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Article

Development of *In Vitro* and *Ex Vivo* Biofilm Models for the Assessment of Antibacterial Fibrous Electrospun Wound Dressings

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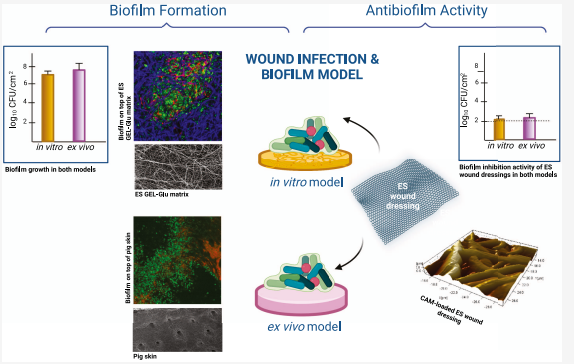
ABSTRACT: Increasing evidence suggests that the chronicity of wounds is associated with the presence of bacterial biofilms. Therefore, novel wound care products are being developed, which can inhibit biofilm formation and/or treat already formed biofilms. A lack of standardized assays for the analysis of such novel antibacterial drug delivery systems enhances the need for appropriate tools and models for their characterization. Herein, we demonstrate that optimized and biorelevant *in vitro* and *ex vivo* wound infection and biofilm models offer a convenient approach for the testing of novel antibacterial wound dressings for their antibacterial and antibiofilm properties, allowing one to obtain qualitative and quantitative results. The *in vitro* model was developed using an electrospun (ES) thermally crosslinked gelatin−glucose (GEL-Glu) matrix and an *ex vivo* wound infection model using pig ear skin. Wound pathogens were used for colonization and biofilm development on the GEL-Glu matrix or pig skin with superficial burn wounds. The *in vitro* model allowed us to obtain more reproducible results compared with the *ex vivo* model, whereas the *ex vivo* model had the advantage that several pathogens preferred to form a biofilm on pig skin compared with the GEL-Glu matrix. The *in vitro* model functioned poorly for *Staphylococcus epidermidis* biofilm formation, but it worked well for *Escherichia coli* and *Staphylococcus aureus*, which were able to use the GEL-Glu matrix as a nutrient source and not only as a surface for biofilm growth. On the other hand, all tested pathogens were equally able to produce a biofilm on the surface of pig skin. The developed biofilm models enabled us to compare different ES dressings [pristine and chloramphenicol-loaded polycaprolactone (PCL) and PCL−poly(ethylene oxide) (PEO) (PCL/PEO) dressings] and understand their biofilm inhibition and treatment properties on various pathogens. Furthermore, we show that biofilms were formed on the wound surface as well as on a wound dressing, indicating that the demonstrated methods mimic well the *in vivo* situation. Colony forming unit (CFU) counting and live biofilm matrix as well as bacterial DNA staining together with microscopic imaging were performed for biofilm quantification and visualization, respectively. The results showed that both wound biofilm models (*in vitro* and *ex vivo*) enabled the evaluation of the desired antibiofilm properties, thus facilitating the design and development of more effective wound care products and screening of various formulations and active substances.



KEYWORDS: *ex vivo biofilm model, in vitro biofilm model, skin wound infection, electrospinning, wound dressings, antibacterial, antibiofilm*

1. INTRODUCTION

Biofilms are reported to occur in many areas of medicine, being present in 80% of all known human infections. In wound care, microbial biofilms are recognized as one of the main causes of ineffective treatment and wound chronicity.[1] It is proposed that at least 78% of chronic wounds contain biofilms.[2] Mature biofilms develop already during the first 10 h and persist indefinitely when the wound remains untreated. Despite the increasing understanding of the mechanisms of biofilm formation in skin wounds, current strategies for wound biofilm and infection treatment are still far from ideal.

Clinical decisions for treatment are usually made based on planktonic bacteria in the wound, and this approach enables the treatment of acute wound infections.[1,3,4] However, to successfully treat wound infections in nonhealing wounds, the biofilm needs to be removed from the wound via surgical debridement.[2] As the next step, local antimicrobial therapy is needed to kill the released bacteria and attack the bacteria still residing in the biofilm residuals. Because bacterial biofilms in chronic and nonhealing wounds continue to be a medical challenge, the search for local therapies against biofilm formation and the treatment of formed biofilms are becoming increasingly important.[2]

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Electrospinning (ES) is a popular method for creating advanced fibrous wound care preparations (e.g., wound dressings) for local antimicrobial drug delivery and wound healing. ES allows the incorporation of one or more antibacterial or antimicrobial agents into the fibrous matrices, and the drug release can be modified and controlled.[5−7] Polymeric ES dressings have shown potential for the treatment of chronic wounds, and there are different ES wound matrices already available in the market, such as Restrata and Phoenix.[8−10] However, the effectiveness of antibacterial ES fiber dressings has mainly been demonstrated with planktonic bacteria and using standardized antimicrobial assays, such as the Kirby−Bauer disk diffusion or broth microdilution methodologies and time−kill assays (e.g., EUCAST disk diffusion, American Association of Textile Chemists and Colorists (AATCC) methods; ASTM E2315-16). The mentioned antimicrobial assays are usually used to evaluate and screen the effect of wound care matrices against infection development. The most popular *in vitro* test for assessing the antimicrobial properties of wound dressings is the Kirby−Bauer disk diffusion method and its modifications. The prerequisite for the suitability of the agar plate method is that the drug must be released from the carrier and diffused into the agar. It has been shown that the carrier can vary with the formulation used, and it also differs between selected antimicrobial agents.[11] We have shown previously that ES fibrous matrices can have very different morphological, physicochemical, and mechanical properties, which consequently can modify the drug release and its antibacterial efficacy.[12,13] The surface structure and morphology of fibers and fibrous matrices highly influence biofilm formation.[14,15] Therefore, the antibiofilm activity of wound dressings needs to be separately demonstrated.

Various *in vitro* biofilm assays have been developed and compared.[16,17] Even a standardized method for the evaluation of biofilm resistance properties of tube, yarn, and fiber specimens has been proposed (ASTM E3151-18). These tests provide valuable information about the growth of biofilms and their quantification, and they have been used as such and/or with some modifications for the testing of wound dressings including ES dressings.[18−20]

There are various *in vitro*, *ex vivo*, and *in vivo* biofilm and wound infection models available for evaluating the antibacterial efficacy and antibiofilm properties of wound care products.[21,22] Indeed, *in vivo* wound infection models on animals (e.g., mice, rat, rabbit, and pig) have been reported,[23−25] but their use may be very expensive, and they provide low throughput for the initial formulation screening phase due to the high biological variability between animals. Furthermore, *in vivo* animal data cannot always be directly translated to humans, and there are differences in how biofilms are formed and how they interact with their hosts, not to mention the ethical aspects in line with the 3R rule. Therefore, to develop an effective wound care therapy, including antibiofilm therapy, cost-effective standardized and reproducible models that aim to mimic the clinical situation are required.[2] The use of *in vitro* and *ex vivo* infection and biofilm models has found its place. *In vitro* biofilm models have been developed, which enable one to study biofilm treatment properties of wound dressings under more wound-like conditions.[26−28] Also, various *ex vivo* wound infection models have been proposed where tissues or organs are extracted from animals and/or humans and further cultured under *in vitro* conditions while preserving their three-dimensional structure.[29−32] Different *ex vivo* models such as the burn wound infection model[33] or the wound biofilm model[32,34] on pig skin have been recently developed and published. Nevertheless, none of them are validated for the testing of ES wound dressings. Currently, there is a lack of appropriate protocols and biorelevant models or easy-to-use screening methods suitable for the characterization of such novel antimicrobial drug delivery system (DDS)-based wound dressings. Moreover, to test the antimicrobial and antibiofilm efficacy of a wound dressing, biofilm models with relevant pathogens (depending on the actual clinical problem) are needed.

The aim of the present study was to develop *in vitro* and *ex vivo* biofilm models and protocols for assessing the antibacterial and antibiofilm properties of ES wound dressings. Models were designed suitably for these novel fibrous DDSs, taking into consideration the formulation aspects such as materials and their concentrations (e.g., polymers and antibacterial agents), which determine the physicochemical as well as biopharmaceutical properties of the ES matrix, including its drug release behavior and efficacy against specific bacteria or biofilms. The created models were designed to test the effectiveness of ES wound dressings using different relevant wound pathogens and compare them with each other to find suitable wound-dressing candidates for further *in vivo* testing in an animal model and/or in humans.

2. EXPERIMENTAL SECTION

2.1. Materials. *2.1.1. Drugs, Polymers, and Supplies.* Polycaprolactone (PCL) (M_n 80,000), poly(ethylene oxide) (PEO) (M_w 900,000), chloramphenicol (CAM) (PubChem CID: 5959), gelatin type A from porcine skin (GEL), anhydrous D-(+)-glucose (Glu), methanol (MET) (gradient grade), glycerol, chloroform (CHF), and acetic acid (99.8−100.5%, puriss p.a.), were purchased from Sigma-Aldrich Inc. (Darmstadt, Germany). All materials were of reagent grade or better and were used as received without any further purification.

2.1.2. Bacterial Strains and Growth Media. Three types of pathogenic bacteria relevant for skin and wound infections were used in the biofilm study: Gram-negative bacterium *Escherichia coli* DSM 1103 (ATCC 25922) and Gram-positive bacteria *Staphylococcus aureus* DSM 2569 (ATCC 29213) and *Staphylococcus epidermidis* DSM 28319 (ATCC 35984). For sterility testing, *E. coli* laboratory strain MG1566 (ATCC 700926) for aerobic conditions and *Fusobacterium nucleatum spp polymorphum* for anaerobic conditions were used as positive controls. All bacterial strains were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and stored at −80 °C. All bacteria were grown in BD Difco Lennox lysogeny broth (LB) (Becton, Dickinson and Company, Le Point de Claix, France) culture medium. For the biofilm studies, Dulbecco's modified Eagle medium DMEM/F-12 (Sigma-Aldrich, Gillingham, United Kingdom), with 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, without L-glutamine and phenol red, was used together with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Sao Paulo, Brazil). Phosphate-buffered saline (1 × PBS), pH adjusted to 7.4, was used in the biofilm assay. For some additional testing also 10 mM or 50 mM HEPES buffer was used after dilution from 1 M HEPES buffer solution (Corning, Manassas, USA). For European Pharmacopoeia

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(Ph.Eur, 10.0) sterility testing (monograph 2.6.1.), two different dehydrated culture media, soybean casein digest medium (also known as tryptic soy broth (TSB), Sigma-Aldrich, Bangalore, India) and fluid thioglycolate medium LAB025 (Lab M Limited, Lancashire, United Kingdom), were used. To create anaerobic conditions, anaerobic gas generation bags from BD GasPak, EZ Anaerobe Container System (Benex Limited, Dublin, Ireland) were used.

