Indifferent effect is defined when $1 < FIC < 2$ and antagonism behaviour for FIC values ≥ 2 (EUCAST, 2000).

2.6. Statistical analyses

Results were expressed as an arithmetic mean ($n = 3$) ± standard deviation (SD), unless mentioned otherwise. Statistical significance of fiber diameter distributions between different formulations was calculated by applying one-way ANOVA and post hoc *t*-test (two-sample assuming unequal variances) with Microsoft Excel v.16.67 software ($p < 0.05$). A non-linear regression model (four-parameter logistic curve) was fitted using absorbance values (OD_{600}) of drug solution controls for bacterial strains. Data analysis was performed using GraphPad Prism 9 and Microsoft Excel v.16.67.

3. Results and discussion

3.1. Preparation of electrospun pleurocidin-loaded fiber matrices and their initial physicochemical characterisation

Electrospun fibrous dressings are known to act as local drug carriers for the treatment of wounds providing desired and controlled release of the AMPs incorporated within them (Dart et al., 2019; Kielholz et al., 2022; Yüksel et al., 2016). Therefore, pleurocidin was incorporated into fibrous wound dressing via coaxial electrospinning. Coaxially electrospun matrices are known to provide protective environment for the peptide during electrospinning and storage (Jiang et al., 2014) and therefore it was hypothesised that higher peptide concentrations within matrices could be obtained using coaxial electrospinning. Hence, here we use coaxial electrospinning of pleurocidin to achieve a theoretical drug concentration up to 0.7% w/w in solid state, almost three times higher than previously reported (Wang et al., 2015). The coaxial electrospinning produces nanofibers with a smooth surface (Fig. 1). The mean pleurocidin-loaded fiber diameters are 0.54 ± 0.09 μm, significantly lower ($p < 0.05$) than pristine matrices (0.60 ± 0.17 μm) (Fig. 1). Clear size distribution differences were also observed in profiles, as size variation ranges were much narrower for pleurocidin-loaded fibers compared with pristine. Suitable PVA concentrations were found during process optimization; 16% w/w is used for the core solution and 15% w/w for the shell solution. These concentrations provide good and reproducible electrospinnability (consistent stream, without solution dripping nor formation of globs) and nanofiber formation (homogeneous fibers without beads). The electrospinning process is more stable for the preparation of pleurocidin-loaded PVA formulations than for non-loaded PVA formulations, resulting in more homogeneous fibers (with lower fiber size distribution) and fewer electrospinning errors in the matrix (Fig. 1).

