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An antibiotic potentiator retains its activity after being immobilized on silicone and prevents growth of multidrug-resistant *Pseudomonas aeruginosa* biofilms

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ABSTRACT

Device-Associated Healthcare-Associated Infections (DA-HAI) are a major threat to public health worldwide since they are associated with increased hospital stays, morbidity, mortality, financial burden, and hospital overload. A strategy to combat DA-HAI involves the use of medical devices endowed with surfaces that can kill or repel pathogens and prevent biofilm formation. We aimed to develop low-toxic protease-resistant anti-biofilm surfaces that can sensitize drug-resistant bacteria to sub-inhibitory concentrations of antibiotics. To this end, we hypothesized that polymyxin B nonapeptide (PMBN) could retain its antibiotic-enhancing potential upon immobilization on a biocompatible polymer, such as silicone. The ability of PMBN-coated silicone to sensitize a multidrug-resistant clinical isolate of *Pseudomonas aeruginosa* (strain Ps4) to antibiotics and block biofilm formation was assessed by viable counting, confocal microscopy and safranin uptake. These assays demonstrated that covalently immobilized PMBN enhances not only antibiotics added exogenously but also those incorporated into the functionalized coating. As a result, the functionalized surface exerted a potent bactericidal activity that precluded biofilm formation. PMBN-coated silicone displayed a high level of stability and very low cytotoxicity and hemolytic activity in the presence of antibiotics. We demonstrated for the first time that an antibiotic enhancer can retain its activity when covalently attached to a solid surface. These findings may be applied to the development of medical devices resistant to biofilm formation.

1. Introduction

Biofilms have been reported to be involved in 80% of all human infections [1]. Under the biofilm growth mode, pathogens become extremely adept at colonizing medical devices (e.g., catheters, prostheses, valves, pacemakers), and when this happens, they often lead to Device-Associated Healthcare-Associated Infections (DA-HAIs) [2]. DA-HAIs are considered a major threat to public health worldwide for both developed and undeveloped countries [3]. The International Nosocomial Infection Control Consortium (INICC) reported that the rates of DA-HAIs in developing countries are 3 to 5 times higher than rates reported for developed countries [3,4]. Such infections usually cause an increase in hospital stays, morbidity, mortality, financial burden, and hospital overload [5]. Effective clearance of DA-HAIs often requires implant removal which subjects the patient to additional surgeries and in some cases, prolonged stays in intensive care units [6,7].

Treatment of DA-HAIs is very challenging because bacteria become highly resistant to antibiotics (even 1000 times more) when they switch to the biofilm growth mode [8]. Moreover, DA-HAIs are often caused by bacteria intrinsically resistant to many antibiotics, thus further reducing the effective therapeutic choices [9]. This situation is prompting an intense search for antimicrobial compounds alternative to antibiotics. In

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previous studies, we and others showed that antimicrobial peptides (AMPs) efficiently kill biofilms formed by *Pseudomonas aeruginosa* [10,11]. AMPs are well known for having broad-spectrum of antibacterial activity, reduced tendency to induce resistance and the ability to kill metabolically inactive microbes including the persister cells present in biofilms [12,13]. In addition, activity of antibiotics can be greatly enhanced in the presence of sub-inhibitory concentrations of some AMPs [14,15], a feature particularly relevant for this study.

Among others, preventive strategies against DAI-HAIs involve the use of medical devices endowed with surfaces that can kill or repel pathogens and block biofilm formation [16,17]. Different molecules including antibiotics, surfactants, AMPs and nanoparticles carrying bactericidal compounds were used to coat medical devices in order to prevent bacterial attachment and hence biofilm formation [16,18,19].

Concerning AMPs, it has been reported that some of these compounds efficiently prevent biofilm formation when they were used to coat different surfaces such as titanium [20] and silicone [21]. Interestingly, it has been repeatedly shown that AMPs retain their anti-biofilm activity even after their covalent immobilization on a surface [22–24]. This strategy improves the attractiveness of AMPs for in vivo anti-biofilm applications because immobilized peptides are more resistant to proteolytic cleavage than their unbound counterparts [25]. However, to the best of our knowledge, synergy between AMPs and antibiotics were never exploited by combining these two compounds on a functionalized surface. In addition, the ability of a surface-immobilized peptide to enhance antibiotic activity has never been reported in the scientific literature.

Polymyxin B (PMB), the best characterized AMP, has potent bactericidal activity against planktonic and biofilm cells of many Gram-negative bacteria [26–28]. However, this lipopeptide has been reported to be nephrotoxic [29], a fact precluding its systemic administration in antibiotherapy [30,31]. Notably, the non-acylated derivative of Polymyxin B, PMB nonapeptide (PMBN) has an acute toxicity in mice almost five times lower than that of its parent compound [32]. Although PMBN has poor antibacterial activity, it displays a very potent synergism with antibiotics [33–35]. In addition, we recently showed that PMBN greatly enhances the antimicrobial activity of efflux pumps and beta-lactamase inhibitors (EPI and BLI) against biofilms formed by multidrug-resistant *P. aeruginosa* [36,37].

We hypothesized that PMBN could retain its antibiotic-enhancing potential upon immobilization on a surface. If so, this feature could be applied to the development of protease-resistant low-toxic surfaces that might sensitize drug-resistant microbes to antibiotics, EPI and BLI thereby preventing biofilm formation.