2.1.3. Histology Solutions. Tissue-freezing medium (Leica, Richmond, IL) was used for embedding and freezing the skin samples. Histological stains used were hematoxylin solution according to Harris (Carl-Roth, Karlsruhe, Germany) and eosin solution prepared according to the manufacturer's instruction using eosin Y (Acros Organics, Geel, Belgium) (hematoxylin and eosin (H&E) staining).

2.1.4. Fluorescent Stains. To visualize biofilm formation by confocal fluorescence microscopy (CFM), nucleic acid stain Syto 9 (Invitrogen, Eugene) and the EBBABiolight 630 (Ebba Biotech AB, Solna, Sweden) stain binding to cellulose and amyloid components were used.

2.2. Preparation Methods. *2.2.1. Preparation of Electrospun (ES) Artificial Skin and Fibrous Wound Dressings. 2.2.1.1. GEL-Glu Matrix Artificial Skin.* For the *in vitro* model, a previously published thermally crosslinked ES GEL-Glu matrix[35,36] was used as artificial skin. For the preparation of the ES solution, in 10M acetic acid, GEL 25% (w/v) was dissolved, and Glu 10% (w/w solid state) was added to the mixture as a crosslinking agent.[35] The ES solution was obtained after 24 h of stirring at room temperature (24 ± 2 °C) (RT). ES was carried out using an ESR200RD robotized ES system (NanoNC, Seoul, Republic of Korea). ES parameters were the following: needle 23G, voltage 18−19 kV, flow rate 15 μL/min, distance between the needle and the collector 15 cm, and roller speed 20 mm/min. The GEL-Glu matrix was crosslinked for 3 h at 170 °C after ES to achieve better mechanical properties and stability in aqueous conditions as shown in previous studies.[35−37]

2.2.1.2. Wound Dressings. Model wound dressings were prepared by ES to test the suitability of the *in vitro* and *ex vivo* models for analyzing the antibiofilm activity of ES drug delivery systems. The ES solution compositions, conditions, and method parameters of all four different types of wound dressings (two pristine PCL and PCL/PEO dressings and two antibacterial drug CAM-loaded CAM-PCL and CAM-PCL/PEO dressings) have been previously published by Preem et al. In brief, ES solutions were prepared by dissolving the polymer PCL 12.5% (w/V) alone or PCL 10% (w/V) together with PEO 2% (w/V) in a chloroform/methanol (CHF/MET) (3:1 V/V) solvent mixture. The concentration of the model drug CAM within drug-loaded dressings was 4% (w/w) based on the dry weight of the polymer(s).[38] To minimize the possible contamination of the produced ES wound dressings, clean preparation techniques were used. The folio for fiber collection was autoclaved before use; also, the ES robot chamber was cleaned with 70% ethanol solution before ES. Fibrous dressings were collected onto an aluminum foil and stored in ziplock bags at RT and 0% relative humidity (RH).

2.2.2. Preparation of Bacteria. The bacteria used in this study were stored in glycerol stocks at −80 °C. Then, the required bacteria were thawed and plated on LB agar plates for overnight incubation at 37 °C. For the biofilm assay, one bacterial colony was inoculated into 3 mL of liquid LB broth and grown for 20 h at 37 °C, with continuous movement at 200 rpm. Before every assay, the exact bacterial concentration in the overnight culture was fixed and confirmed by counting the colonies of colony forming unit (CFU) plating of 10-fold dilutions. For bacterial dilution, sterile distilled water was used.

2.2.3. Pig Ear Skin Collection, Preparation, and Storage. An *ex vivo* model was developed using pig ear skin, received from the local slaughterhouse (Rotaks-R Oy, Tartu, Estonia). All pigs were washed in a hot-water bath, during which superficial burn wounds were developed, and most of the hair on the skin was already removed. The ear skin was collected manually by separating the skin from the cartilage using surgical scalpels and cut into 10 × 10 cm pieces. In case needed, ears were first washed with 1 × PBS, and the remaining hair on the skin was removed by shaving. Cut pieces of the skin were treated differently for the histological study to determine the best skin preparation option (minimizing the modification of the skin). Half of the samples were placed in a 20% (V/V) glycerol/PBS solution for 2−4 h for cryoprotection purposes[39] and then placed into ziplock bags (LDPE); the other half of the samples were placed directly into ziplock bags. Liquid nitrogen for snap freezing of samples was used followed by γ sterilization. Sterilized samples were stored at −80 °C, as advised by the Central Tissue Bank. For the biofilm assay, the pig skin was defrosted and cut into 1 × 1 cm pieces. Pig ear skin had a uniform thickness of about 4 ± 1 mm. Before the test, pig ear skin samples were held in sterile 1 × PBS on ice for up to 2 h.

2.3. Sterilization. γ-Irradiation at a dose of 50 kGy was used to sterilize ES wound dressings,[40] GEL-Glu matrices, and pig ear skin. Sterilization was performed by the Scandinavian Clinics Estonia OÜ. Frozen pig ear skin samples on top of dry ice pellets were irradiated immediately after sample collection. The pig ear skin was subjected to different sterilization treatments (e.g., UV light for 30 min on both sides and 70% ethanol treatment), but skin samples were sterile only after γ-irradiation. In addition to the chemical indicators provided by the company, we also added biological controls. *Bacillus sp.* spores were prepared by first culturing bacteria on solid media, then suspending the solid-medium-grown cells in 50% ethanol, and incubating for 1 h to kill all vegetative cells.[41] The remaining spores were washed and suspended in distilled water. The number of spores in suspension was determined by plating and counting of CFUs. No growth after sterilization confirmed the efficacy of the methods. All of the liquids (growth media, 1 × PBS, distilled water) used in the sterility and antibacterial/antibiofilm studies were sterilized by filtration (filter membrane pore size of 0.22 μm) or autoclaved according to the manufacturer's instruction. Autoclaving with the standardized program for 15 min at 121 °C was also used to sterilize filter paper disks (used in a biofilm model setup) and all other equipment and supplies needed for the sterility and antibiofilm tests.

2.4. Characterization Methods. *2.4.1. Sterility Testing.* After γ-irradiation, all samples (ES pristine wound dressings, ES GEL-Glu matrix, and pig ear skin) were tested in triplicates for sterility under aerobic and anaerobic conditions according to the Ph.Eur. (10.0, monograph 2.6.1.) guidelines. Testing was carried out under aseptic conditions. TSB was used for aerobic bacteria and fungi, and the fluid thioglycolate medium used for anaerobic bacteria was hydrated with distilled water before use according to the manufacturer's instructions. Thereafter, previously cut and sterilized 1 × 1 cm samples in triplicate were inoculated into the test tubes with sterile

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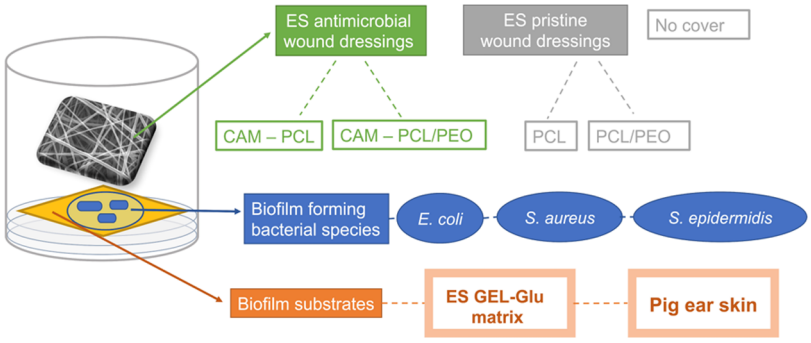

Figure 1. Schematics of the experimental work conducted for the development and validation of the biofilm models. Key: CAM, chloramphenicol; ES, electrospinning; GEL, gelatin; Glu, glucose; PCL, polycaprolactone; PEO, polyethylenoxide.

forceps under a laminar-flow hood. The test samples were incubated for 14 days at RT and protected from light for aerobic bacteria and fungi and at 30 °C under anaerobic conditions for anaerobic bacteria. For anaerobic conditions, test tubes were placed in a plastic bag together with anaerobic gas generation bags and sealed airtight. According to the Ph.Eur. guidelines, samples were considered sterile if no growth was detected after 14 days of incubation. Negative controls, pure growth medium without any inoculation, had to remain clear, and positive controls, Gram-negative *E. coli* MG1655 for aerobic conditions and *F. nucleatum* spp for anaerobic conditions, inoculated at the same time as the samples, had to show bacterial growth. Results are documented as images.

2.4.2. Biofilm Assay. 2.4.2.1. Biofilm Inhibition Model. A biofilm assay was designed to study the potential inhibition of biofilm formation in sterile nontreated flat-bottom 24-well plates (VWR International, LLC, Shanghai, China), and a one-well-based biofilm model was set up as follows. At the bottom of the well, three sterile filter paper disks cut to fit the well properly (diameter 13 mm) were placed. Dulbecco's modified Eagle medium (DMEM/F-12) without L-glutamine and phenol red supplemented with heat-inactivated FBS (10% (v/v)) was used as a medium to mimic the wound exudate. Then, 250 μL of the medium was added into every well, which was an optimal amount to immerse the filter papers and keep the setup moist. Then, either a 1 × 1 cm GEL-Glu matrix (cut under aseptic conditions under a laminar-flow hood) as artificial skin for the *in vitro* setup or 1 × 1 cm pig skin for the *ex vivo* setup was positioned on top of the filter papers. A schematic representation is shown in the Results (Figure 1).

