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ENGINEERING A COLLAGEN-BASED WOUND-MIMETIC PLATFORM FOR IN VITRO P. AERUGINOSA BIOFILM GROWTH AND DNASE I–CIPROFLOXACIN POST-TREATMENT

Hinal B Joshi[1], Sheetal Sambar[2], Ankita P Singh[3*]

[1]Department of Food Processing Technology, A D Patel Institute of Technology, The Charutar Vidya Mandal (CVM) University, New Vallabh Vidya Nagar, Behind Vitthal Udyog Nagar, Anand, Gujarat, India- 388121. Research Scholar, Email ID: phd.joshihinal@adit.ac.in,
[2]Department of Food Processing Technology, A D Patel Institute of Technology, The Charutar Vidya Mandal (CVM) University, New Vallabh Vidya Nagar, Behind Vitthal Udyog Nagar, Anand, Gujarat, India- 388121. phd.sambarsheetal@adit.ac.in
[3*]Former Assistant Professor , Department of Food Processing Technology, A D Patel Institute of Technology, The Charutar Vidya Mandal (CVM) University, New Vallabh Vidya Nagar, Behind Vitthal Udyog Nagar, Anand, Gujarat, India- 388121. ankitapagedar@gmail.com

ABSTRACT:
Biofilm-associated wound infections caused by Pseudomonas aeruginosa are difficult to eradicate due to the protective extracellular polymeric substance (EPS), which restricts antibiotic penetration and promotes antimicrobial resistance. The present study aimed to develop a collagen-based wound-mimetic biosimilar surface that closely simulates the in vivo wound microenvironment for in vitro biofilm formation and to evaluate the efficacy of DNase I-assisted antibiotic therapy against pre-formed biofilms. The biosimilar surface was developed using rat-tail collagen Type I and simulated wound fluid (SWF) and optimized by Response Surface Methodology (RSM) using Design-Expert 13.0 software. The optimized formulation consisted of 90.09 µL SWF and 30.23 µL collagen Type I, producing the highest absorbance (0.5684, $p < 0.05$). P. aeruginosa formed significantly greater biofilm biomass on the collagen-based biosimilar surface than on conventional polystyrene plates, demonstrating its suitability as a wound-mimetic model. The synergistic activity of DNase I and ciprofloxacin was optimized using Combenefit software, and the selected combination reduced the minimum biofilm inhibitory concentration (MBIC) of ciprofloxacin from 256 to 128 µg/mL. Furthermore, the combination therapy achieved 92.21% biofilm inhibition within 10 min and markedly reduced biofilm thickness, as confirmed by bright-field microscopy, scanning electron microscopy, and confocal laser scanning microscopy. These findings demonstrate that the developed collagen-based biosimilar surface provides a reliable in vitro wound biofilm model and that DNase I-mediated matrix disruption significantly enhances ciprofloxacin efficacy against pre-formed P. aeruginosa biofilms, offering a promising therapeutic strategy for chronic wound infections.

KEYWORDS: Collagen-based biosimilar surface; Pseudomonas aeruginosa; Wound-mimetic biofilm model; DNase I; Ciprofloxacin