2. Material and methods

2.1. Chemicals

PMBN, doxycycline, levofloxacin, azithromycin, vancomycin, bicine buffer, phosphate buffer saline (PBS), fetal bovine serum (FBS), penicillin-streptomycin, amphotericin B and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (Darmstadt, Germany) and prepared and stored according to manufacturer's recommendations. CellTiter 96® AQ_{ueous} One Solution Reagent (MTS) was obtained from Promega Corporation (Madison, Wisconsin, USA). Polysorbate 80 (tween 80) was purchased from Becton, Dickinson and Company (New Jersey, USA).

2.2. Bacterial strain and culture mediums

The *P. aeruginosa* strain used in this study was the multidrug-resistant clinical isolate Ps4 [38]. For routine procedures, bacteria were grown at 37 °C in Tryptic Soy Broth (TSB; BioMérieux, Spain) or in TSB supplemented with 16 g/L agar (TSA; Pronadisa, Spain). Mueller-Hinton cation-adjusted (MHCA) broth (Difco Laboratories, Michigan, USA) was used in experiments involving growth of planktonic and biofilm-forming cells.

2.3. Minimal inhibitory concentration (MIC)

Conventional minimal inhibitory concentration (MIC) testing was done using the broth microdilution assay as recommended by the Clinical and Laboratory Standards Institute (CLSI) [39]. MIC was defined as the lowest antimicrobial concentration yielding no visible growth after 18 h of incubation. Briefly, serial 1/2 dilutions of the antimicrobials (levofloxacin, doxycycline, azithromycin and PMBN) were prepared in a 96-well plate. A diluted bacterial suspension of a fresh culture of Ps4 (final cell density of 5×10^5 CFU/mL) was added to each well before incubating the plates for 18 h at 37 °C. In each assay, wells containing inoculated medium without antimicrobials and uninoculated medium were included as growth and sterility controls, respectively.

2.4. Two-dimensional synergy testing

Potential synergistic interactions between PMBN and antibiotics were assessed by the checkerboard assay using MHCA broth as described before [38]. First, 2-fold serial dilutions of the antibiotic were prepared in a 96-well plate. In separate tubes, a serial dilution of the peptide was prepared. Thereafter, a fresh culture of Ps4 was added to the peptide dilutions after being adjusted to 0.5 McFarland standard (equivalent to 10^7 CFU/mL) and diluted 1:50 with MHCA to obtain a 5×10^5 CFU/mL suspension. Aliquots of this suspension were transferred into the wells of the 96 well plate and mixed with an equal volume of antimicrobial solution. The antibiotic concentration range was selected according to the previously determined MICs and combined with 6 different concentrations of PMBN. Microplates were first incubated at 37 °C for 18–20 h and then inspected visually to determine turbidity in each well. In each assay, wells containing inoculated medium without antimicrobials and uninoculated medium were included as growth and sterility controls, respectively. The fractional inhibitory concentration index (FICI) for each double antimicrobial combination (e.g. compounds A and B) was calculated as follows [40].

$$FICI_{A/B} = \frac{MIC_{A(combination)}}{MIC_{A(alone)}} + \frac{MIC_{B(combination)}}{MIC_{B(alone)}}$$

FICIs were calculated using the concentrations in the first non-turbid well found in each row and column along with the turbidity/non-turbidity interface. Combinations were considered as synergistic (FICI $\leq$ 0.5), additive (0.5 < FICI < 1), indifferent (1 < FICI < 4), and antagonistic (FICI > 4) [41].

2.5. Immobilization of PMBN

2.5.1. Preparation of PDMS surfaces

Polydimethylsiloxane (PDMS) polymer was synthesized using a two-component kit Sylgard 184 (Dow Corning, USA); according to the manufacturer's instructions. Concisely, base and curing agents were mixed at 10(base):1(curing) (w/w) by mixing 9 mL silicone elastomer base and 1 mL silicone elastomer curing agent and then poured into a 90-mm Petri dishes and left to dry for 48 h. The resulting silicone polymer was cut into cylindrical coupons (5 mm diameter and 3 mm height) using a hole puncher. Shaped coupons were sterilized by subjecting them to a 5 min sonication in a commercial detergent at room temperature followed by 10 min sonication in pure methanol at room temperature under sterile conditions. Based on a published two-step procedure [42] PDMS coupons were initially functionalized with polymerized dopamine (dopamine HCL, Sigma-Aldrich, Darmstadt, Germany) after which the peptide was covalently bound to the polydopamine (PDA) coated surface. Specifically, the PDMS coupons were first incubated in a 2 mg/mL dopamine HCL solution under basic conditions (Bicine buffer, 10^{-2} M, pH = 8.5) and constant shaking for

18 h at room temperature. Then, they were washed with distilled water and incubated in a 2 mg/mL PMBN solution in bicine buffer (pH = 8.5) and constant shaking for 2 h at room temperature.

2.5.2. Fluorescamine assay

PMBN coating efficiency was determined using a fluorescamine assay (Sigma-Aldrich, France). The amount of unattached peptide in the buffer solution retrieved immediately after completing the coating process was quantified by measuring fluorescence intensity. The supernatants containing unattached peptides were mixed with fluorescamine solution (3 mg/mL in acetone) at a 1:3 ratio in a 96-black-well plate. After 15 min of incubation at room temperature with shaking, the fluorescence intensity of each sample was measured using a microplate reader (FLUOstar OPTIMA Microplate Reader, BMG Labtech, Germany) at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. Finally, the amount of unattached PMBN in the solution was calculated from the standard curve of PMBN and this value was used to deduce the amount of immobilized peptide by subtraction. Immobilization efficiency was expressed as the ratio between the amount of immobilized peptide and the total amount of loaded peptide. This assay was also used to quantify the amount of PMBN released from the PDMS surface after 1, 2 and 3 days of incubation in water.