2.4.2.2. Biofilm Formation on Top of the Substrate. Different pathogenic bacteria were tested to create different monobacterial biofilms in the *in vitro* and *ex vivo* models. Namely, *E. coli* DSM 1103 (ATCC 25922), which is a clinical isolate, *S. aureus* DSM 2569 (ATCC 29213) isolated from wounds, and *S. epidermidis* DSM 28319 (ATCC 35984) isolated from catheter sepsis were used. An overnight culture (20 h, 37 °C, 200 rpm) in LB broth was diluted using 1 × PBS to the optimal dilution (10^6 CFU/mL) immediately before the experiment, and the exact concentration was fixed in every assay by CFU plating. About 10 μL of bacterial dispersion was added on top of the pig skin for the *ex vivo* setup or on the GEL-Glu matrix for the *in vitro* setup. These *in vitro* and *ex vivo* models were incubated for 24 and 48 h at 37 °C. For incubation, well plates were closed with parafilm and placed in ziplock bags to avoid drying.

To assess whether the selected bacterial strains use gelatin (GEL) or glucose (Glu) in the GEL-Glu matrix for nutritional purposes, an additional test was designed and performed. Bacteria were inoculated in 10 mM HEPES buffer (1 mL) alone and/or together with the GEL-Glu matrix (size of 1 × 1 cm). The buffer chosen was the same as that used in the medium for the developed biofilm assay. The bacterial concentration at time point 0 was 10^4 CFU/mL. Bacterial growth at all tested time points (24, 48, 72, and 96 h) was measured by CFU plating. After 1 week, biofilm disruption manipulations (as described in the following paragraph Biofilm Disruption and Quantification: (30 s sonication + 30 s vortexing) × 6) were performed on the samples to check for any biofilm formation.

In addition, to study the effect of a thicker layer of the GEL-Glu matrix in the test for biofilm formation, one modification was made to the *in vitro* biofilm model. The *in vitro* model was created as described above, but a thicker artificial skin was created by positioning three GEL-Glu matrices on top of each other. An *S. epidermidis* culture dispersion was used to create a biofilm, which was incubated for 24, 48, and 72 h. After incubation, the steps described in the following paragraph (Biofilm Disruption and Quantification) were used to disrupt and quantify the biofilm.

2.4.2.3. Biofilm Disruption and Quantification. Subsequently, planktonic bacteria were removed by washing the samples (S = 1 cm^2) twice with 1 mL of 1 × PBS solution and placed into a new buffer (1 mL). Biofilm disruption was carried out similarly to that in previously published studies.[27,42] Shortly, vortexing (Vortex-Genie 2, Scientific Industries) of the samples for 30 s and sonification (Bandelin Sonorex digital 10 P, operating at 20% of the maximum power) for 30 s were performed 6 times. When not handling the samples, they were placed on ice. After that, 100 μL aliquots were used to make 10 times dilutions, plated on LB agar plates, and incubated overnight at 37 °C. All experiments were carried out in triplicate, and the mean value was obtained. Also, technical replicates were used for CFU plating. Data are presented as CFU/cm^2, which is equal to the CFU of bacteria from the biofilm grown on a 1 × 1 cm sample.

2.4.2.4. Biofilm Model Validation. CAM-loaded ES wound dressings (CAM-PCL and CAM-PCL/PEO) and pristine wound dressings (PCL and PCL/PEO) were cut into 1 × 1 cm samples under aseptic conditions under a laminar-flow hood. All drug-loaded dressings were weighed on an analytical balance (RADWAG Wagi Elektroniczne XA 210.4Y, Radom, Poland) before the experiment. The average CAM-loaded dressing weight was 2 ± 0.7 mg; hence, the drug content in

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dressings (solid state 4%) was 0.08 ± 0.03 mg. All CAM-loaded dressings were always weighed before the experiment to calculate the exact drug content in every sample.

In the assay, ES wound dressings were placed on top of the GEL-Glu matrix or pig skin immediately after the bacterial dispersion. To ensure close and comparable contact between the ES wound dressing and the pig skin or GEL matrix, well plate inserts (CellCrown 24, Scaffdex Oy, Tampere Finland) were placed on top of the ES dressing which allows proper hydration of the wound dressing. For the biofilm assay, drug-loaded samples were always studied in comparison with pristine wound dressings and with uncovered substrates (GEL-Glu matrix or pig skin). For this, in every assay, three samples were covered with drug-loaded ES wound dressings, three with pristine wound dressings, and three were left uncovered, and they were incubated as described above. For biofilm quantification, the wound dressings were removed from the substrate, and both the substrate and the dressing were separately placed into 1 mL of 1 × PBS and washed twice with 1 × PBS; biofilm quantification was carried out as described above.

2.4.2.5. Biofilm Treatment Model. The biofilm model was modified to determine whether the model can be used to study the effect against already formed biofilms. A monobacterial biofilm was created on top of the GEL-Glu matrix or pig skin and incubated for 24 h. After 24 h, ES wound dressings were applied on top of the pregrown biofilm. To ensure direct contact, a Cell Crown insert was placed on top of the wound dressing, and an additional 250 μL of cell culture medium was added to provide nutrients for bacteria and ensure the desired wettability of the wound dressing. Incubation was performed for an additional 24 or 48 h, followed by biofilm quantification as described above.

2.4.2.6. Contamination Detection Controls. All sterile liquids (growth media, 1 × PBS, distilled water) were plated on agar plates before the first use and after the last use, which allowed us to determine whether the solutions were sterile and remained sterile during the assay. Also, the sterility of filter paper disks, the GEL-Glu matrix, pig skin, pristine ES wound dressings, and inserts was tested. For this, the tested object was placed on top of three filter paper disks, and 250 μL of medium was added. No bacterial dispersion was added to the controls, but otherwise the controls were incubated similarly to the samples. All manipulations performed with the samples were similarly performed with the controls. The sample results were considered to be valid only when controls were free of contamination.

2.5. Microscopic Analyses. *2.5.1. Histological Evaluation of Pig Skin.* Cryosections were made of differently treated pig ear skin samples for histological evaluation and confirmation of epidermis removal during heat treatment (development of superficial skin burn wounds). Skin samples (1 × 1 cm) were embedded perpendicularly to the surface of the tissue-freezing medium (Leica, Newcastle, United Kingdom) such that the sections were cut longitudinally through the epidermis and dermis using a cryostat (Leica, CM1850, Nussloch, Germany). Serial cross-sections, 50 μm thick, were cut at −23 °C. Sections were washed with 1 × PBS to get rid of the excess freezing medium. After that, H&E staining was carried out, and the samples were fixed with a cover glass on top of microscope slides. Micrographs were recorded using a Zeiss Stemi 508 light microscope together with a Zeiss Axiocam 208 color microscope camera (Suzhou, China), using program Zen 3.4. (Zen Lite).

2.5.2. Biofilm Imaging by Scanning Electron Microscopy (SEM). SEM was used to visualize the formed bacterial biofilms. Micrographs were recorded by SEM (Zeiss EVO 15 MA, Germany) under 9000× and 2000× magnifications. Microscopy sample preparation for biofilms grown on both substrates (GEL-Glu matrix and pig skin) after the biofilm assay (24 h) was carried out. After incubation, substrates were rinsed twice with 1 × PBS and then fixed with 4% formaldehyde solution in 1 × PBS for 30 min at RT, and then rinsed again twice with 1 × PBS and dehydrated in increasing concentrations of ethanol (30, 60, and 96%). In the ethanol solution with the highest concentration, the samples were kept for 5 min. Dried samples were mounted on aluminum stubs using carbon tape and sputter coated with platinum in an argon atmosphere.

2.5.3. Biofilm Imaging by Confocal Fluorescence Microscopy (CFM). Confocal fluorescence microscopy (CFM, LSM710, Carl Zeiss, Germany) was performed to visualize biofilm formation on top of the GEL-Glu matrix and pig skin at different time points (0, 24, and 48 h). Samples were prepared similarly to uncovered and untreated samples in the biofilm assay. For imaging, nucleic acid stain Syto 9 (Invitrogen, Eugene), an excitation laser of 488 nm, and emission recorded in the range of 503−542 nm were used to visualize bacteria and the stain EBBABiolight 630 (Ebba Biotech AB, Solna, Sweden), an excitation laser of 514 nm, and emission recorded in the range of 545−657 nm were used to visualize the biofilm matrix formed by *E. coli*. The EBBABiolight 630 orange optotracer molecule binds to cellulose and amyloid components in the biofilm matrix. For visualizing the GEL-Glu matrix fibers, an excitation laser of 405 nm and emission in the range of 410−502 nm were used to record autofluorescence. Stains were diluted 100× using 50 mM HEPES buffer, which was obtained by diluting 1 M HEPES buffer solution (Corning, Manassas). Samples for microscopy were prepared using 2 μL of both diluted stains, which were placed on top of the sample. Zen software was used for the analyses (Zeiss, Oberkochen, Germany).

2.5.4. Atomic Force Microscopy (AFM) of ES Wound Dressings. The surface topography and morphology of ES wound dressings were investigated with AFM (Autoprobe CP, ThermoMicroscopes). The surface topography images of the ES samples were obtained over a 20 μm × 20 μm area. The AFM mapping was performed in the contact mode with a cantilever of 0.12 N/m spring constant (Silicon cantilever, CSCH21A, NT-MDT Ltd., Russia) at a scan rate of 0.6 Hz. The measurements were carried out at RT using a large-area scanner (100 mm lateral scan size).

2.6. Statistical Analysis. Data are presented as the arithmetic mean (n = 3) ± standard deviation (SD). Statistical analyses were performed using either the pairwise t-test or the independent t-test. The variance was calculated using the f-test. CFU data were $\log_{10}$-transformed before statistical analyses. The log reduction was determined by subtracting the log of the bacterial concentrations in treated samples from the values quantified in untreated biofilms. The data were analyzed using Microsoft Excel (version 16.58). Scientifically significant differences were found using p-values, and a p-value of 0.05 was considered significant. In the case of multiple comparisons, p-values were adjusted using the Holm−Bonferroni method.