INTRODUCTION

It is well-established that bacteria primarily exist in sessile clusters referred to as biofilms, rather than as individual planktonic cells, which significantly contribute to the persistence of chronic wounds (1). Approximately 80% of clinically relevant infections, including those in cystic fibrosis, endocarditis, osteomyelitis, and chronic wounds, are linked to biofilms (2,3). Biofilms are highly resistant to antimicrobial treatments and the immune system, with their Extracellular Polymeric Substance (EPS) acting as a barrier that limits antimicrobial penetration (4,5), (6). Biofilms are estimated to contribute to around 60-80% of chronic wound infections (7). Biofilm-related infections, common in conditions like diabetic foot ulcers and pressure sores, hinder wound healing and promote persistent infections due to their resistance to antimicrobial therapies and the immune system, posing a significant medical challenge. Moreover, in-depth studies of the distribution of infectious organisms in chronically infected wounds have revealed variations in the depth at which microbial biofilm clusters are embedded within the wound tissue (8–10). For instance, one study showed that S. aureus is typically located in the superficial layers near the wound surface, while P. aeruginosa is mostly found in the deeper layers of the chronic wound bed (11). The types of bacteria present in significant quantities, such as Enterococcus faecalis, Proteus species, P. aeruginosa, S. aureus, and anaerobic bacteria, can vary depending on the wound type. For instance, chronic venous leg ulcers are found to contain Staphylococcus aureus in 93.5% of cases, Enterococcus faecalis in 71.7%, Pseudomonas aeruginosa in 52.2%, coagulase-negative staphylococci in 45.7%, Proteus species in 41.3%, and anaerobic bacteria in 39.1% (12). Biofilms can also contribute to approximately 6% of acute wound infections (13), as observed in animal models of acute burn wounds (14) and surgical site infections (15). The antimicrobial resistance characteristics of biofilms can result in extended inflammation, slower healing, and a heightened risk of infection.

In vitro, biofilms can be formed in microtiter plates, static or flow cell systems, bioreactors, various surface coatings, and agar plates, all of which consist of solid surfaces or materials such as glass, plastic, and stainless

steel (16–18). In contrast, in vivo microbial infections involve biofilm formation on semi-solid surfaces, like wound tissue, or within the surrounding environment. In most in vitro studies on biofilms or antibiofilm strategies, experiments are conducted on polystyrene surfaces using optimized media and clinically irrelevant microbial species, which do not accurately mimic real wound infection models (19,20). Acknowledging this limitation, recent research has included serum or plasma in in vitro growth conditions to better simulate the host environment (21–26). To mimic the in vivo conditions of wound infections, collagen-based surfaces and wound-like environments have been designed for studying biofilm growth (21,25,27,28). However, there are limited studies in this area.

In this study, we developed a collagen-based wound biofilm model comprising Simulated Wound Fluid (SWF), rat-tail collagen Type I, mixed-species bacterial biofilm, and other components. The formulation of this model is built upon the composition of collagen-based wound surface models described in earlier studies (21). For this study, we have selected S. aureus, K. pneumoniae, and P. aeruginosa as they cause infection in diabetic foot ulcers (29), burn wounds (30), bedsores (31), surgical site infections (32), venous leg ulcers (33), traumatic wounds (34), and so on.

MATERIALS AND METHODS

Chemicals and reagents

Reagents for collagen matrix development: The collagen matrix was developed using rat-tail collagen Type I, Fetal Bovine Serum (FBS), sodium hydroxide (NaOH), acetic acid, and SWF. SWF was prepared by combining NaCl, FBS, and peptone water (PW). All chemicals and reagents were sourced from Hi-Media Laboratory, Mumbai, India.

Other media & reagents: Tryptic Soy Broth (TSB), crystal violet, antibiotic (ciprofloxacin) and glacial acetic acid. All media and reagents were sourced from Hi-Media Laboratory, Mumbai, India.

Microorganisms & growth conditions

The microbial strain Pseudomonas aeruginosa (MTCC 3541) was procured from the Microbial Type Culture Collection (MTCC) in Chandigarh, India. The culture was grown overnight in Tryptic Soy Broth (TSB), and optical density was adjusted to 1 at 620 nm. Subsequently, the culture was preserved as 50% glycerol stocks and stored at -20°C.