2.5.3. Water contact angle measurements

The wettability of the functionalized surfaces was analyzed by means of the water contact angle using an optical tensiometer. First, a 2 μL water droplet was poured on the surface, allowing it to rest on it for 5 min. Then, a video-based optical contact angle measuring instrument (OCA 15EC Data physics GmbH, Filderstadt, Germany) was used to obtain digital images that were analyzed with the dynamic contact angle analyzer.

2.6. Impregnation of silicone with antibiotics:

PMBN-coated coupons were immersed in 1.5 mL of a solution containing either levofloxacin (0.5 mg/mL dissolved in 9:1 acetone/water) or doxycycline (2.5 mg/mL dissolved in 9:1 acetone/water) and incubated for 24 h at room temperature in aseptic conditions. Then, coupons were removed from the solution and left to dry overnight. Finally, coupons were washed with sterile distilled water before testing their anti-biofilm activity.

2.7. Anti-biofilm activity

2.7.1. Biofilm generation

Freshly prepared suspensions of Ps4 containing 5×10^5 CFU/mL in MHCA were used as inoculum in these experiments. PMBN-coated coupons (n = 3 per well of a 24-well plate) were dipped into the bacterial suspension at a final volume of 700 μL/well and incubated at 37 °C for 24 h in the presence and absence of antibiotics (2 μg/mL of levofloxacin or doxycycline). Uncoated coupons were subjected to the same steps and used as controls. An identical procedure was applied for the inoculation of antibiotic-impregnated coupons. For the latter experiment, coupons soaked in acetone/water 9:1 ratio were used as controls.

2.7.2. Safranin staining

After 24 h of incubation with Ps4 at 37 °C, the biofilm formed on the surface of coupons was stained with 0.1% safranin for 10 min. The excess stain was removed by gently rinsing the coupon surface with sterile ultra-pure water. The safranin associated with the biofilm was dissolved in sterile ultra-pure water by scratching the surface with a pipette tip and then by subjecting the coupon to intense vortexing until the silicone became colorless. The absorbance of this final solution was measured by spectrophotometry (Genesys 20, Thermo Scientific, Waltham, USA) at 490 nm.

2.7.3. Biofilm viable cell counts

For cell counting experiments, the tested coupons were placed inside plastic bags with PBS added and subjected to mechanical disruption using Stomacher® 80 Biomaster (Seward, United Kingdom) to detach all the biofilm grown on the surface. The solutions were then sonicated for 10 min and a serial 10-fold dilution was made. Then, 100 μL of the appropriate dilutions were plated on TSA and the plates were incubated at 37 °C overnight. Colony-forming units grown on TSA plates were counted and results were expressed as log CFU/cm^2 of coupon.

2.7.4. Confocal microscopy imaging

Tested coupons were stained using bacterial viability LIVE/DEAD Backlight kit (ThermoFisher Scientific, Spain) according to the manufacturer's protocol. Imaging of the surface of the coupons was performed using a confocal laser scanning microscope (Cell Observer Z1 microscope, Zeiss, Oberkochen, Germany) equipped with a 63× objective. Image acquisition was done with the Zeiss software package, and image processing with ImageJ (ImageJ/Fiji 1.46, National Institution of Health, USA).

2.8. Killing assay

Kinetics of killing of planktonic bacterial cells exposed to combinations of PMBN-coated coupons and antibiotics was determined by viable counting. To this end, Ps4 was grown in 24-well plates along with functionalized coupons exactly as described above and culture samples were obtained at 0.25, 0.5, 1, 2, 3 and 4 h after the beginning of growth. Samples were then diluted and cultured on TSA plates at 37 °C overnight and a colony counting was performed as described above

2.9. Biocompatibility assays

2.9.1. Cytotoxicity

Toxicity of PMBN-coated silicone in the presence of antibiotics was determined on the 3T3 fibroblast cell line [43]. First, 3T3 cells were cultured in DMEM supplemented with 5% FBS. Cells were seeded in a 96-well plate at a concentration of 2×10^4 cells per well and then plates were incubated at 37 °C overnight in a humidified atmosphere of 5% CO_2. One silicone coupon was added to each well containing the attached cell layer and DMEM such that the functionalized surfaces were in close proximity to the cell layer (i.e. 1 mm, approximately) to prevent contact-dependent mechanical cell detachment, as described elsewhere [44]. Simultaneously, cells were also exposed to the antibiotic concentration that had been previously shown to be active on biofilms (2 μg/mL of doxycycline or levofloxacin). After 24 h of incubation at 37 °C, coupons were removed and 100 μL MTS reagent was added to each well and incubation was resumed at 37 °C for 2 h. Finally, the cell-mediated generation of reduced MTS was determined by spectrometry at 490 nm. In each assay, untreated 3T3 cells and cells treated with 2% DMSO were used as negative and positive cytotoxicity controls, respectively.