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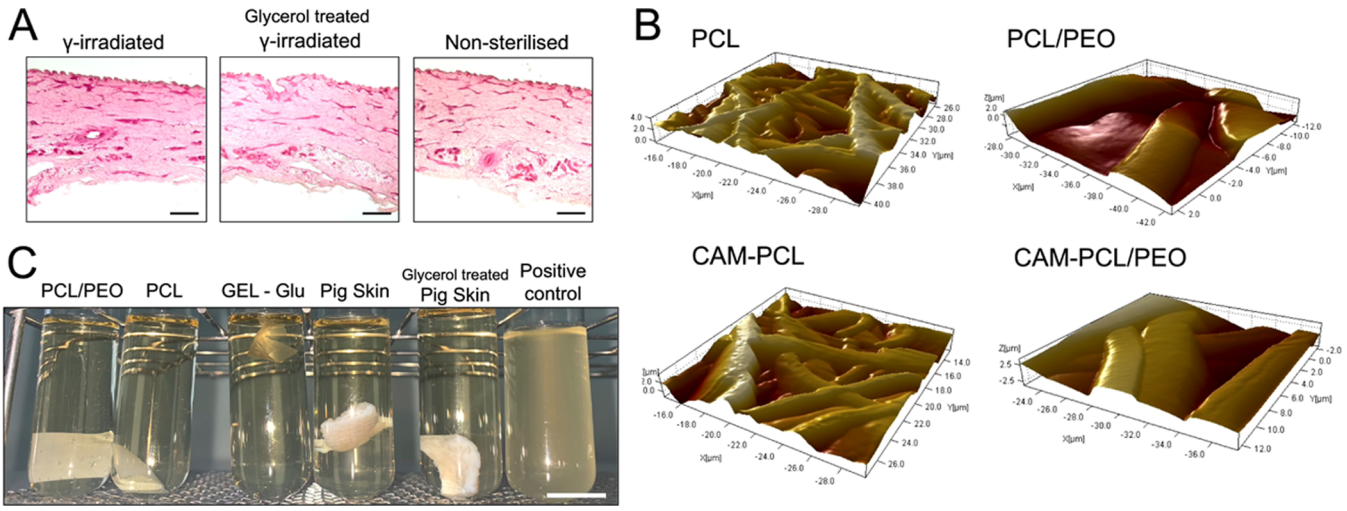

Figure 2. (A) Micrographs of cross-sections of heat-treated pig ear skin (with superficial burn wounds) with and without γ-irradiation and glycerol treatment; H&E stained. Scale bar: 500 μm. (B) AFM micrographs of ES fibrous wound dressings. (C) European Pharmacopoeia 10.0 sterility test results after 14 days of incubation of γ-irradiated (50 kGy) samples (ES pristine fibrous wound dressings and infection model substrates) under aerobic conditions at room temperature (RT) in the dark. Positive bacterial control (*E. coli*). Key: GEL, gelatin; Glu, glucose; PCL, polycaprolactone; PEO, polyethylenoxide. Scale bar 1 cm.

3. RESULTS AND DISCUSSION

3.1. Preparation, Storage, and Sterility Testing of Biofilm Substrates. The aim of the present study was to develop and compare *in vitro* and *ex vivo* biofilm models for assessing the antibiofilm properties of electrospun (ES) antibacterial wound dressings (Figure 1).

The *in vitro* model was created using a thermally crosslinked ES porcine gelatin and glucose (GEL-Glu) matrix as artificial skin. The ES GEL-Glu matrix displays a skin ECM-like fibrous structured surface with an average fiber diameter of 535 nm ± 60 nm; the degree of swelling was 290 ± 30%, and the thickness of the matrix was approximately 0.08 mm.

The *ex vivo* model was developed using pig ear skin as it resembles human skin the most.[43,44] The skin was subjected to a superficial burn wound in a hot-water bath (temperature 60 °C), collected, frozen using liquid nitrogen, and sterilized using γ-irradiation. To confirm the presence of a superficial burn wound (epidermis removal), skin samples were subjected to histological analyses by microscopy (Figure 2A). It can be seen that the epidermis layer was removed or partially removed during the thermal treatment in a water bath.

Different pathogenic bacteria isolated from infected wounds were used to develop monobacterial biofilms: Gram-negative bacterium *E. coli* DSM 1103 (ATCC 25922) and Gram-positive bacteria *S. aureus* DSM 2569 (ATCC 29213) and *S. epidermidis* DSM 28319 (ATCC 35984). Biofilm formation on top of the substrates (GEL-Glu matrix vs pig ear skin) was studied at first, and then, for model validation, four different previously developed and characterized ES wound dressings were used.[38] Two of them contained the antibacterial drug chloramphenicol (CAM) and the other two were without a drug. The biofilm model was used, and data were obtained at two time points: 24 and 48 h.

To visualize these ES wound dressings and better understand their interactions with bacteria, AFM micrographs were recorded (Figure 2B). All four ES dressings had uniform smooth fibers; for PCL and PCL/PEO dressings, the average fiber diameter was less than the micrometer range (fiber diameter varied across the mat), whereas the PCL/PEO dressing consisted of microfibers (fiber diameter was homogeneous across the mat). It has been previously shown that the porosity of ES wound dressings is the same in all cases (between 87 and 89%)[38] PCL dressings have more hydrophobic properties as the degree of swelling is only 5%, whereas PCL/PEO dressings are more hydrophilic, which allows the dressing to swell up to 250%.[38] Good wetting properties are important for sufficient drug release from wound dressings.

The design and development of a successful biofilm model require sterility. In this study, a combination of three sterilization methods was used, namely, filtration, autoclaving, and γ-irradiation. Filtration was used to sterilize all of the liquids (growth media, 1 × PBS, distilled water) used in the assay. Autoclaving was used to sterilize the filter paper disks and the equipment (scissors, forceps, pipettes, and pipette tips) used. In our preliminary experiments, UV treatment for 30 min on both sides for the sterilization of filter paper disks and GEL-Glu matrices was unsuccessful, and samples showed contamination. All ES wound dressings and GEL-Glu matrices were therefore sterilized using γ-irradiation. γ-Irradiation may cause changes in the molecular structures of the polymers and therefore affect the properties of ES wound dressings.[45] Therefore, γ-irradiation with a dose of 50 kGy was chosen, as it was previously shown to be suitable without any harmful effects and no reduction in the CAM content (no degradation of CAM).[40] The effectiveness of γ-sterilization was confirmed using the Ph.Eur sterility test, which showed that no bacterial growth occurred after 14 days of incubation (Figure 2C).

Identifying a suitable sterilization method for pig skin samples was not an easy task. The sterilization itself should successfully remove the contamination but should not damage the structure of the skin used for the model development. Preliminary testing showed that just wiping the surface of the skin gently with 70% ethanol solution was not enough for disinfection (Supporting Information, Figure S1). This has also been shown in previous studies.[33,46−48] Although even rinsing the pig skin samples with ultrapure water has previously been reported to be effective,[49] in our work, contamination was not removed using ultrapure water or ethanol. Often, 70% ethanol

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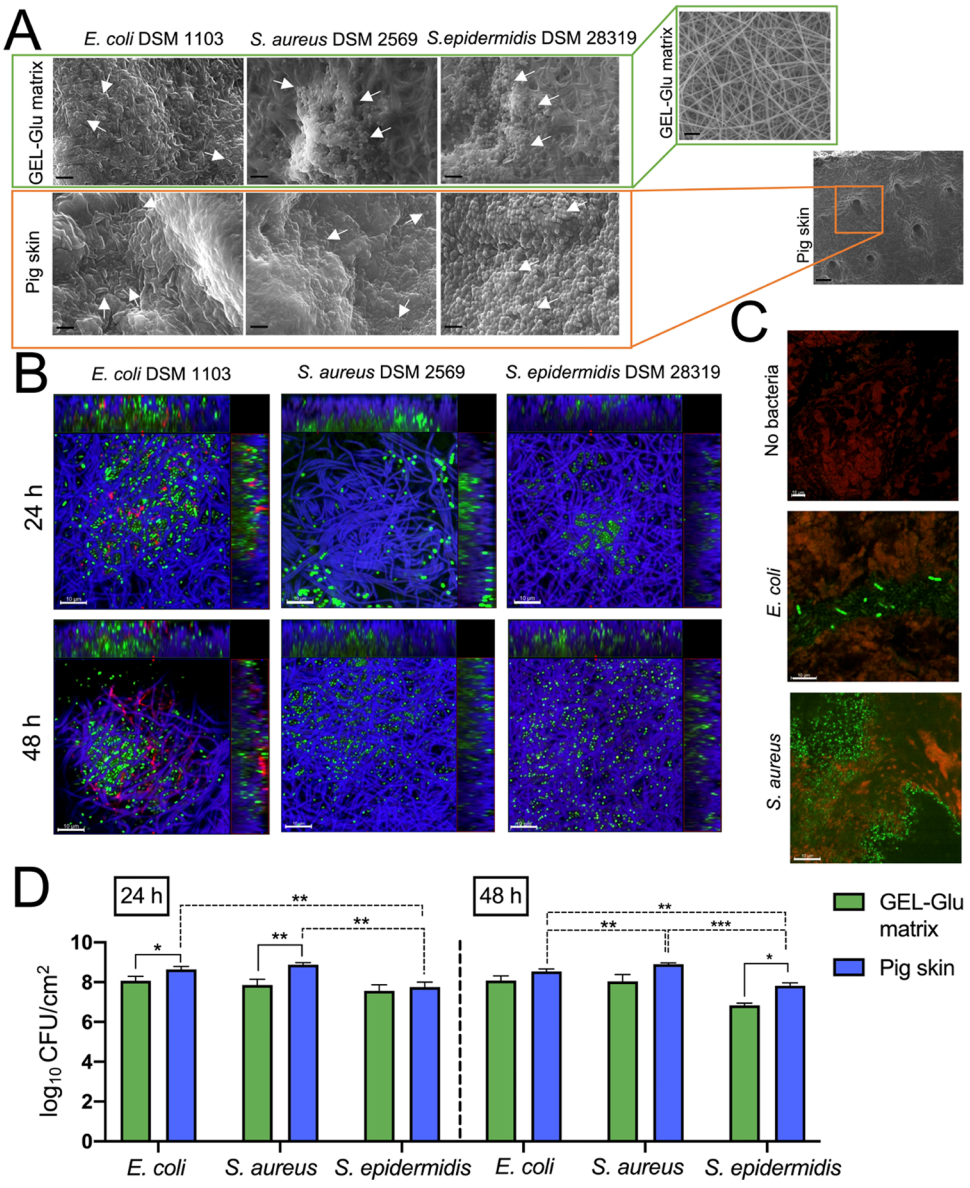