Formation of collagen-based biosimilar surface

SWF and Type I rat-tail collagen were employed to develop an in vitro collagen-based wound model, referred to as the biosimilar surface. SWF was prepared by mixing equal parts (50%) of FBS and sterile physiological NaCl in 0.1% sterile PW. The biosimilar surface was constructed in polystyrene cell culture dishes using Type I rat-tail collagen, following the manufacturer's protocol for eukaryotic cell culture (35). To prepare 10 mL of collagen solution at a concentration of 2 mg/mL, 1 mL of 0.1% acetic acid was combined with 2 mL of collagen stock solution (10 mg/mL) and maintained on ice. Subsequently, 6.0 mL of cold SWF was added, followed by 1 mL of 0.1 M NaOH. After thorough mixing, 1 mL of the collagen solution was distributed into cell culture dishes, and 50 µL was dispensed into each well of 96-well plates. The plates were then incubated at 35°C for 1 hour to ensure complete polymerization of the collagen (21). This protocol was optimized using Design Expert (DE) 13.0 software, with the Central Composite Design of Response Surface Methodology (RSM) suggesting 13 different combinations (Table 1).

Fixation: Following the incubation of the collagen-based biosimilar surface, the matrices were stabilized by treating them with a 4% formaldehyde solution.

After fixation, the collagen-based biosimilar surfaces in all wells were stained with a 1% crystal violet solution. The response in terms of absorbance was taken at 595nm.

Biofilm formation assay

The biofilm-forming potential of P. aeruginosa was evaluated using the outlined method (36). Briefly, 200 µL of Tryptic Soy Broth (TSB), containing 10% inoculum was dispensed into each well of a sterile 96-well polystyrene plate (Axiva Biotech, New Delhi, India), where the biosimilar surface has already been developed in each well. Biofilms were allowed to develop at 37°C for 24 hours. After incubation, the well contents were discarded, and the wells were washed 3–4 times with sterile Phosphate Buffered Saline (PBS) to remove any loosely adhered cells. The wells were gently blotted on paper towels and air-dried. The biofilms were stained with 1% crystal violet, rinsed 3–4 times in water within a large Petri dish to remove excess stain, blotted again, and air-dried. To quantify the biofilms, the bound crystal violet was solubilized with 33% glacial acetic acid, and the absorbance was measured at 595 nm using a microplate reader (EPOCH 2c, BIOTEK, USA).

Synergistic approach between DNase I and antibiotics for pre-formed biofilm inhibition

Synergistic optimization of DNase I and ciprofloxacin

Several commercially available antibiotics are used for the treatment of biofilm-associated infections. Among them, ciprofloxacin was selected because of its proven inhibitory and biofilm-eradicating efficacy against P. aeruginosa in accordance with CLSI guidelines (37). The combination therapy involving DNase I and ciprofloxacin was optimized using Combenefit 2.02v software. During the analysis, the concentrations of the first drug were arranged in columns, while those of the second drug were arranged in rows. The software utilizes three standard reference models—Bliss, Loewe, and Highest Single Agent (HSA)—to assess the synergistic interactions between the two agents (Di Veroli et al., 2016). This tool enables the quantification of synergistic or antagonistic

effects between antibiotics and DNase I against biofilms formed by different pathogens. In the generated plots, a darker blue colour indicates a higher degree of synergism between the two agents. DNase I and ciprofloxacin were evaluated within concentration ranges of 0–20 µg/mL and 0–1024 µg/mL, respectively. For the present study, the Bliss and Loewe models were applied for interaction analysis.

Evaluation of DNase I & ciprofloxacin against pre-formed P. aeruginosa biofilm

Pre-formed P. aeruginosa biofilms developed on collagen-based biosimilar surfaces as well as directly on 96-well polystyrene plate were treated with ciprofloxacin alone and in combination with DNase I to determine the Minimum Biofilm Inhibitory Concentration (MBIC). The MBIC assay was performed using the broth microdilution method in a 96-well flat-bottom microplate format adapted from the Clinical & Laboratory Standards Institute (CLSI) guidelines (38). Briefly, the bacterial suspension was diluted in TSB broth to obtain an absorbance of 1.0 at 600 nm, corresponding to an inoculum density of 1×10^8 CFU/mL, for biofilm formation. After 24 h of incubation, the contents of the wells were discarded, followed by washing with PBS 3–4 times to remove loosely attached cells. Subsequently, the wells were treated with ciprofloxacin alone for 1 h contact time, while treatment with ciprofloxacin in combination with DNase I was carried out for 10 min contact time. Thereafter, the biofilm formation assay was performed as described previously to evaluate the inhibitory effect of ciprofloxacin alone and in combination with DNase I. Uninoculated wells containing only TSB were used as negative controls, treated similarly to the test wells, and their absorbance values were subtracted from the corresponding test readings.