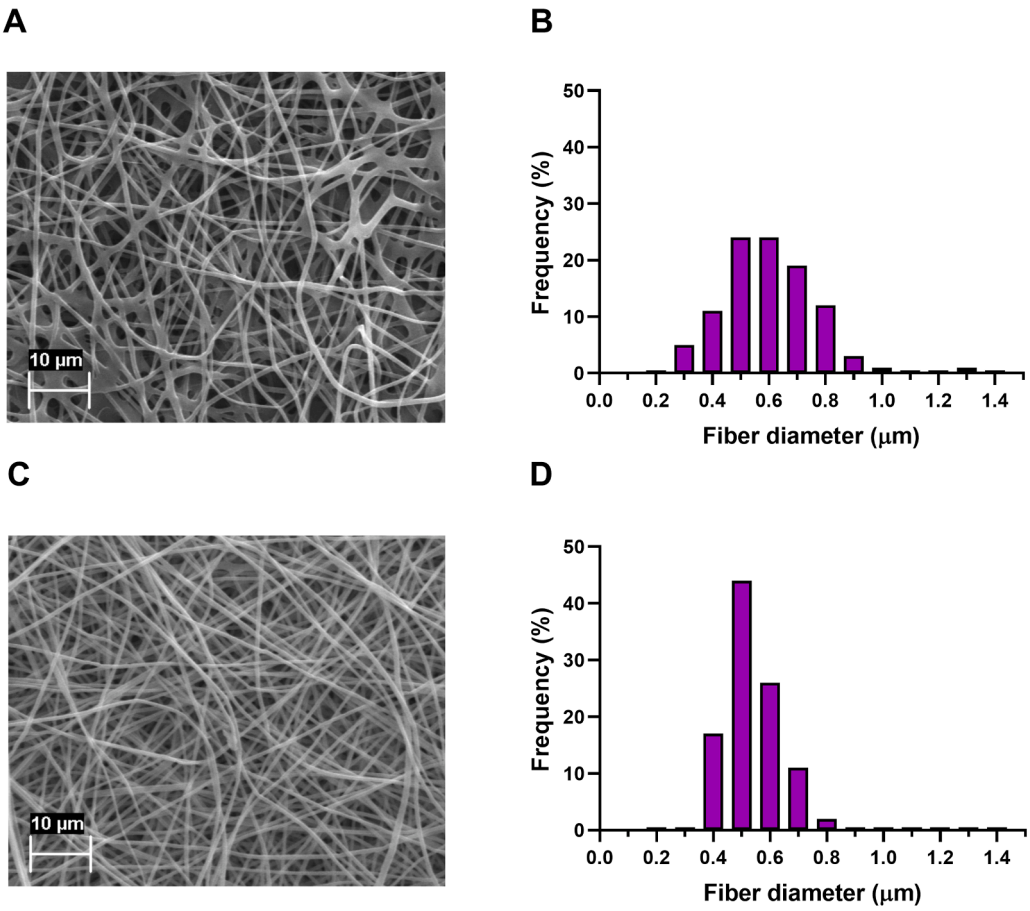

Fig. 1. Morphology of pleurocidin – loaded and pristine polyvinyl alcohol (PVA) electrospun fiber matrices developed by coaxial electrospinning. Scanning electron microscopy (SEM) images **(A; C)** together with fiber diameter size distribution histograms **(B; D). A-B.** Pristine PVA coaxially electrospun matrix. **C-D.** Pleurocidin-loaded PVA coaxially electrospun matrix.

Good aqueous solubility of pleurocidin as well as PVA (with the help of heating for PVA) affords clear electrospinning solutions and the electrospinning process could be performed under optimised conditions. The latter means that during electrospinning no dripping of the solution takes place, and the fibers are uniformly collected on the grounded collector plate. Furthermore, these electrospun matrices possess relatively homogeneous matrix thickness (approximately 0.05 mm). The average weight for 0.6 cm diameter circle of coaxially electrospun matrix was 529 ± 56 μg, weight variations are mainly due to the sample removal and cutting differences between the samples. Measured pleurocidin content within the fiber matrix after electrospinning was approximately 0.8% w/w. This shows that the peptide was not degraded during electrospinning and the measured concentrations match with the theoretical concentrations (0.7% w/w). The small deviation is most likely related to the analysis method. The stability of the peptide during electrospinning may also be related to the lack of harsh solvents and the use of PVA, which is a well-known hydrophilic, biocompatible and water-soluble polymer (Aslam et al., 2018). Considering that antimicrobial peptides in a powder or in an aqueous solution are known to degrade fast at room temperature (within 24 h) it is important to assess peptide stability within the electrospun PVA fiber matrices. After 6 months of storage at room temperature and 0% RH the amount of pleurocidin measured from coaxially electrospun fiber matrix was 0.50% ± 0.04% w/w, compared with 0.80% ± 0.01% measured immediately after electrospinning, indicating that the matrices maintain sufficient peptide to remain active.

3.2. *Antibacterial activity of electrospun pleurocidin-loaded matrices*

When conditions for reproducible production of pleurocidin containing nanofibers had been developed, the release of functional peptide was confirmed next by testing the antibacterial activity and potency of pleurocidin-loaded fiber matrices. First, the MIC values of pleurocidin in solution were determined against a panel of eight pathogenic bacterial strains (MIC values and dose-response curves in Fig. 2**A**). These results were then compared with the growth inhibition caused by pleurocidin-loaded PVA matrices, based on the theoretical pleurocidin content and drug amount released from the matrices (Fig. 2). According to variations in weight of the 0.6 cm diameter matrices used, the maximum theoretical amount of the peptide that can be released into each well ranges between 16.5 – 20.5 μg/mL (assuming 100% release from fiber matrix). Fiber matrices release enough antimicrobial peptide to completely inhibit the growth of all the following strains: *P. aeruginosa* PAO1, methicillin-resistant *S. aureus* EMRSA-15 (NCTC 13616), *K. pneumoniae* M6 and NCTC 13368, *A. baumannii* strains AYE and ATCC 17978, and *E. coli* NCTC 12923 (Fig. 2; **Supplementary Figure S1**). Pleurocidin concentrations obtained following the release from 0.7% w/w pleurocidin-loaded PVA matrices are therefore all well above the MIC for these strains and bacterial growth is completely inhibited. For *P. aeruginosa* strain NCTC 13437, the drug amount released from the matrices is not always able to inhibit the growth, being dependent on matrix weight variations (Fig. 2**B**). We show therefore that the amount of pleurocidin within the fibers plays a critical role in determining the activity of electrospun fiber matrix against more difficult-to-treat wound pathogens.