2.9.2. Hemocompatibility

The potential hemolytic activity of PMBN-coated coupons was evaluated as described by Oren and Shai [45] in the presence of antibiotics. Human red blood cells (hRBCs) from a healthy volunteer were concentrated by centrifugation (10 min at 900 ×*g*, 4 °C) and the resulting pellet was resuspended in a volume of PBS 5 times that of the original blood sample. Coupons (n = 3) were dipped into 2 mL of this hRBCs suspension in the presence or absence of antibiotics (2 μg/mL) and incubation was carried out for 1 h at 37 °C, with shaking at 100 rpm. Finally, hRBCs solutions were centrifuged at 1500*g* for 5 min and the amount of hemoglobin present in the supernatant was quantified by spectrometry at 540 nm. Negative and positive controls of hemolysis were prepared by suspending the hRBCs solution in PBS or 1% Tween 80, respectively.

Table 1
In unbound form, PMBN sensitizes *Pseudomonas aeruginosa* CUN 4158-02 Ps4 to Levofloxacin, Doxycycline and Azithromycin.

PMBN[a] μg/mL	MIC[b] of Levofloxacin at the indicated PMBN concentration (μg/mL)	FICI[c]	MIC of Doxycycline at the indicated PMBN concentration (μg/mL)	FICI	MIC of Azithromycin at the indicated PMBN concentration (μg/mL)	FICI
0	16	–	64	–	128	–
1	16	1.002	32	0.502	32	0.251
2	0.06	0.006	1	0.018	16	0.127
4	0.06	0.008	0.5	0.012	16	0.129

(Combinations were considered synergistic if FICI ≤0.5.) MIC of PMBN against Ps4 was >512 μg/mL.
[a] Polymyxin B nonapeptide
[b] MIC: Minimal inhibitory concentration.
[c] FICI: fractional inhibitory concentration index.

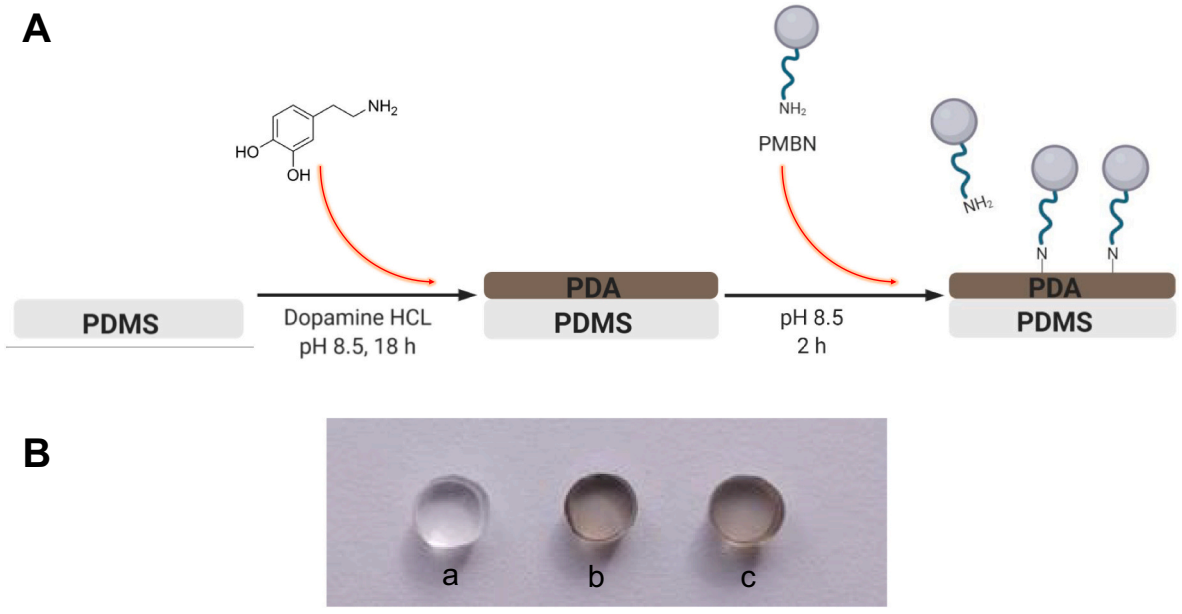

Fig. 1. A. Schematic of PMBN two-step tethering process on a PDMS surface. The PDMS coupons were first functionalized with a layer of polydopamine (PDA) by incubating the surfaces with dopamine under alkaline conditions. Step two allows the covalent binding of the peptide (PMBN) to the functional groups exposed on the PDA layer after incubation of PDMS-PDA with PMBN under alkaline conditions. B. Top view of the silicone coupons at different stages of the immobilization process. Note the change in color after treatment with dopamine: a) PDMS, b) PDMS-PDA, c) PDMS-PMBN.

Table 2
Relevant features of the functionalized surfaces used in this study.

Surface[a]	Water contact angle (θ_{W}°)[b]	Amount of peptide immobilized (μg/mm^2)[c]	Peptide immobilization efficiency[d]	Stability of PMBN attachment[e]
PDMS	110.42 ± 1.13	–	–	
PDMS-PDA	78.01 ± 4.67	–	–	
PDMS-PDA-PMBN	77.54 ± 5.01	3.3 ± 0.31	43%	100%

[a] PDMS: Polydimethylsiloxane; PDMS-PDA: PDMS conjugated to polydopamine; PDMS-PDA-PMBN: PDMS-PDA conjugated to polymyxin B nonapeptide.
[b] Angles were measured using an optical tensiometer fitted with a dynamic contact angle analyzer. Lower values correspond to more hydrophilic surfaces.
[c] Quantified by fluorescamine assay. Values are means ± SD. The amount of peptide bound to the surface was estimated by subtracting the value measured after the immobilization process from the amount originally added.
[d] Corresponds to the ratio of immobilized peptide to the amount of peptide originally added.
[e] Assessed by fluorescamine assay. The amount of peptide released from the surface was determined by substraction considering the amount before incubation as 100%.