Figure 3. (A) SEM micrographs of biofilm formation after 24 h on top of the substrates—gelatin−glucose matrix (GEL-Glu matrix) and pig skin. Arrows point out the bacteria. Three different wound bacteria were used: *E. coli* DSM 1103, *S. aureus* DSM 2569, and *S. epidermidis* DSM 28319. The scale bar is 3 μm for the overview image of the pig skin surface, and it is 10 μm for all other SEM micrographs. (B) CFM micrographs of biofilm formation (24 and 48 h) on top of the GEL-Glu matrix in the *in vitro* model. Biofilm matrix visualized using EbbaBiolight 630 is in pink; ES gelatin−glucose matrix (GEL-Glu matrix) fibers visualized by autofluorescence are in blue; and bacteria stained using SYTO-9 are in green. Three different wound bacteria were used: *E. coli* DSM 1103, *S. aureus* DSM 2569, and *S. epidermidis* DSM 28319. Scale bar: 10 μm. The sample depth shown is 14.4 μm. (C) CFM micrographs of biofilm formation (24 h) in the *ex vivo* model. Pig skin had nonspecific red autofluorescence; bacteria were stained with SYTO-9 in green. Two different wound bacteria were used: *E. coli* DSM 1103 and *S. aureus* DSM 2569. Scale bar: 10 μm. (D) Biofilm formation after 24 and 48 h on top of the substrates—gelatin−glucose matrix (GEL-Glu matrix) and pig skin. Three different wound bacteria were used: *E. coli* DSM 1103, *S. aureus* DSM 2569, and *S. epidermidis* DSM 28319. Results are shown in the logarithmic scale as the number of colony-forming units (CFUs), with standard deviation bars (n = 3). Statistical significance is shown as follows: *p < 0.05; **p < 0.01; and ***p < 0.001. Experiments were performed using at least three technical replicates. Key: GEL, gelatin; Glu, glucose.

treatment is combined with the soaking of samples in an antibiotic solution.[50] This approach was not used in our study to avoid misinterpretation of the results later. Bathing the skin samples in ethanol for a longer time period seemed to be too destructive to the tissue, and for the same reason, UV sterilization was avoided. The chosen sterilization method for pig skin in this study was γ-irradiation as it has been previously used and recommended for the sterilization of pig ear skin samples for the *ex vivo* model.[51,52] The standard γ-irradiation dose for sufficient sterilization of biological samples is 25 kGy.[52,53] Unfortunately, for our samples, it was not effective enough. It has been previously established by Johnston et al. that the sterility assurance level is extremely dependent on the initial bioburden, and the use of standard irradiation levels will require a method to reduce the bioburden before irradiation.[39] In our study, pigs were placed in a hot-water bath after slaughter, which can also reduce the bioburden in addition to the formation of superficial burn wounds and the removal of hair. After this, sterilization with a higher dose of irradiation (50 kGy) was used. The effect of γ-irradiation is based on the

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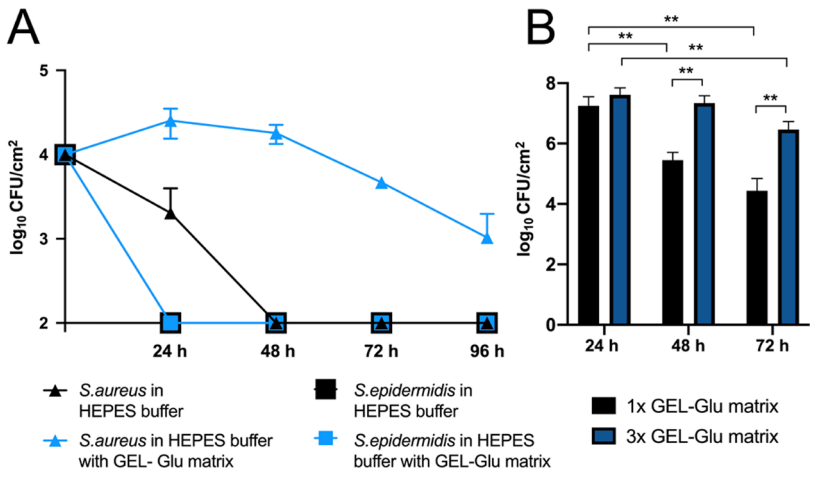

Figure 4. (A) Use of the gelatin−glucose matrix (GEL-Glu matrix) as a nutritional substrate in HEPES buffer. Bacteria were inoculated into 10 mM HEPES buffer alone and/or together with the GEL-Glu matrix for up to 96 h. *S. aureus* DSM 2569 and *S. epidermidis* DSM 28319 were used. The number of colony-forming units (CFUs), with standard deviation bars (n = 3), are shown in the logarithmic scale. Experiments were performed using at least three technical replicates. The detection limit of the assay is 2 $\log_{10}$ CFU/cm^2. (B) *S. epidermidis* DSM 28319 biofilm formation on top of a single-layered gelatin−glucose matrix (1 × GEL-Glu) and triple-layered gelatin−glucose matrices (3× GEL-Glu matrix) in HEPES-buffered DMEM/F-12 growth media at different time points (24, 48, and 72 h). The number of colony-forming units (CFUs), with standard deviation bars (n = 3), are shown in the logarithmic scale. Statistical significance is shown as $^{**}P < 0.01$. Experiments were performed using three technical replicates. Key: GEL, gelatin; Glu, glucose.

water content of the samples; its absence leads to the breaking of collagen polypeptide chains and its presence leads to hydroxyl radical formation, which causes the crosslinking of collagen fibers.[51,54] Higher doses of γ-irradiation may also reduce tissue integrity. To understand the effect of γ-irradiation on the skin and confirm its suitability for sterilization, the initial skin samples and γ-sterilized samples were subjected to histological analyses by microscopy (Figure 2A). Comparing the micrographs, it was seen that the skin integrity was not reduced by γ-irradiation. No major differences were observed between the γ-sterilized and nonsterilized skin samples. It is likely that due to the protective measures taken, the skin integrity was preserved: samples were placed in 20% glycerol solution before sterilization and deeply frozen after sterilization, according to Rooney et al.[53]

During the biofilm model development, the relevance of sterility and detection of contamination was confirmed. For this reason, a contamination detection protocol for the assay was created, as described in the Methods section. Furthermore, negative controls without added bacterial dispersion were prepared in every assay in the same well plate to test the sterility of the filter paper disks, pig skin, and GEL-Glu matrix. These were incubated with the media similarly to the samples used in the assay. The 1 × PBS buffer and growth media used were also tested for sterility by CFU plating.

3.2. Biofilm Formation on Top of Different Substrates—ES GEL-Glu Matrix (*In Vitro* Model) vs Pig Skin (*Ex Vivo* Model). The initial step in the model development was to determine whether monobacterial biofilms form in both models, using different pathogenic bacteria and at different time points. CFU enumeration was chosen to obtain appropriate and comparable results for biofilm formation. SEM micrographs were obtained to confirm the findings, and CFM results helped visualize the live biofilm matrix on different substrates (artificial skin vs pig skin).

The results showed that after 24 h, all three selected bacteria (*E. coli* DSM 1103, *S. aureus* DSM 2569, and *S. epidermidis* DSM 28319) had already adhered on these substrates and formed a biofilm consisting of up to 10^9 CFU/cm^2 bacteria, being also visible in SEM and CFM micrographs (Figure 3).

SEM micrographs showed that both substrates were colonized with different bacteria already after 24 h (Figure 3A). It was also seen that in the *in vitro* model, bacteria grew between and inside the fibers, and in the *ex vivo* model, bacterial colonies were found more inside the skin pores. CFM micrographs also confirmed that all tested bacteria preferred to grow inside the GEL-Glu matrix, and by making z-stacks of our samples, the depth of colonized bacteria could be determined (Figure 3B). Differences between bacteria showed that *E. coli* microcolonies were formed more on the surface of the fibrous matrices, whereas *S. aureus* and *S. epidermidis* formed colonies far deeper inside the fiber matrix. CFM microscopy together with Ebba Biolight staining also provided additional and relevant information about live biofilm formation, which allowed us to visualize the biofilm matrix formed by *E. coli* on top of the developed artificial skin (GEL-Glu matrix) (Figure 3B). It was seen that the biofilm matrix was formed on top of the colonizing bacteria. For *S. aureus* and *S. epidermidis*, the biofilm matrix was not stained with Ebba Biolight, but larger microcolonies of both bacteria were observed at both time points (Figure 3B). Furthermore, the microcolonies were more frequently observed in the substrate after 48 h in comparison with after 24 h of testing.

The visualization of bacteria and the biofilm matrix on top of the pig skin by CFM was challenging. Stained pig skin without bacteria itself had a nonspecific red autofluorescence (Figure 3C). The biofilm matrix-specific red fluorescence was not detected on any of the CFM micrographs. Overall, it was challenging to visualize even colonizing bacteria on top of the pig skin by CFM due to their growth occurring mainly inside the skin pores, which are not easily reachable for visualization using CFM. SEM micrographs of *E. coli* and *S. aureus* colonies were successfully obtained on pig skin only at the edges of skin pores. For the *ex vivo* model, the SEM images were much more informative and can be used for better visualization of bacteria on top of pig skin (Figure 3A).