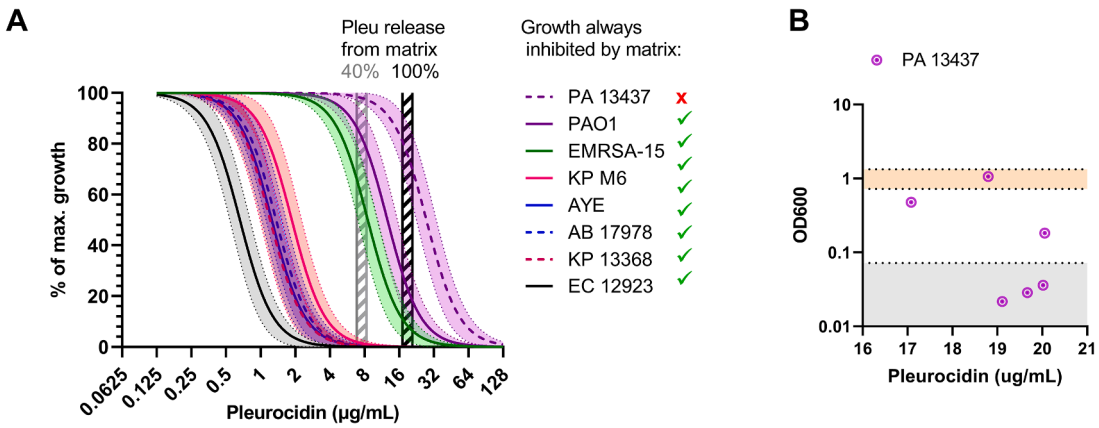

Fig. 2. A. Efficacy of pleurocidin solution and pleurocidin containing PVA fiber matrices against selected bacterial pathogens. Drug concentration response curves were obtained using a non-linear regression model (four-parameter logistic curve) using percentage of maximal growth based on absorbance values (OD_{600}) from control pleurocidin solution. Pleurocidin concentration range released from the matrix (40% and 100% shown) and available in the well, has been calculated from an average 0.6 cm diameter piece of pleurocidin loaded PVA fiber matrix based on the theoretical content. **B.** Efficacy of pleurocidin containing PVA matrices against PA NCTC 13437. Key: orange area- control growth; grey area- growth inhibition is defined as $OD_{600} < 0.1$ above the background noise (less than 10% of control). Exact pleurocidin concentration for each matrix has been calculated using each weight and it is based on a 100% release of the theoretical concentration. For specific bacterial strains please see Table 2. Key: Pleu- pleurocidin.

In previous work, others have shown that approximately 40% pleurocidin is released after 15 min from monoaxial PVA fibers containing 0.25% w/w pleurocidin (Wang et al., 2015). Similar sized pieces of matrix would, therefore, produce concentrations of between 2.4 and 2.9 µg/mL. This would be sufficient to inhibit only half the strains in the panel: *E. coli* NCTC 12923, the two *A. baumannii* strains and *K. pneumoniae* NCTC 13368. Although the fibers and electrospinning conditions used in the present study (coaxial electrospinning) differ from previous study by Wang et al. (2015) (monoaxial electrospinning), if the same level of pleurocidin release were to occur here from 0.7% w/w pleurocidin loaded PVA matrices, then concentrations of between 6.6 and 8.2 µg/mL would be expected. This concentration range would be sufficient for the inhibition of all strains except both *P. aeruginosa* strains and EMRSA-15. Consequently, the range of isolates from the panel used here that are actually inhibited by 0.7% w/w pleurocidin loaded coaxially electrospun PVA matrices suggests that the functional pleurocidin released from the matrices is close to its maximum content.

Further analyses were carried out using both *P. aeruginosa* strains to study the relationship between the weight of each pleurocidin-loaded fiber matrix and its antimicrobial activity. Both fresh pleurocidin solution and pleurocidin-loaded PVA matrix produce dose-dependent changes in growth of both *P. aeruginosa* strains correlating with weight of matrix pieces (Fig. 3). Growth of *P. aeruginosa* NCTC 13437 is completely inhibited using a pleurocidin matrix weighing 500 µg, while a matrix weighing 400 µg only partially inhibits growth (Fig. 3**A**). In contrast, *P. aeruginosa* PAO1 growth is completely inhibited using a pleurocidin matrix weighing 400 µg (Fig. 3**B**). The observed dose-dependent behaviour of the pleurocidin-loaded fiber matrices confirms that the peptide is released and maintains its activity after its incorporation on electrospun matrices.