2.10. Statistical analysis

Results were obtained from three independent experiments each with a triplicate repetition (N = 3). Statistical analysis was performed using student unpaired *t*-test. All statistical analyses were calculated using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA). Data were expressed as the mean ± standard deviation (SD) and *p*-values of <0.05 were considered significant.

3. Results

3.1. Unbound PMBN enhances antibiotic activity

Prior to investigating whether surface-bound PMBN can enhance antibiotic activity, we first characterized this property using non-apeptide free in solution. For this purpose, we measured the capacity of PMBN to sensitize a multi-drug resistant strain of *Pseudomonas aeruginosa* (Ps4) to levofloxacin and doxycycline using a checkerboard assay. As shown in Table 1, addition of 2 μg/mL of PMBN sufficed to reduce 256 times the MIC value of levofloxacin (from 16 μg/mL to 0.0625 μg/mL). As expected, this translated into a very low value (i.e. 0.0057) of fractional inhibitory concentration index (FICI), thereby implying potent synergism (i.e. index value <0.5). Similar results were obtained with doxycycline and azithromycin (Table 1) although the enhancement

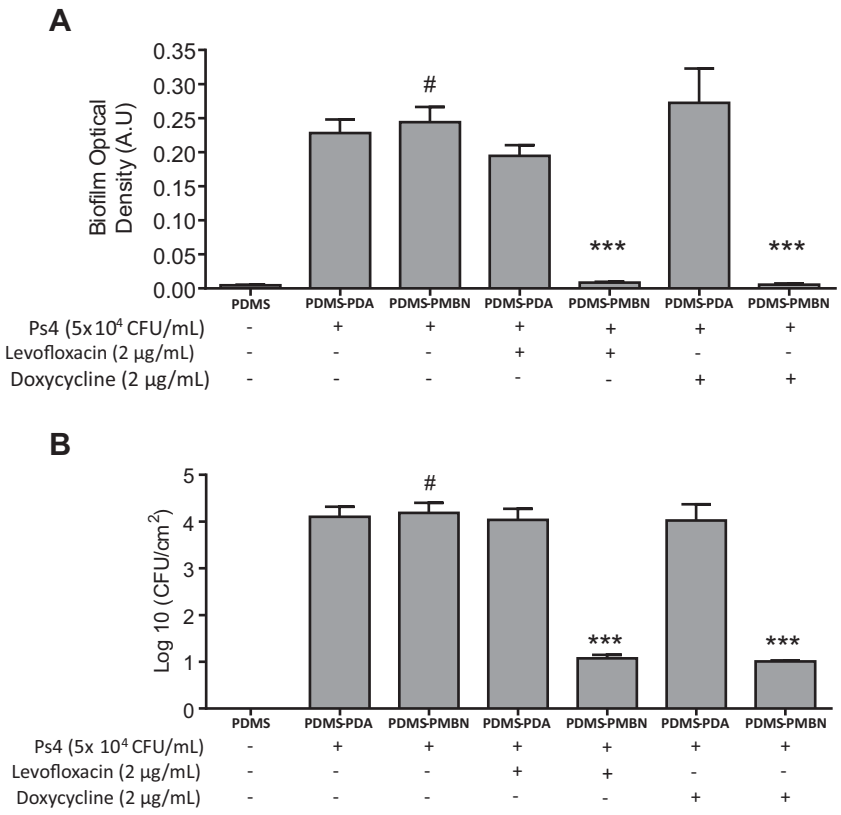

Fig. 2. Biofilm preventive capacity of PMBN-coated coupons in the presence of antibiotics. The biofilm formed by *Pseudomonas aeruginosa* CUN 4158-02 Ps4 on the coupons after 24 h of incubation at 37 °C in MHCA supplemented with the indicated antibiotics was first stained with safranin and then suspended in water and quantified by spectrophotometry at 490 nm (A) or by colony counting (B). Results are the mean ± S.D. of 3 independent experiments performed in triplicate. Obtained results were analyzed using student *t*-test and statistical differences were significant (***; $p < 0.001$) compared to the untreated control (#).

was of lower magnitude in these cases (FICI = 0.0176 and 0.12, respectively). In contrast, PMBN by itself had no activity against Ps4 (MIC >512 µg/mL).

3.2. Immobilization of PMBN on silicone surface

Immobilization of PMBN was performed on the surface of small discs ("coupons") excised from polydimethylsiloxane (PDMS; Fig. 1). Prior to the surface immobilization of PMBN, we tested whether the protocol followed to produce the PDA [42] was successful by conducting the dopamine polymerization in solution, under the same conditions as those used to functionalize the coupons. The FTIR-ATR spectra of the freeze-dried product were compared to that of the monomer in SI, Supplementary Fig. S1, showing remarkable differences between both, especially in the fingerprint region, which confirm the formation of the polymer by using this procedure [46]. Thereafter, PMBN was bound to PDMS using polydopamine (PDA) as linker (Fig. 1A). Dopamine mediated peptide attachment resulted in a color change (Fig. 1B) and an increase in hydrophilicity, as deduced by the reduction in water contact angle (Table 2). The functionalized surface was also analyzed by FTIR-ATR. To this effect, the surface of the coupon was gently scraped with a blade and the spectrum was recorded (Supplementary Fig. S2). Along with the bands of polydimethylsiloxane (790, 1030, and 1257 cm^{-1}), which indicate certain content of silicone in the scrapings, the presence of PMBN is revealed in the broad, poorly-resolved region between 1620 and 1260 cm^{-1}, in which the amide I and II bands of the peptide, overlapping the PDA spectrum, can be detected. The amount of PMBN immobilized on the surface was then estimated by fluorescamine assay, resulting in 3.3 ± 0.31 µg/mm^2. Overall, we achieved an immobilization efficiency of 43% (Table 2).