Microscopic analysis for P. aeruginosa biofilm on biosimilar surface

The morphology of treated and untreated P. aeruginosa biofilms developed on the collagen-based wound model was analyzed using light microscopy, confocal laser scanning microscopy (CLSM) at ICMR-NIRRCH, Mumbai, Maharashtra, India and scanning electron microscopy (SEM) at NIPER-A, Gandhinagar, Gujarat, India. For microscopic evaluation, biofilms treated with ciprofloxacin alone were exposed for 1 h, whereas biofilms treated with the combination of ciprofloxacin and DNase I were given a contact time of 10 min. Furthermore, the inhibitory effect of the DNase I–ciprofloxacin combination against pre-formed Pseudomonas aeruginosa biofilms was quantified by fluorescence microscopy, specifically CLSM, using Green Fluorescent Protein (GFP)-tagged bacterial cells. GFP tagging of P. aeruginosa was carried out by SLS Research Pvt. Ltd., Surat, Gujarat, India. Furthermore, dead bacterial cells were detected by using DAPI dye which represents the dead cell by red colour.

Statistical analysis

All experiments were conducted independently in three separate trials; each performed in triplicate. The results are reported as averages, with the standard deviation from the mean calculated using Microsoft Excel 2021. Statistical analysis was performed using XL-Statistics v4.5, applying Student's t-test with Bonferroni post hoc analysis and Analysis of Variance (ANOVA), with a significance threshold set at $p<0.05$.

RESULTS

Biosimilar surface development

After developing the collagen-based biosimilar surface according to the combinations suggested by the DE 13.0 software, the absorbance values obtained were entered into the software, as presented in Table 1. The software analysed these values using a significant quadratic model with no lack of fit ($p < 0.05$). Furthermore, a 3-D surface plot was generated by the software, illustrating the optimum concentrations of SWF and rat tail collagen Type I that produced the highest absorbance at 595 nm (Fig-1). The software recommended using 90.09 µL of SWF and 30.23 µL of rat tail collagen Type-I. This combination produced an absorbance value of 0.5684, which closely matched the actual absorbance obtained ($p<0.05$). Therefore, the combination of SWF and rat tail collagen Type-I showing the highest absorbance was selected for further studies.

Table 1: Optimization for collagen-based biosimilar surface development

Run	A: collagen	B: SWF	Absorbance
	(µL)	(µL)	at 595nm
1	30	90	0.6824
2	50	150	0.506333
3	50	30	0.409
4	30	90	0.654
5	1.71573	90	0.5925
6	30	90	0.658333
7	30	90	0.5741
8	30	5.14719	0.379

9	10	30	0.437
10	10	150	0.534667
11	30	90	0.58412
12	30	174.853	0.494667
13	58.2843	90	0.559