The half maximal inhibitory concentration (IC_{50}) of pleurocidin is different depending on whether the peptide was placed in solution or within matrices (assuming maximal release). IC_{50} values of *P. aeruginosa* NCTC 13437 were 19.42 µg/mL and 13.98 µg/mL for pleurocidin solution and pleurocidin-loaded matrices, respectively. In the case of *P. aeruginosa* PAO1, IC_{50} values are 13.68 µg/mL and 11.67 µg/mL. Notably, in both cases, the inhibition achieved with the pleurocidin-loaded PVA fiber matrices substantially exceeds what would be expected and consequently outperforms freshly made pleurocidin solution. Such a possibility has been suggested in previous work (Wang et al.,

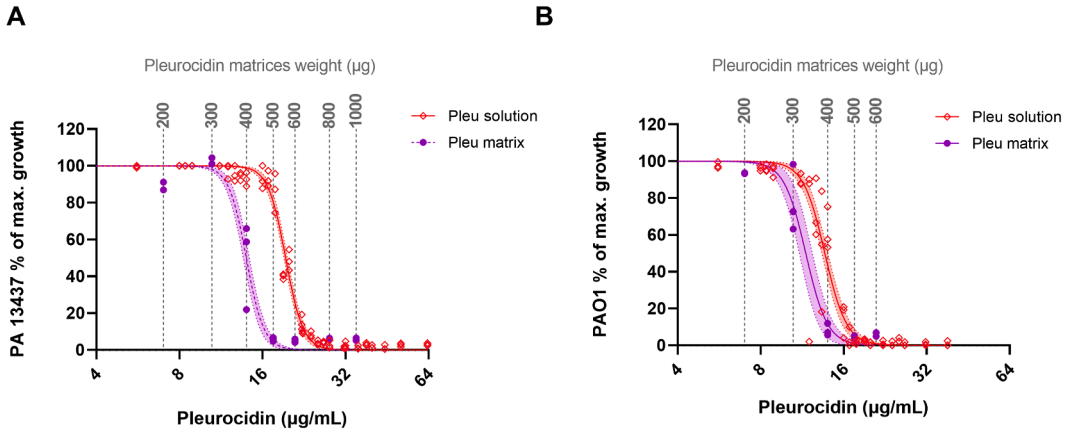

Fig. 3. Pleurocidin loaded PVA fiber matrices outperform pleurocidin solutions. Pleurocidin-dose-dependent, normalized bacterial growth for bacterial strains *P. aeruginosa* NCTC 13437 **(A)** and *P. aeruginosa* PAO1 **(B)** comparing pleurocidin solution and theoretical maximum concentrations achievable with release from pleurocidin-loaded electrospun PVA fiber matrices of varying weights. A non-linear regression model (four-parameter logistic curve) was fitted using % of maximal growth based on absorbance values (OD_{600}) at different pleurocidin solution concentrations for both *P. aeruginosa* bacterial strains. 95% confidence intervals of fitted curves are shown as shaded areas.

2015), but the present study design enables us to present strong evidence that this is indeed the case. This effect may be related to the protection given by the matrix as pleurocidin carrier, reducing its degradation in contact with the environment. Moreover, a better interaction between the drug and pathogens may occur when the latter attach on the surface of the electrospun matrix. It is evident that antimicrobial peptides need to be released from the electrospun matrix in sufficient concentration in order to kill the pathogen, but it is not always easy to determine the exact amount of released antimicrobial peptide *in vitro*. Previously, drug release experiments have been conducted using antimicrobial peptide containing fiber matrices and distilled water as a release environment (Wang et al., 2015) or *in vitro* agar diffusion plates (Han et al., 2017). These experimental differences may impact results and their interpretation since the activity of antimicrobial peptide is heavily affected by the surrounding environmental conditions (Abbate et al., 2013; Lan et al., 2010). Indeed, we have shown that release of more simple drugs, such as chloramphenicol, from electrospun fiber matrices may be affected by the drug release environment and drug release model system (Preem et al., 2019). As mentioned before, previous study has shown 40% release of pleurocidin from the PVA matrices to distilled water (Wang et al., 2015), this is the estimation we also used in our study. We quantified the maximum amount of pleurocidin released into phosphate buffer solution by HPLC which did not exceed 40%, but the quantification was variable and future work involves developing a more robust and reproducible assay that is able to detect the burst release as well as avoid possible readsorption of peptide onto the fiber matrix. In the case of larger peptide molecules, such as antimicrobial peptides, the testing and understanding of release and activity is complicated and needs a separate and deeper investigation.

3.3. Antibacterial activity of common biocide solutions used for wound cleaning

Biocides are widely used in the prevention and treatment of wound infections. Usually, biocide formulated as solutions or creams are used, and frequently also antimicrobial wound patches consisting of biocides like silver are used in case of infected wounds (Haidari et al., 2020; May et al., 2022). However, when novel wound dressings containing antimicrobial peptides are used, these will most likely be used together with the standard wound care approaches, such as flushing the wound with biocide solutions (European Wound Management Association (EWMA), 2006). Recently published guidelines for the treatment of burn infections suggest using topical biocides, such as silver sulfadiazine, specifically for second- and third-degree burns (Yoshino et al., 2020). Therefore, we investigated the effect of biocides on the antibacterial activity and potency of pleurocidin.