3.3. Anti-biofilm activity of the functionalized surface in combination with antibiotics

To study if PMBN can enhance antibiotic activity when bound to a surface we compare the ability of Ps4 to colonize PMBN-coated coupons in the absence or the presence of antibiotics added at sub-inhibitory concentrations. After incubation, planktonic cells adhered to the coupon surface were removed by washing and biofilms formation was assessed by safranin staining and viable counting. As shown in Fig. 2, neither the antibiotic by itself nor the immobilized peptide without the antibiotic reduced the ability of Ps4 to colonize the silicone surface. In marked contrast, addition of sub-inhibitory concentrations of doxycycline or levofloxacin to the PMBN-coated surface resulted in a 25-fold reduction in biofilm mass (Fig. 2A) which translated approximately into a 1.000-fold decrease in the number of viable biofilm-forming cells (Fig. 2B). In contrast, PMBN failed to enhance azithromycin under equivalent conditions (data not shown), suggesting that potentiation was dependent upon the relative potency of each antibiotic against Ps4 (Table 1).

In independent experiments, biofilm formation on the silicone surface was assessed by confocal microscopy using the LIVE/DEAD Backlight kit as a fluorochrome. Consistent with our previous data (Fig. 2), the immobilized peptide failed to prevent bacterial colonization in the absence of antibiotics (Fig. 3B) and resultant biofilms seemed to contain mostly viable (i.e. green) cells. Additional controls demonstrated that antibiotics by themselves could not inhibit biofilm growth when added on a non-functionalized surface (Fig. 3C and E). In contrast, no bacterial growth was detected on the PMBN-coated surface incubated with sub-inhibitory concentrations of either doxycycline or levofloxacin (Fig. 3D and F).

Interestingly, in the previous assays, we observed that solutions containing both antibiotics and peptide coated coupons remained uncloudy during the entire experiment. On the contrary, when one of these components was tested separately (i.e. either the antibiotic alone