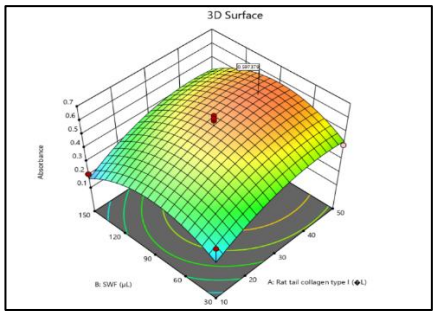

Fig-1: 3D surface graph of optimization for biosimilar surface development

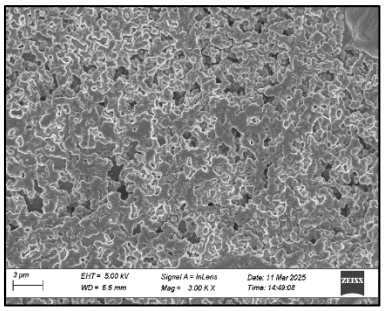

Fig-2: SEM image of developed collagen-based biosimilar surface

Biofilm formation assay

When P. aeruginosa was allowed to develop a biofilm on a 96-well polystyrene surface, the absorbance obtained was 2.148. In contrast, a higher absorbance (i.e., 2.524) was observed when the biofilm was formed on the collagen-based biosimilar surface, owing to the inherent contribution of the collagen-based biosimilar surface to the overall absorbance. Therefore, the absorbance contributed by the collagen-based biosimilar surface itself was subtracted from the total absorbance value, considering the O.D. of the biosimilar surface as 0.05684.

Anti-biofilm effect of DNase I and ciprofloxacin for pre-formed biofilm inhibition

The interaction between DNase I and ciprofloxacin was evaluated using Combenefit version 2.02 according to the Bliss independence and Loewe additivity models. The optimized combination identified by the software was 64 µg/mL ciprofloxacin with 10 µg/mL DNase I. For conciseness, the corresponding optimization plots are not presented. These findings align with those reported by Sharma and Pagedar Singh, 2018 (39). Consequently, this specific combination was selected for targeting pre-formed P. aeruginosa biofilms. While ciprofloxacin monotherapy yielded a Minimum Biofilm Inhibitory Concentration (MBIC) of 128 µg/ml, the addition of DNase I successfully reduced the MBIC to 64 µg/ml. Furthermore, the combination treatment enhanced biofilm formation inhibition, achieving an 88.39% reduction in just 10 min. of contact time compared to 78.32% with ciprofloxacin alone at 128 µg/ml (Fig-3).

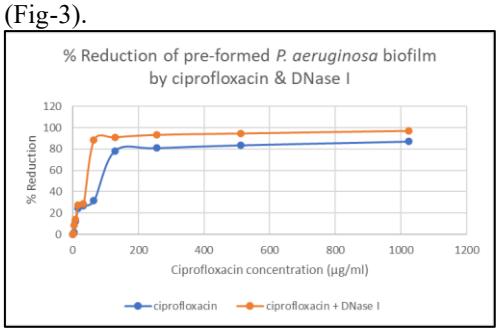

Fig-3: Post anti-biofilm treatment using ciprofloxacin with and without DNase I

Microscopic observation of inhibited pre-formed P. aeruginosa biofilm on biosimilar surface

Biofilm biomass reduction following treatment was evaluated using bright-field microscopy, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM) utilizing GFP-tagged bacterial cells. Both bright-field and SEM imaging revealed a distinct reduction in the biofilm's surface canopy upon treatment with ciprofloxacin alone (128 µg/ml). However, the addition of DNase I resulted in significantly lower surface coverage (Fig-5), despite using a less contact time of DNase I i.e., 10 mins (Fig-5). Compared with the untreated control (Fig. 4), the DNase I-treated preformed biofilm exhibited substantially reduced surface coverage, supporting the hypothesis that DNase I disrupts and weakens the biofilm matrix.