First, MIC assays were conducted for different biocides currently used for wound infection prevention and treatment, using the same bacterial strains as for testing the antibacterial activity of pleurocidin-loaded fiber matrices. Based on MIC testing results, in comparison with biocide concentrations in wound care solutions, decisions can be made about the efficacy of the biocides. As expected, all biocides were effective against the tested Gram-negative bacteria (Table 3).

Similarly to the pleurocidin MIC assay, *P. aeruginosa* strain NCTC 13437 is the least susceptible to some of the tested biocides, notably benzalkonium chloride. All these biocides were more potent against *A. baumannii* strains compared with *P. aeruginosa* strains. These biocides have different mechanisms of action against bacteria, thus different concentrations for an effective killing are to be expected. Octenidine was shown to be the most effective biocide among those biocides (Table 3). Silver nitrate and silver sulphadiazine showed similar MIC results and were also effective against tested bacterial strains. Chlorhexidine activity against the tested strains appeared to be rather similar to octenidine and benzalkonium chloride. However, although these three biocides share a similar mechanism of action - destabilizing cell membranes - much higher benzalkonium chloride concentrations were required to inhibit the growth of two *P. aeruginosa* strains compared with the *A. baumannii* strains. This lower sensitivity of *P. aeruginosa* has been previously discussed and is thought to be related to a failure of antimicrobials to efficiently penetrate the outer bacterial membrane (Gilbert and Moore, 2005). Povidone iodine is known to be highly effective for wound infection treatment as it can eliminate ESKAPE (Rice, 2008) pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) and biofilms, with maintained efficacy in the presence of blood (Barreto et al., 2020). Although it is one of the most used antiseptics and it is also widely used for the treatment of wound infections, its potency compared to other biocides tested is quite low (Table 3).

Table 3
Minimum inhibitory concentration (MIC) of different biocides. At least three independent experiments were performed in triplicate. Results are shown as a range when there were fluctuations. For comparison, common biocide formulations and their concentrations are provided.

Biocide	Bacterial strain	Biocide concentration (μg/mL)	Common biocide conc. in use	References
Silver nitrate	*A. baumannii* AYE	8	5000 μg/mL (0.5% solution)	(Moyer et al., 1965)
	A. baumannii ATCC 17978	8		
	P. aeruginosa NCTC 13437	4–16		
	P. aeruginosa PAO1	8–16		
Silver sulfadiazine	*A. baumannii* AYE	8–32	10 μg/mg (1% cream)	(White and Cooper, 2005)
	A. baumannii ATCC 17978	16–32		
	P. aeruginosa NCTC 13437	32		
	P. aeruginosa PAO1	32		
Octenidine	*A. baumannii* AYE	4	500 - 1000 μg/mL (0.05 - 0.1% solution)	(Maillard et al., 2021)
	A. baumannii ATCC 17978	4		
	P. aeruginosa NCTC 13437	4		
	P. aeruginosa PAO1	4		
Povidone iodine	*A. baumannii* AYE	2500	100 000 μg/mL (10% solution)	(Williamson et al., 2017)
	A. baumannii ATCC 17978	2500		
	P. aeruginosa NCTC 13437	2500		
	P. aeruginosa PAO1	5000		
Chlorhexidine	*A. baumannii* AYE	4–8	5000 - 40 000 μg/mL (0.5 – 4% solution)	(Williamson et al., 2017)
	A. baumannii ATCC 17,978	4		
	P. aeruginosa NCTC 13437	4–8		
	P. aeruginosa PAO1	4–16		
Benzalkonium chloride	*A. baumannii* AYE	8	13,000 μg/mL (0.13% solution)	(Pereira and Tagkopoulos, 2019)
	A. baumannii ATCC 17978	8		
	P. aeruginosa NCTC 13437	256		
	P. aeruginosa PAO1	64		

3.4. Pleurocidin antibacterial activity in the presence of common biocides

Often the treatment of wound infections consists of various agents in order to provide a broader antibacterial spectrum and successfully inhibit or kill the pathogens. The use of combinations may be provided due to different topical treatments performed sequentially with carry-over or systemic administration of antibiotics used together with topical application of biocides (Percival et al., 2014). However, the use of combinations may not always be suitable and the use of biocides together with antimicrobial peptides may lead to unexpected outcomes. Consequently, we tested whether and to what extent the use of biocides affects the antibacterial activity of pleurocidin.

In general, we find weak synergism as well as some indifference when pleurocidin is used in combination with the biocides (Fig. 4).

There are no differences in the fractional inhibitory concentrations (FIC-s) observed between the two *A. baumannii* strains, but the two *P. aeruginosa* strains differ. Most striking is the difference in FIC observed for the combination of pleurocidin and benzalkonium chloride; a modest synergy is observed when challenging *P. aeruginosa* NCTC 13437 but a strongly antagonistic effect is observed for the same combination against *P. aeruginosa* PAO1 (Fig. 4**C** and 4**D**).