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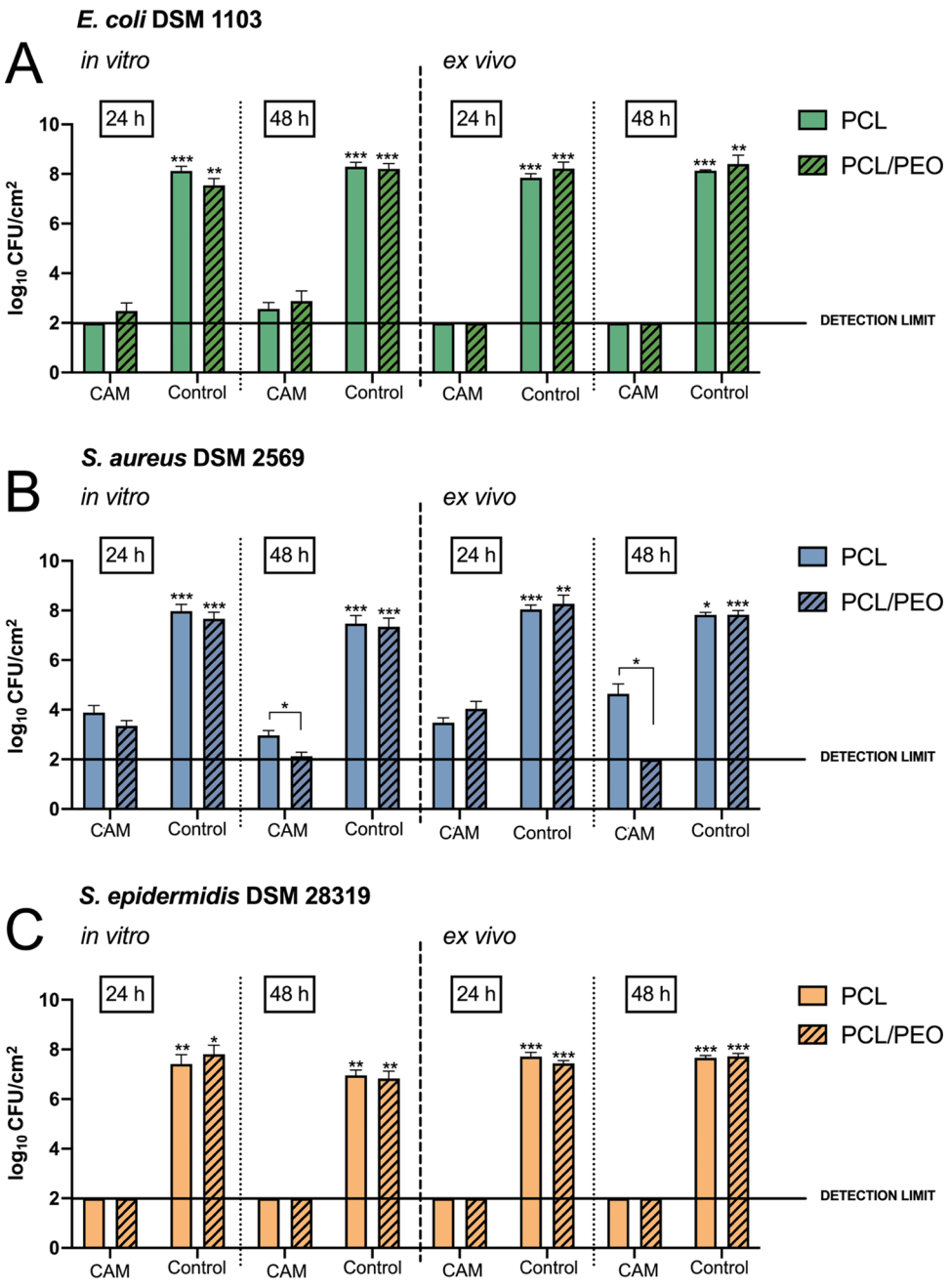

Figure 5. Model validation using electrospun (ES) wound dressings. For the *in vitro* model, the GEL-Glu matrix was used as artificial skin, and for the *ex vivo* model, pig skin was used. Three different bacteria were used: (A) *E. coli* DSM 1103, (B) *S. aureus* DSM 2569, and (C) *S. epidermidis* DSM 28319. Results are shown in the logarithmic scale as the number of colony-forming units (CFUs) cm^2, with standard deviation bars ($n = 3$). The detection limit of the assay is 2 $\log_{10}$ CFU/cm^2. CAM-loaded ES wound dressings were compared with pristine control wound dressings. A comparison between the PCL vs PCL/PEO formulations is also presented. Statistical significance is shown as follows: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Key: CAM, chloramphenicol; PCL, polycaprolactone; and PEO, polyethylenoxide.

The results in Figure 3D indicate that all selected bacterial strains can be successfully used in the developed biofilm assays. Differences between the *in vitro* and *ex vivo* models in the amount of bacterial biofilm (measured as the number of biofilm-forming bacteria) formed on top of the substrates were observed after 24 h. For *E. coli* and *S. aureus*, there were statistically significant differences in the biofilm formation on the GEL-Glu matrix (*in vitro* model) compared with pig skin (*ex vivo* model), with a higher formation observed in the latter model. At the 48 h time point, the differences were not statistically relevant anymore for *E. coli* and *S. aureus* (Figure 3D). Differences between the *in vitro* and *ex vivo* models at the 48 h time point were observed with *S. epidermidis*, wherein the biofilm formation on top of the GEL-Glu matrix was significantly lower than that on top of the pig skin (Figure 3D). Overall, pig skin was the most favored substrate for biofilm formation for all three bacteria at any time point. Nevertheless, differences in the number of biofilm-forming bacteria among the three selected bacteria were seen only in the *ex vivo* model (Figure 3D). For example, in the *ex vivo* model, *S. epidermidis* formed less biofilm on pig skin compared with *E. coli* and *S. aureus* at both time points.

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It is known that for a closed model, no continuous flow of the medium is possible, which leads to changes in experimental conditions as the nutrient content is reduced and metabolic products build up.[55] The latter results in a higher biofilm growth rate at the beginning of the assay when there are enough nutrients.[56] Nevertheless, with *S. epidermidis*, lower numerical values of biofilm bacteria were found in the *in vitro* biofilm model using the GEL-Glu matrix at the 48 h time point. This suggests that other bacteria (*E. coli* and *S. aureus*) might use other substances of the matrix for nutritional purposes. To confirm this statement, an additional test was performed, where *S. aureus* and *S. epidermidis* were inoculated into 10 mM HEPES buffer alone or together with the GEL-Glu matrix, and the use of the GEL-Glu matrix as a nutritional substrate was assessed (Figure 4A).

S. aureus survived in the testing environment when the GEL-Glu matrix was present for at least 96 h, but no bacteria were detected in pure HEPES buffer after 48 h (Figure 4A). For *S. epidermidis,* no bacteria were observed already after 24 h in both environments. These results enable us to explain the lower concentrations of the *S. epidermidis* biofilm bacteria on the GEL-Glu matrix in the developed *in vitro* biofilm model, as *S. epidermidis* does not use the GEL-Glu matrix as a nutritional substrate, unlike *S. aureus*. This is most likely due to the different virulence of these bacteria. *S. aureus* as well as *E. coli* have several virulence factors and are both pathogens capable of initiating infections in a human body, whereas *S. epidermidis* usually does not have aggressive virulence factors.[57]

Indeed, one of the possible reasons for the higher bacterial numbers on top of pig skin might also be related to the thickness of the substrate (5 mm for pig skin vs 0.08 mm for the GEL-Glu matrix). Taking this into consideration, a modification in the *in vitro* model was made. Instead of one GEL-Glu matrix, three GEL-Glu matrices on top of each other were used (a total thickness of 0.27 mm), and biofilm formation was tested with *S. epidermidis* (Figure 4B). To show the effect more clearly, we prolonged the study up to 72 h. In this assay, the *S. epidermidis* biofilm formation on top of the single-layered GEL-Glu matrix significantly decreased with every time point. Differences between the single- and triple-layered GEL-Glu matrices were not significant after 24 h but were significant in the subsequent time points. The results showed that when using triple-layered GEL-Glu matrices, the biofilm formation does not reduce with time at the same rate as it provides a larger three-dimensional surface for bacteria to grow. However, the number of biofilm-forming bacteria is still significantly lower after 72 h when compared with the 24 h time point (Figure 4B).

3.3. Biofilm Model Validation Using Drug-Loaded ES Fibrous Wound Dressings. Both *in vitro* and *ex vivo* biofilm models were used to study the effect of previously developed and characterized antibacterial ES wound dressings by Preem et al. on biofilm formation.[12,38,40]

The results showed that CAM-loaded wound dressings drastically inhibited biofilm formation on top of the substrates compared with non-CAM-loaded control dressings (Figure 5). Results were statistically significant in both models, showing up to 6 log reductions in the number of biofilm-forming bacteria at the 24 and 48 h time points. Both models behaved similarly, meaning that no statistically significant differences between the *in vitro* and *ex vivo* models in the number of biofilm-forming bacteria were found at any time point. As *ex vivo* models are mainly developed and used as a bridge between *in vitro* and *in vivo* models, it is a great finding to have similar results obtained using either artificial skin (GEL-Glu matrix) or pig skin. The *ex vivo* model is known to provide more valuable features for mimicking real "skin wound" conditions as most of the wound bed components are naturally occurring components in the skin. *Ex vivo* models do not require any supplementary agents to be added into the growth medium compared with *in vitro* models.[33] In our study, the GEL-Glu matrix in the *in vitro* model also provided nutritional supplements to some bacterial strains, acting similarly to the pig skin.

As expected, no effect was revealed with non-CAM-loaded control dressings, which showed biofilm formation up to 8 $\log_{10}$ CFU/cm^2, similar to the nontreated dressing (uncovered substrate) (Figures 3D and 5). As shown by the results of our study, biofilm inhibition was somewhat less efficient against *S. aureus* as compared with *E. coli* and *S. epidermidis*, which might be due to the higher effective concentration of CAM needed. Nevertheless, antibacterial ES wound dressings had a statistically significant inhibiting effect on biofilm formation in both models at all time points and with all selected bacterial strains.