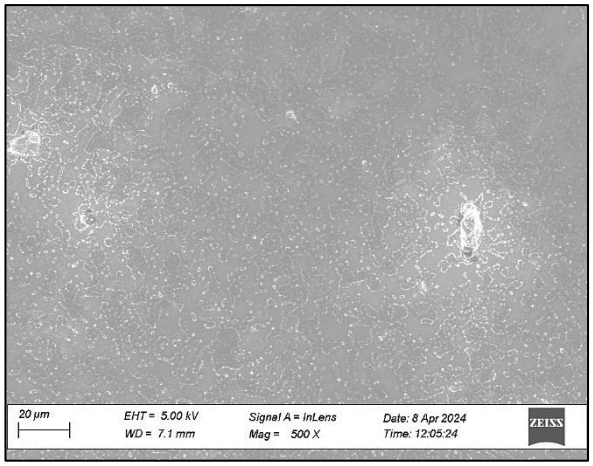

Fig-4: SEM image of untreated (control) mixed spp. biofilm

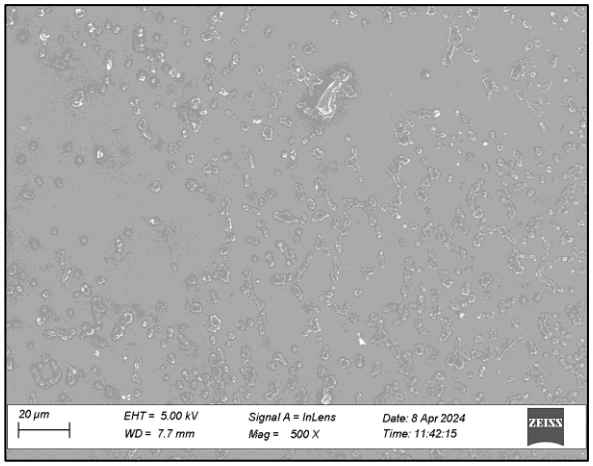

Fig-5: SEM image of post-treated mixed spp. biofilm with DNase I (contact time: 10 mins)

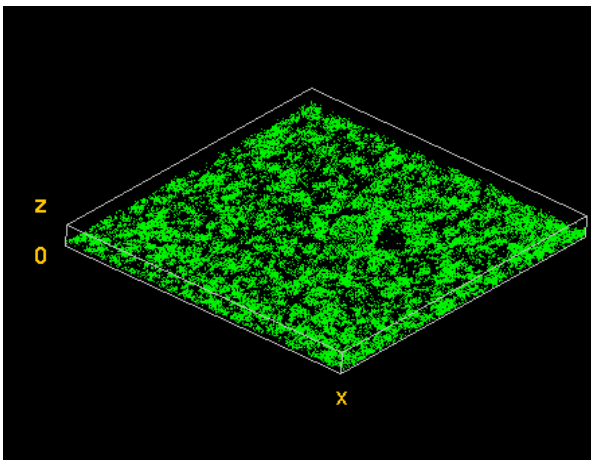

Fig-6: CLSM image of untreated preformed biofilm (control) on biosimilar surface; H = 180 µm

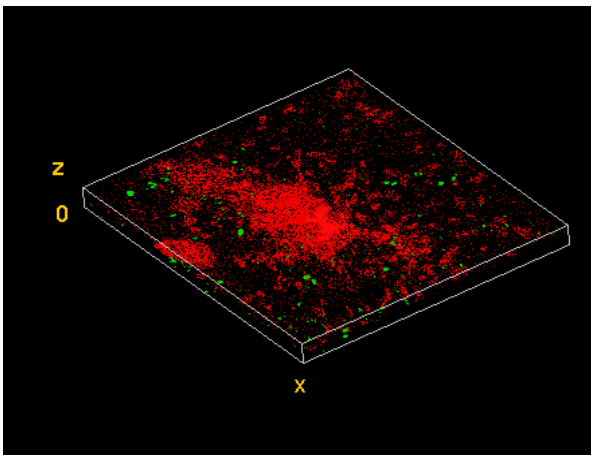

Fig-7: CLSM image of post-treated biofilm by ciprofloxacin on biosimilar surface (contact time: 1 hr); H = 170 µm

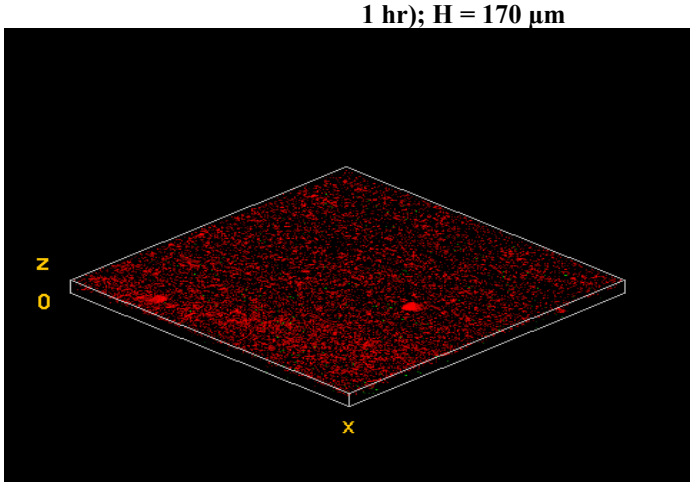

Fig-8: CLSM image of post-treated biofilm by ciprofloxacin + DNase I on biosimilar surface (contact time: 10 mins); H = 110 µm