In order to give a better overview of the co-activity of pleurocidin and benzalkonium chloride combinations against bacterial strains, microdilution checkerboard analysis results are shown (Fig. 5).

It was expected that a lower drug concentration from both molecules would to be needed when used in combination to achieve the desired antimicrobial activity. This was the case for most bacterial strains tested against benzalkonium chloride, which resulted in weak synergy (Fig. 5). However, the opposite behaviour was observed in the case of *Pseudomonas* PAO1 (Fig. 5**F**). Similarly, chlorhexidine combinations with pleurocidin against both *P. aeruginosa* strains showed lower growth inhibition compared when each drug was separately used (**Supplementary Figure S2**). This antagonistic phenomenon was not observed with other strains nor biocide/pleurocidin combinations tested.

It has been shown that antibiotics and biocides when used in combination may lead to different physiological effects on *P. aeruginosa* (Pietsch et al., 2021). The effects are specific to the antibiotic-biocide combination, and here we find biocides with similar mechanisms of action show consistent interaction effects with different antibiotics, which holds true here in the case of silver nitrate and silver sulfadiazine (Fig. 4). Moreover, inter-strain variation in response to antibiotic/biocide combinations has been described before. Most notably, differences in synergy/antagonism were observed for combinations of gentamicin and chlorhexidine, when tested against 36 *P aeruginosa* strains (Barnham and Kerby, 1980), while similar behaviour has been observed for combinations of silver nanoparticles and meropenem