The aim of these developed *in vitro*/*ex vivo* models was not only to derive an assay to study the antimicrobial/antibiofilm properties of wound dressings but also to compare different formulations for their efficacy under more biorelevant conditions and predict their *in vivo* behavior. In this study, we on purpose tested ES wound dressings made from the hydrophobic polymer PCL and another one, where, in addition to the PCL hydrophilic polymer, PEO was added. As previously shown, the drug release kinetics into 10 mL 1 × PBS differ between the two wound dressings, although no differences in the disk diffusion assay (release into the gel) were seen.[38] Interestingly, previously reported biofilm assays revealed that the CAM-PCL dressing was more effective compared with the CAM-PCL/PEO dressing on *E. coli* CFT073, most likely due to the different release kinetics.[38] In the current assay, both formulations with the same drug load exhibited a similar antibiofilm effect, as a 6-log difference was observed in bacterial numbers for *E. coli* and *S. epidermidis* (Figure 5A,C). The effect was visible at all time points and in both models. The log reduction was the same or higher in *ex vivo* models compared with *in vitro* models, which can be explained by the overall higher bacterial numbers in the *ex vivo* model.

However, using the same dressings with *S. aureus*, differences between formulations were seen after 48 h (Figure 5B). The CAM-PCL/PEO dressing was statistically more effective in inhibiting biofilm formation than the CAM-PCL dressing. The drug load in the used ES wound dressings is approximately 80 μg, but the amount of drug released at specific time points from the matrix differs. As shown before for the CAM-PCL/PEO dressing, 90% of the drug is released in the first 60 min, but for the CAM-PCL dressing, the amount of drug released is only 40% after 24 h and up to 50% after 48 h.[38] Therefore, the available drug concentration after 24 h of treatment is different, being higher when using the CAM-PCL/PEO dressing.

3.4. Effect of Pristine ES Wound Dressings on Biofilm Formation and Bacterial Adhesion. It was also of interest to determine whether control dressings with no drug (pristine polymer dressings) have any effect on biofilm formation as carrier polymers can potentially inhibit or induce biofilm formation. The search for antibacterial/antibiofilm polymers and smart textiles with the same properties is evolving rapidly;[58−61] therefore, novel assays are needed to test their

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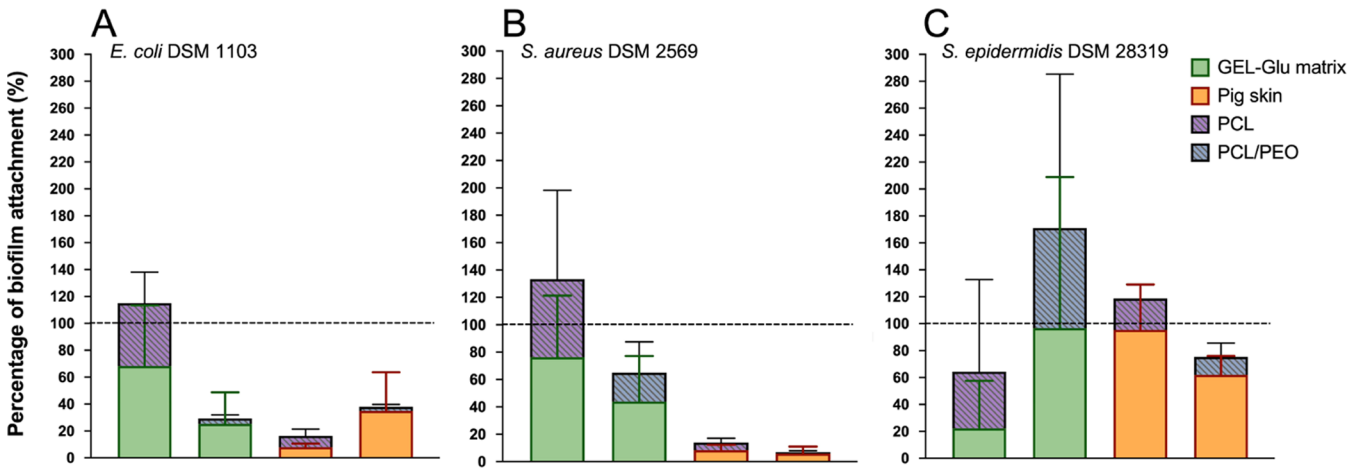

Figure 6. Bacterial biofilm adhesion on top of the substrate−gelatin−glucose matrix (GEL-Glu matrix) or pig skin or onto the covering wound dressing (ES pristine wound dressings). Substrates were covered with pristine wound dressings and incubated for 24 h, after which the substrate and wound dressing were separated, and the biofilm formed on top of each part was studied independently. Three different bacteria were used: (A) *E. coli* DSM 1103, (B) *S. aureus* DSM 2569, and (C) *S. epidermidis* DSM 28319. Two different formulations were tested—PCL wound dressing and PCL/PEO wound dressing (control dressings); 100% is shown to mark the arithmetic mean of biofilm formation on top of the different uncovered substrates. Key: GEL, gelatin; Glu, glucose; PCL, pristine wound dressings made from polycaprolactone; PCL/PEO, pristine wound dressings made from polycaprolactone and polyethylenoxide.

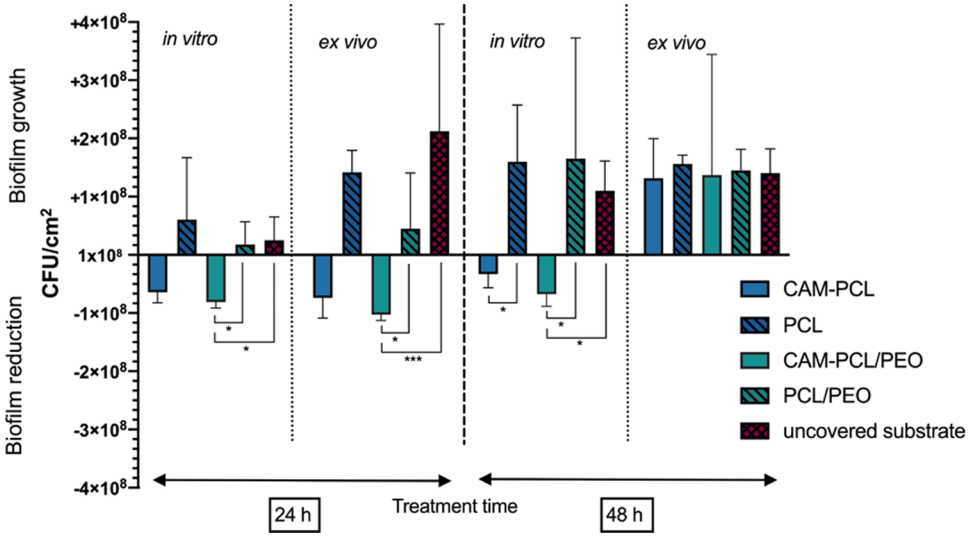

Figure 7. Biofilm treatment model. Effect of the model CAM-loaded wound dressings (CAM-PCL and CAM-PCL/PEO dressings) on already formed *E. coli* DSM 1103 biofilms (24 h). A comparison is made with pristine wound dressings and uncovered substrates. The *in vitro* setup was created on top of the ES GEL-Glu matrix and the *ex vivo* setup was created on top of pig skin. The number of biofilm-forming bacteria before treatment was 10^8 CFU/cm^2. Results are shown in a linear scale as the number of colony-forming units (CFUs), with standard deviation bars (n = 3). Changes in the threshold value (10^8 CFU/cm^2) are presented. Statistical significance is shown as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Key: CAM, chloramphenicol-loaded wound dressings; PCL, pristine wound dressings made from polycaprolactone; PCL/PEO, pristine wound dressings made from polycaprolactone and polyethylenoxide.

efficacy against biofilms. We proposed that the models developed by us could act as suitable tools for these evaluations. In a current study, all control dressings (pristine wound dressings without any antibacterial agent) were tested against uncovered substrates—GEL-Glu matrix or pig skin (shown as 100%)—to determine whether the number of bacteria in the formed biofilm differed (Figure 6). In addition, it was also investigated whether the bacteria adhered and formed the biofilm preferentially on the substrate or on the ES fibrous pristine wound dressing. For this assessment, the control dressing and the substrate were mechanically separated from each other after incubation, and the amounts of biofilm formed on both surfaces were studied separately.

Results showed that the amount of biofilm on top of the substrate when covered with the pristine control wound dressing does not exceed the amount of biofilm formed on top of the uncovered substrate. However, when the GEL-Glu matrix substrate is covered with the PCL dressing, then the amount of biofilm formed in combination with both (substrate and ES dressing) exceeds the amount of biofilm formed on uncovered substrates. For *S. epidermidis*, the results were different as it was also seen that the GEL-Glu matrix covered with PCL/PEO and pig skin covered with PCL dressing had higher amounts of combined biofilms than the uncovered samples.