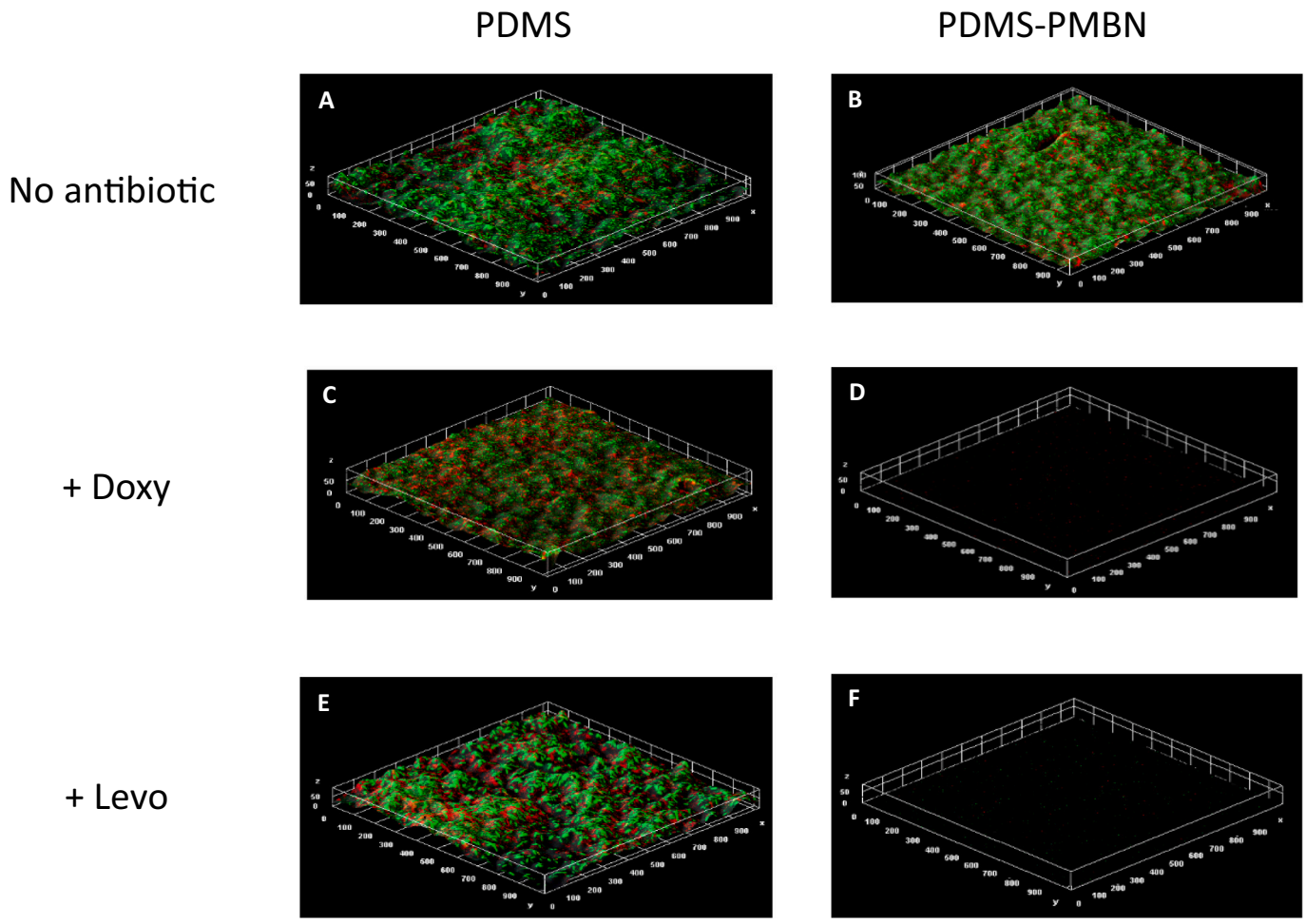

Fig. 3. Tridimensional confocal microscopy images of biofilm formed on the surfaces of PMBN-coated coupons in the presence or absence of antibiotics. PMBN-coated (right column) or uncoated coupons (left column) were incubated with Ps4 for 24 h in MHCA supplemented with 2 μg/mL of either Levofloxacin ("+ Levo") or Doxycycline ("+ Doxy"). For imaging, biofilms were stained with the DEAD/LIVE Backlight kit. This experiment was independently repeated 3 times and in all the cases the observations shown here were reproduced.

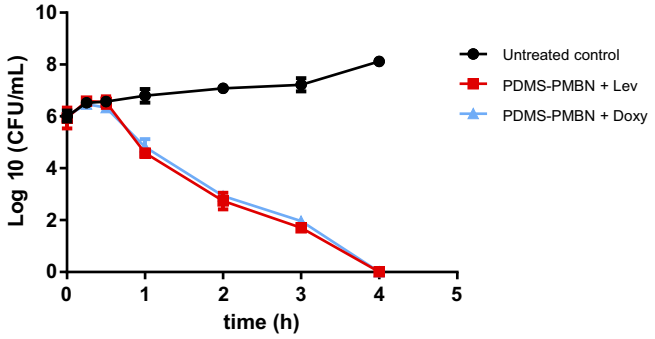

Fig. 4. Kinetics of killing of planktonic cells of *Pseudomonas aeruginosa* CUN 4158-02 Ps4 due to the combined action of PMBN-coated coupons and antibiotics. PMBN-coated coupons (n = 3 per well) were placed into the wells of a 24-well plate filled with MHCA supplemented with antibiotics (final concentration of 2 μg/mL of Levofloxacin or Doxycycline in a 700 μL total volume) and then the medium was inoculated with Ps4. At the indicated times post-inoculation, samples were taken out and plated for viable counting. Results are the mean ± S.D. of 3 independent experiments performed in triplicate. Obtained results were analyzed using Student t-test and statistical differences were significant ($p < 0.05$) compared to the untreated control.

or the PMBN-coated surface without antibiotic) solutions turned cloudy after incubation. This strongly suggests that the coating could prevent not only biofilm formation but also planktonic cell growth in the antibiotic solution. To investigate this possibility, we studied the growth kinetics of the inoculum upon its introduction in the antibiotic solution containing the functionalized coupons. Notably, these assays demonstrated that failure of Ps4 to grow planktonically in the presence of immobilized PMBN and antibiotics was due to a potent bactericidal effect (Fig. 4). Specifically, the decrease in cell count was detectable 1 h after incubation, whereas the complete eradication of viable bacteria required three more hours and resulted in a 6-log viability reduction.

The possibility remained that the observed bactericidal effect could be the consequence of hypothetical leaching of PMBN from the silicone surface. If so, it would not be surprising that unbound PMBN could act in synergy against planktonic cells, as we showed in Table 1. To study the stability of the PMBN-PDA-PDMS linkage we incubated the peptide-coated coupons in water for 3 days at 37 °C and, after water removal, we repeated the assay shown in Fig. 2A. Interestingly, after incubation, the PMBN-coated silicone combined with either doxycycline or levofloxacin displayed the same anti-biofilm preventive activity of the original sample (i.e. prior to incubation in water (Fig. 5)).

3.4. Biocompatibility

To study the biocompatibility of the functionalized surface, 3T3

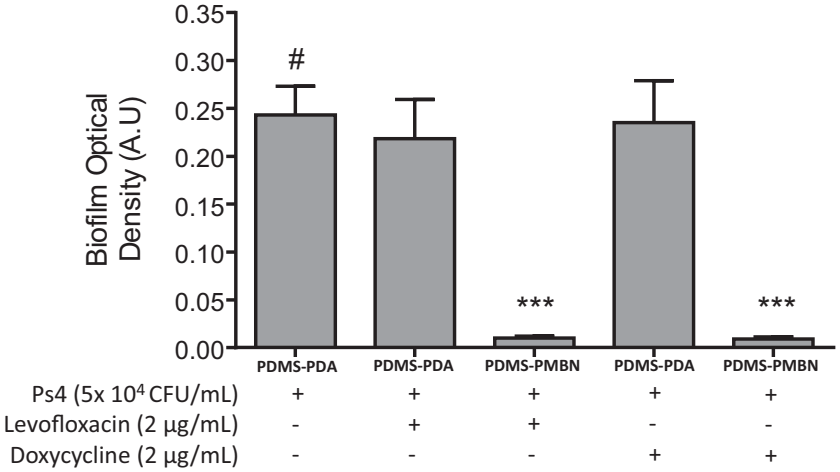

Fig. 5. Biofilm preventive capacity of PMBN-coated coupons that had been previously incubated for 3 days in water. After the 3-day incubation of PMBN-coated coupons in water, coupons were introduced in MHCA medium inoculated with *Pseudomonas aeruginosa* CUN 4158–02 Ps4 and supplemented with 2 µg/mL of either Levofloxacin or Doxycycline. After 24 h of incubation at 37 °C, the biofilm formed on the coupons was first stained with safranin and then suspended in water and quantified by spectrophotometry at 490 nm. Results are the mean ± S.D. of 3 independent experiments performed in triplicate. Obtained results were analyzed using student t-test and statistical differences were significant (***; $p < 0.05$) compared to the untreated control (#).