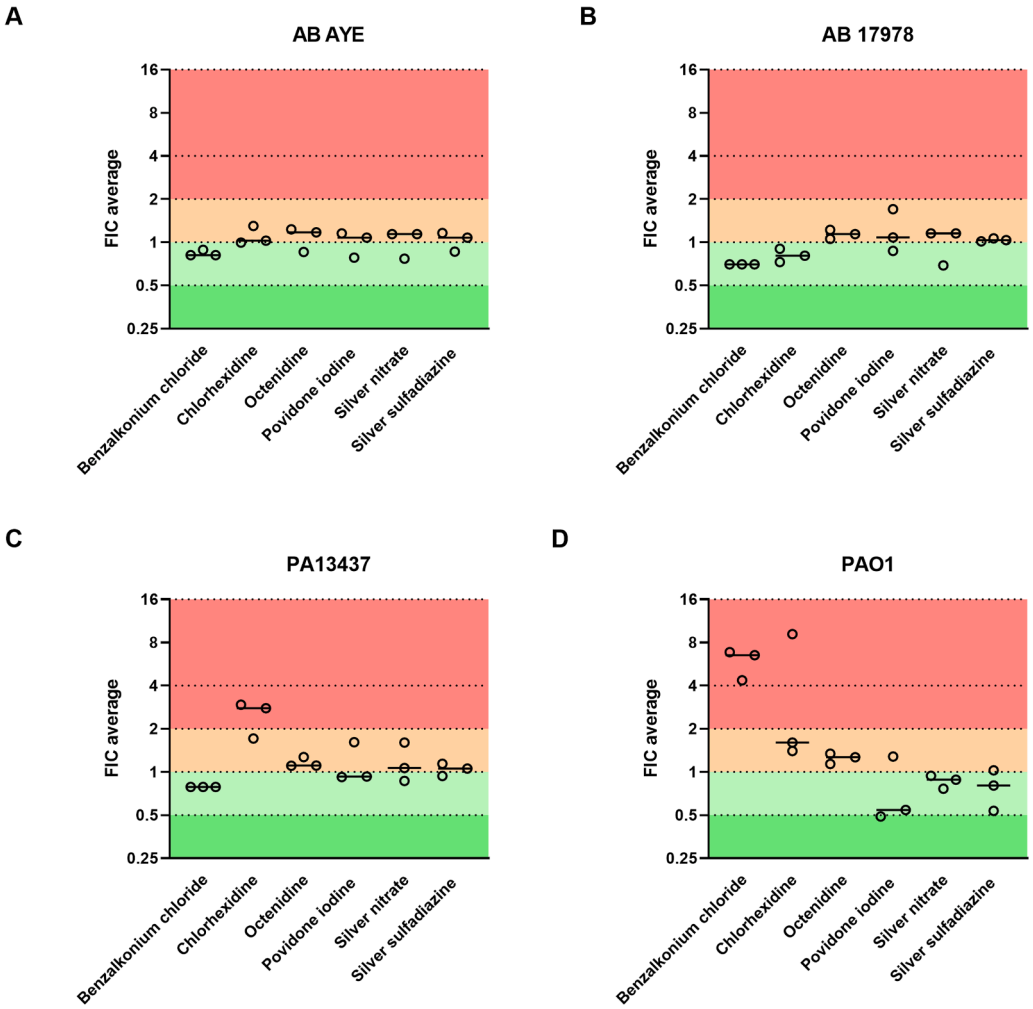

Fig. 4. Compatibility of pleurocidin and six common biocides (benzalkonium chloride, chlorhexidine, octenidine, povidone iodine, silver nitrate and silver sulfadiazine) are presented. The average fractional inhibitory concentration (FIC) was calculated and presented for different bacterial strains: *A. baumannii* AYE (AB AYE) (**A**), *A. baumannii* ATCC 17978 (AB17978)(**B**), *P. aeruginosa* NCTC 13437 (PA13437)(**C**) *P. aeruginosa* PAO1 (PAO1)(**D**). FIC < or equal to 0.5 were considered strongly synergistic, values of 0.5 to 1 were weakly synergistic or additive. FICs from 1 to 2 are defined as indifferent, while those of > 2 are antagonistic. Results are presented as a scatter dot plot, the median is shown with a line.