CLSM analysis further demonstrated a reduction in the height (H) of the pre-formed biofilm grown on the biosimilar surface across all treatment groups. Specifically, ciprofloxacin monotherapy reduced biofilm height to 170 µm after a 1-hour exposure (Fig-7). In contrast, the optimized ciprofloxacin-DNase I combination achieved a more pronounced height reduction to 110 µm within only 10 minutes of contact time (Fig-8). Additionally, CLSM was utilized to visualize dead bacterial cells within the biofilm. Because DNase I functions strictly as a matrix-disrupting agent rather than a bactericidal one, it did not directly kill the bacterial cells. Instead, bacterial mortality was driven solely by ciprofloxacin. Consequently, biofilms treated with DNase I monotherapy did not exhibit red fluorescence (indicative of dead cells) but did display a reduced overall biofilm height due to matrix degradation.

DISCUSSION

In this study, an in vitro Pseudomonas aeruginosa biofilm model was successfully established on a newly developed collagen-based biosimilar surface, with robust growth kinetics quantified via absorbance measurements at OD595nm. Because this biosimilar substratum is primarily composed of rat-tail type II collagen, its structural framework closely mirrors the biochemical environment of human skin, directly corroborating the bioengineered tissue-mimicry concepts established by Kadam et al., 2019 (25). Following colonization, the mature biofilm was subjected to either ciprofloxacin monotherapy or a combination regimen supplemented with DNase I. Extracellular DNA (eDNA) functions as a fundamental structural scaffold within the extracellular polymeric matrix; consequently, its enzymatic degradation is critical to destabilizing this barrier and facilitating deeper antibiotic penetration (40,41). While ciprofloxacin monotherapy yielded a high Minimum Biofilm Inhibitory Concentration (MBIC) of 256 µg/ml, the addition of 10 µg/ml DNase I successfully halved the required antimicrobial dose to 128 µg/ml. This 50% reduction in the therapeutic threshold was structurally validated through multi-modal microscopic analysis (bright-field, SEM, and CLSM). Microscopic imaging of the combination therapy group demonstrated significant reductions in surface area coverage and biofilm height alongside definitive bactericidal action, whereas ciprofloxacin monotherapy achieved only localized bactericidal effects that required an extended 1-hour contact time. Ultimately, these findings demonstrate that combining matrix-degrading enzymes with standard antibiotics represents a highly efficient, fast-acting therapeutic strategy capable of dismantling protective biofilm architectures and significantly lowering the clinical dose requirements of conventional antimicrobials, thereby providing crucial validation for current paradigms in anti-biofilm research (42).

CONCLUSION

This study demonstrates that combining ciprofloxacin with the matrix-degrading enzyme DNase I provides a very efficient, quick-acting method against mature Pseudomonas aeruginosa biofilms on collagen surfaces that imitate skin. DNase I reduced the required dosage of ciprofloxacin from 256 to 128 µg/ml by destabilizing the biofilm scaffold through extracellular DNA degradation. Additionally, this dual-action treatment reduced biofilm height to 110 µm in just 10 minutes by speeding up structural disintegration. In the end, this synergistic platform provides a potent remedy for persistent wound infections, demonstrating the importance of enzymatic matrix disruption in preserving antibiotic activity and reducing clinical treatment thresholds.

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Conflicts of interest

The corresponding author confirms that there is no conflict of interest. The results of the study do not affect any associated parties.

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