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Table 1. Comparison between the Models Developed by Us and Previously Published Wound Infection and/or Biofilm Models[a]

Method/ Testing parameters	DEVELOPED		PUBLISHED						
			In vitro		*Ex vivo*				
	In vitro model	*Ex vivo model*	Brackman et al., 2011[27]	Werthén et al., 2010[28]	McMahon et al., 2020[28]	Steinstraesser et al., 2010[31]	Wilkinson et al., 2016[32]	Yang et al., 2013[30]	Andersson et al., 2021[30]
Biofilm substrate	**ES GEL-Glu matrix**	**Pig skin**	(a) Silicon discs (b) Collagen surface in 15-ml tube	Collagen surface in 15-ml tube	Pig skin	Human skin	Pig skin	Pig skin	Pig skin
Evaluating biofilm inhibition	**Yes**	**Yes**	No	No	No	No	No	No	No
Evaluating biofilm treatment	**Yes**	**Yes**	Yes	Yes	Yes	No	Yes	Yes	No
Evaluating biofilm formation on the tested treatment cover material	**Yes**	**Yes**	No	No	No	No	No	No	No
Use of antibiotics in model set up (not as treatment)	**No**	**No**	No	No	Yes	Yes	No	Yes	No
Visualisation of bacterial growth + Visualised bacteria + Visualised biofilm matrix + Three dimensional (3D) images (z-stack)	**SEM CFM** **Bacteria and biofilm matrix visualised in 3D**	**SEM CFM**	No microscopy	CLSM Light microscopy Bacterial colonies visualised	No microscopy	Fluorescence microscopy Light microscopy No biofilm matrix visualised Single bacteria visualised	SEM Fluorescence microscopy Light microscopy Bacteria and biofilm matrix visualised	SEM Fluorescence microscopy Light microscopy Bacteria and biofilm matrix visualised	SEM Fluorescence microscopy Bacteria visualised
	+++	**++**	-	++	-	+	++	++	+
Model validation + Antibacterial treatment + ES matrices + Wound dressing (solid, semisolid formulation)	**ES antibacterial wound dressings**	**ES antibacterial wound dressings**	Antibiotics Quorum sensing inhibitors	Antibiotics	Wound cleansers (solutions)	No validation	Debriding device	Liquid disinfectant	Topical antimicrobial formulations
	+++	**+++**	+	+	+	-	+	+	++

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Table 1. continued

Method/ Testing parameters	DEVELOPED		PUBLISHED						
			In vitro		*Ex vivo*				
	In vitro model	*Ex vivo model*	Brackman et al., 2011[27]	Werthén et al., 2010[28]	McMahon et al., 2020[28]	Steinstraesser et al., 2010[31]	Wilkinson et al., 2016[32]	Yang et al., 2013[30]	Andersson et al., 2021[30]
Possibility to test ES wound dressings + Not a liquid model + Sample size at least 1x1cm	1x1 cm sample size	1x1 cm sample size	designed in 24-wellplate or 15-ml tube (*allow sample size 1x1 cm)	designed in 24-wellplate (*allow sample size 1x1 cm)	12 mm biopsy piece of dermal tissue – 2 mm in diameter x 1.5 mm - deep	size of the maximum sample (d = 4 mm)	1.3 x 1.3 cm skin squares were used	12 mm biopsy piece of dermal tissue – 3 mm in diameter x 1.5 mm - deep	size of the maximum sample (d = 8 mm)
	++	++	+ (+)	+ (+)	+	+	++	+	+

[a]Key: "+", the listed feature was present in the model. More "+" signs show a better performance of the model for that evaluated parameter. CFM, confocal fluorescence microscopy; CLSM, confocal laser scanning microscopy; ES, electrospinning; GEL, gelatin; Glu, glucose; SEM, scanning electron microscopy. A blue background shows the best of the comparison.

The covered samples were studied further to determine whether the adhesion of the biofilm on top of the pristine wound dressing can be studied in the developed assay. It is known that bacterial biofilms are formed on almost all materials except bioinert and antibiofilm materials. Nanomaterials are no exception.[62] Results show that both PCL and PCL/PEO wound dressings were less favored for biofilm formation than the substrate (Figure 6). Differences are seen between bacteria and between substrates. The standard deviation is also quite wide, showing that the *ex vivo* model would be more favorable for studying the biofilm adhesion on top of the tested materials. Nevertheless, variability in this evaluation remains and most likely arises from the fact that mechanical separation was technically not easily reproducible.

Differences between formulations were evaluated, as it has been shown previously that PEO has antifouling properties that theoretically should avoid bacterial attachment.[63,64] In our formulation, only 2% of the PEO was added into the PCL polymer, but already changes in the biofilm-forming bacterial attachment were seen, showing that a lower amount of biofilm was formed on PCL/PEO dressings (Figure 6).

3.5. Model Modification to Study the Effect of ES Fibrous Wound Dressings on Preformed Biofilms. We also studied whether it is possible to grow the biofilm before applying a drug-loaded wound dressing and test its ability to treat already formed biofilms. For this, a modification was performed to the *in vitro* and *ex vivo* biofilm assays with *E. coli*. After 24 h, the biofilm was quantified (10^8 CFU/cm^2) and then treated for 24 and 48 h with both CAM-loaded and pristine wound dressings. As the bacteriocidal activity of CAM is known to be related to the specific bacterial strain, no major biofilm treatment effect was expected.[65] Because CAM mainly has bacteriostatic effects, the results in Figure 7 are presented in a linear scale as the change in the initial count of biofilm bacteria.

The results clearly show that when substrates were left uncovered, the amount of biofilm increased; the same was seen with pristine wound dressings (Figure 7). For CAM-loaded wound dressings, the decrease in the number of biofilm-forming bacteria was detected after 24 h of treatment in both models and after 48 h of treatment in the *in vitro* model. In the *ex vivo* model, CAM-loaded wound dressings did not have any biofilm-inhibiting effect after 48 h of treatment, which can be explained by the higher growth in general on top of pig skin than that on top of the GEL-Glu matrix. Furthermore, previous studies suggested that the presence of efflux pumps in *E. coli*, upregulated specifically in bacterial biofilms, may remove toxic compounds, including antibiotics, from the bacterial intracytoplasmic space, which could explain the biofilm-specific recalcitrance.[66]

Differences between formulations were also observed: the inhibition of biofilm bacteria was more significant for CAM-PCL/PEO than for CAM-PCL wound dressings (Figure 7). Even though the drug load is the same for both formulations, the faster CAM release from CAM-PCL/PEO wound dressing and therefore higher amount of drug at the site of action at the beginning of the treatment was perhaps beneficial in this case. This might be the reason why a prolonged and sustained release of a higher amount of CAM would be needed for more effective treatment against already formed biofilms.

3.6. Comparison of the Developed Wound Infection and Biofilm Models with Currently Available Models. An increasing number of biorelevant models have been developed and published over the years for studying and evaluating already existing or novelly created wound treatments and wound preparations. Some of them are also validated to study the efficacy of different compounds to treat biofilms. Two previously published *in vitro* models and five *ex vivo* wound and biofilm models for obtaining quantitative results were chosen to be compared with the models developed by us in Table 1.

Most of the published models used a specific substrate (e.g., silicon disks, collagen surface, pig skin, or human skin) to grow biofilms on top and also used microscopy to visualize the biofilms formed on the substrate. For example, the use of immunohistological staining and light microscopy of skin

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cross-sections is quite common. Fluorescence microscopy and confocal laser scanning microscopy images were also obtained from skin cross-sections and collagen surfaces (mimicking the skin surface *in vitro*), respectively. However, three-dimensional images were shown only by Werthen et al.[30] In the *in vitro* wound infection model developed by us, the bacterial biofilm formation on top of the GEL-Glu matrix was easily visualized using CFM, providing informative and easily understandable micrographs, which allowed the visualization of the bacteria and the biofilm matrix separately in three dimensions. Also, we were able to obtain SEM images that were not obtained in any of these published *in vitro* models. In previously published *ex vivo* models, the use of SEM to evaluate biofilms was quite common, together with light microscopy and/or fluorescence microscopy.

None of the previously published wound/biofilm models on substrates were validated for testing antimicrobial ES wound dressings. Hence, in the present study, we aimed to evaluate whether it is possible to use the ES wound dressings for these applications. The published *in vitro* models were promising, allowing a manageable size of the dressing to be used and allowing the properties of the dressings to be investigated. However, compared with the models developed by us, the amount of medium used was 5−10 times higher and the use of 15 mL tubes for one sample is much more inconvenient and operator unfriendly than using one 24-well plate for 24 samples. Among previously published *ex vivo* wound infection models, most of them were designed to use sample sizes (0.2−0.3 cm in diameter) smaller than the 1 × 1 cm squares used in our model. Larger sample sizes in our model provide the following key advantages for testing (considering the advantages for the operator as well as the test sample): (i) easy to reproduce, (ii) sufficient drug load with more biorelevant drug concentrations inside the dressing (closely mimicking the final application conditions), (iii) more precise measurement of the sample weight, and (iv) easy to handle while performing the assay.

Most of the published *ex vivo* models required some specific tools to create the wound (e.g., biopsy needle, electric soldering iron, or dermal tool), which were not necessary for our model as superficial burn wounds were self-created during the washing of pigs in a hot-water bath. For the *in vitro* model presented in this study, ES must be performed to create GEL-Glu matrices. However, this is most likely not a problem for facilities producing ES wound dressings.

One of the most important and key novelties is that our models enabled us to test biofilm inhibition and were validated using ES wound dressings. However, none of the published models were designed to evaluate the biofilm inhibition effect. Most of the published models allowed one to study the treatment of biofilms, similar to our model, quantifying the number of biofilm-forming bacteria after disrupting the biofilm. Sample collection for biofilm disruption was not always technically easy and was often challenging to reproduce. This was also discussed previously, and the latter was due to the variability of cutting out the pieces of the wound from the substrate. In our *ex vivo* model, no additional cutting needed to be performed as pretreated skin and the cut 1 × 1 cm substrate were entirely used to quantify the amount of biofilm formed.

4. SUMMARY AND CONCLUSIONS

In conclusion, we herein demonstrate that the developed biorelevant *in vitro* gelatin−glucose (GEL-Glu) and *ex vivo* (pig ear skin) biofilm models can be successfully used to test the antibiofilm properties of novel ES fibrous antibacterial drug-loaded as well as pristine wound dressings. Depending on the model setup, the inhibition of biofilm formation as well as biofilm treatment can be explored. Pig skin with a superficial burn wound was the preferred substrate for biofilm formation in comparison with the GEL-Glu matrix. All three wound pathogens can be used in these models (*E. coli*, *S. aureus*, and *S. epidermidis*). The selection of pathogenic wound bacteria for model development is important, as *E. coli* and *S. aureus* are able to use the *in vitro* model substrate GEL-Glu matrix for nutrition. The sterility of the models must be assured to conduct successful experiments with wound pathogens. Pig skin is a more universal substrate to use, but its collection, sterilization, and storage are more challenging compared with the *in vitro* model. The electrospun (ES) GEL-Glu matrix can be easily and reproducibly produced and used as a substrate for biofilm growth and visualization, but when tested with ES wound dressings, its interactions (e.g., physical attachment) may affect the model results. This needs to be considered during model selection and design. Nevertheless, both tested chloramphenicol-loaded ES wound dressings exhibited an antibiofilm effect in both tested models. Therefore, it can be concluded that the developed *in vitro* and *ex vivo* models may be used as new diagnostic tools as well as to develop treatments for wound infections.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.2c00902.

Additional information about experimental methods and results; sterility test results (Figure S1) (PDF)

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