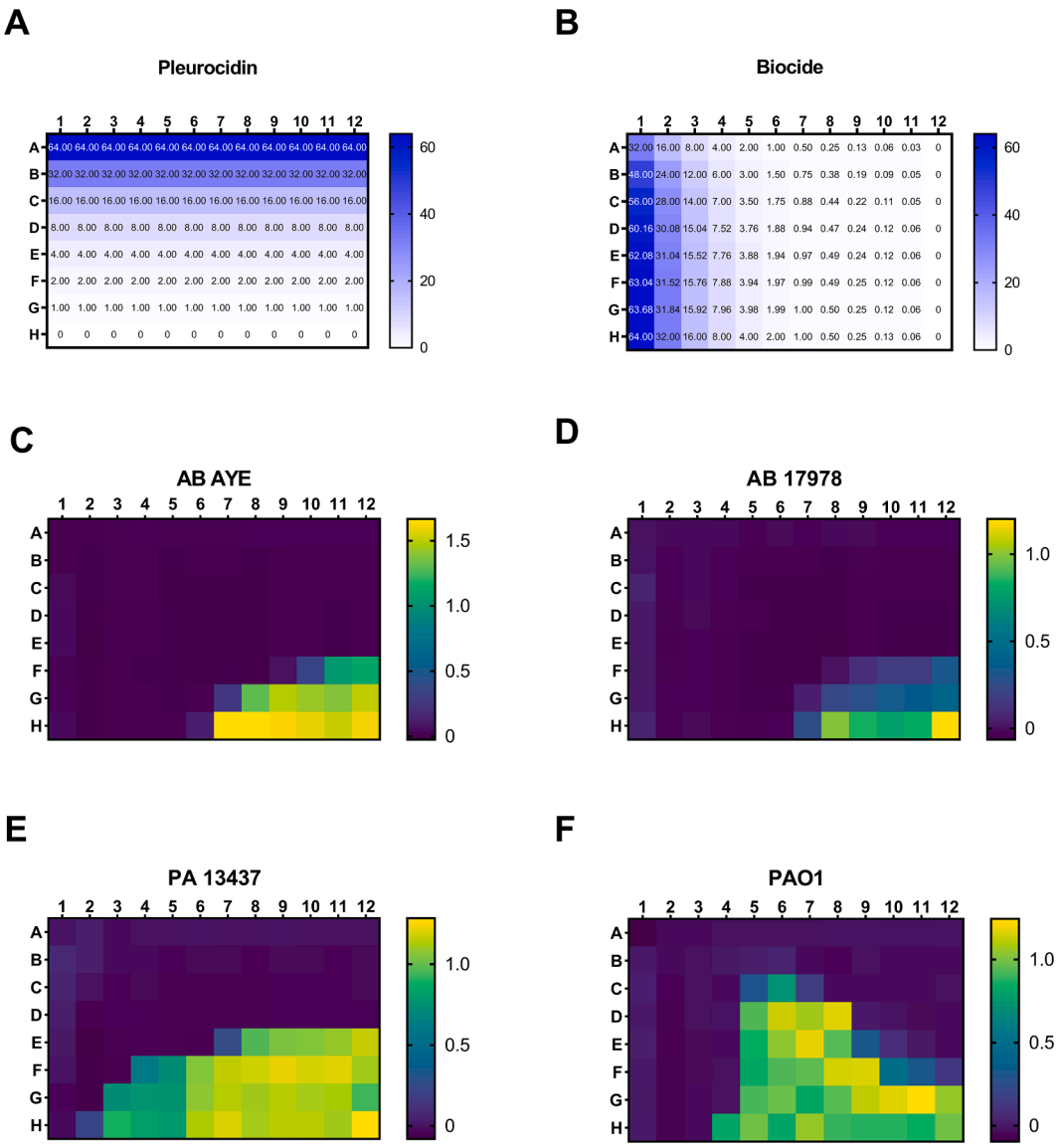

Fig. 5. Microdilution checkerboard analysis revealing the co-activity of pleurocidin and biocides on four pathogenic wound bacterial strains. Schematics of microdilution checkerboard concentrations for pleurocidin (**A**) in combination with different biocide concentrations (**B**). Co-activity of pleurocidin and biocides on four pathogenic wound bacterial strains was estimated by measuring bacterial growth after 24 h. Last column and last row correspond to pleurocidin only MIC and biocide only MIC, respectively. The extent of inhibition is shown as a heat plot, full bacterial growth (measured at OD_{600}) is represented by the lightest/yellow colour. Tested bacterial strains are: *A. baumannii* AYE (AB AYE) (**C**), *A. baumannii* ATCC 17978 (AB17978)(**D**), *P. aeruginosa* NCTC 13437 (PA13437)(**E**) and *Pseudomonas* PAO1 (PAO1)(**F**). One representative experiment of three biological replicates is shown.

(Markowska et al., 2014; Pietsch et al., 2021).

Although further confirmation will be required, considering the interactions between pleurocidin and biocides for both *P. aeruginosa* and *A. baumannii*, pleurocidin may be expected to be broadly compatible with silver sulphadiazine, silver nitrate and povidone iodine, showing weak synergism or an additive effect in most cases (Fig. 4). However, due to the great variability in effect found between the two different *P. aeruginosa* strains, with antagonism observed between pleurocidin and benzalkonium chloride and also with chlorhexidine when challenging one or other strain (Figs. 4 **and** 5**E/F**) it will be essential to study a broader panel of *P. aeruginosa* isolates to understand how widespread such interactions may be.

4. Conclusions and future perspectives

Polyvinyl alcohol (PVA)-based fiber matrices, with antimicrobial peptide pleurocidin content up to 0.7% w/w in solid state, were successfully electrospun using coaxial electrospinning. Pleurocidin preserved its functionality during and after electrospinning. The potency and antibacterial activity of pleurocidin depended on the exact amount of drug incorporated into PVA fiber matrices, showing a stronger effect than pleurocidin used in solution. PVA matrix facilitates pleurocidin killing against several pathogens relevant for causing wound infections including *A. baumannii* and *P. aeruginosa* as well as other ESKAPE pathogens (EMRSA-15, *K. pneumoniae* and *E. coli)*. For successful antibacterial activity the exact and optimised pleurocidin concentration in the matrix, matrix weight, and thus, the amount of peptide released are crucial. Although for *P. aeruginosa*, antagonistic effects were observed when pleurocidin was used in combination with benzalkonium chloride or chlorhexidine, combinations of pleurocidin and silver sulphadiazine, silver nitrate and povidone iodine, showed mainly indifferent or weakly synergistic interactions. Therefore pleurocidin-loaded fibrous wound matrices, obtained using electrospinning, have considerable potential to be used for the successful treatment of wound infections, though the

decision about the use of biocide needs to be performed based on the coactivity of the peptide and biocide combination providing optimized activity.

Antimicrobial peptides have the advantage of killing the bacteria fast, but our study shows that sufficient concentration of antimicrobial peptide is needed in order to provoke the killing and exhibit the desired antibacterial efficacy. It is a threshold concentration as it is seen that using lower concentrations, we may be below the required working antimicrobial concentrations for several pathogenic strains. However, much higher concentrations may lead to problems with drug solubility, electrospinnability as well as bacterial resistance development due to the overuse. Future studies will enable to get a better understanding of the release kinetics of antimicrobial peptides from electrospun matrices while comparing different biorelevant approaches together with antimicrobial activity testing with bacteria. And similarly, important mechanisms behind the observed enhanced potency of antimicrobial peptide in electrospun matrices will be investigated in more depth.

CRediT authorship contribution statement

Celia Ramos: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Kairi Lorenz:** Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Marta Putrinš:** Methodology, Formal analysis, Visualization, Supervision, Writing – review & editing. **Charlotte K. Hind:** Methodology, Investigation, Supervision, Writing – review & editing. **Andres Meos:** Methodology, Investigation, Writing – review & editing. **Ivo Laidmäe:** Methodology, Supervision, Writing – review & editing. **Tanel Tenson:** Methodology, Resources, Supervision, Writing – review & editing. **J. Mark Sutton:** Resources, Supervision, Writing – review & editing. **A. James Mason:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration. **Karin Kogermann:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

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.

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Notes. The authors declare no competing financial interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106648.

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