fibroblasts were exposed to PMBN-coated silicone for 24 h and then cell viability was assessed using the MTS assay. Incubation with the functionalized surface was performed in presence of the antibiotic concentration that had been previously shown to be active on biofilms (2 µg/mL of doxycycline or levofloxacin). As shown in Fig. 6A, the percentage of viability of fibroblasts exposed to combinations of PMBN-coated silicone and antibiotics was higher than 90%.

The potential hemolytic activity of the functionalized surface was evaluated by exposing human red blood cells to PMBN-coated coupons for 1 h at 37 °C, with constant shaking and in the presence of 2 µg/mL of doxycycline or levofloxacin. Fig. 6B shows that all tested combinations and controls exhibited negligible hemolytic activities compared to the level of hemolysis caused by Tween 80.

3.5. Anti-biofilm activity of a silicone coating integrating antibiotics and immobilized PMBN

In previous experiments, we showed that immobilized PMBN can enhance externally added antibiotics. To try to increase the clinical applicability of our functionalized surface, we sought to design a surface incorporating the two components required for anti-biofilm activity. To this end, we first attached PMBN to a silicone surface as described above and then we used the swell-encapsulation method [47] to incorporate either levofloxacin or doxycycline into the silicone matrix. To test the antibiofilm activity of this coating we used the same assay shown in Fig. 2A (i.e. safranin staining) without adding exogenous antibiotics to the coupons. Interestingly, the amount of levofloxacin or doxycycline released from the coating did not suffice to prevent Ps4 colonization in the absence of PMBN (Fig. 7), suggesting that the drugs did not reach their corresponding minimum inhibitory concentration at the coupon surface. In contrast, when the same experiment was carried out using PMBN-coated coupons, the growth inhibition was complete in the case of the levofloxacin containing silicone (100% reduction) and very significant (60% reduction) in doxycycline impregnated coupons (Fig. 7).

4. Discussion

In this study, we demonstrated that an antibiotic enhancer can retain its activity after being immobilized on a surface. To the best of our knowledge, this observation has no precedent in the scientific literature and opens new possibilities for the design of surfaces with antibiotic-enhancing activity. Several antibiotic potentiators, such as SPR741, are in clinical trials for systemic use [48] and this field is gaining momentum due to the constant increase in antibiotic resistance. In this work, we showed that potentiators could also be used to prevent colonization when immobilized on a surface, a property that might be applicable for the development of medical devices resistant to biofilm colonization.

Other authors described functionalized surfaces containing mixtures of antibiotics and other compounds that acted in synergy with antibiotics, such as silver-based compounds, antimicrobial peptides, enzymes and other agents (reviewed by Zhu et al. 2019 and Shahrour et al. 2019). However, in most of these cases, the agent acting as antibiotic enhancer was designed to be released to the lumen of the biomaterial to exert its activity and only one recent study has shown the potential of AMPs to synergize with another peptide after being covalently attached to a surface [49]. However, even in this case, synergism did not involve any antibiotic but just two different classes of AMPs.

Currently, several indwelling medical devices reported to prevent biofilm formation are commercially available [50]. Due to its potency and broad-spectrum of antimicrobial activity, silver derivatives are the most popular microbicidal agents used in these devices. However, there is an active research to develop alternatives to silver, since this heavy metal exhibits toxicity toward human and animal cells [51,52]. In contrast, PMBN was reported to be 100 times less cytotoxic than its parent molecule, the antibiotic PMB [53], and immobilization of the nonapeptide is expected to reduce even more its toxicity. Although in vivo experimentation is needed, the low cytotoxicity and hemolytic activity displayed by the PMBN-coated surface is a preliminary indication of its adequate biocompatibility.

Importantly, we showed that immobilized PMBN can enhance not only antibiotics present in the functionalized coating, but also those added exogenously. This observation suggests that the nonapeptide-bearing surface might be able to prevent in vivo biofilm formation in the presence of systemically administered antibiotics. If this holds true, the same functionalized surface could be used to simultaneously enhance both antibiotics present in the coating and those given systemically.

On the other hand, we showed that PMBN-mediated enhancement occurred when antibiotics were present at sub-inhibitory concentrations. This observation may have interesting implications at the toxicological and pharmacological levels for systemically administered antibiotics. First, it suggests that achieving concentrations higher than the MIC during antibiotic therapy may not be necessary to induce enhancement in vivo and prevent biofilm formation on a PMBN-coated surface. This may reduce the potential toxicity concerns normally associated with antibiotherapy, which always implies the administration of antibiotic levels sufficient to reach supra-MIC concentrations in vivo [54]. Furthermore, our strategy may broaden the concentration range at which antibiotics are therapeutically useful, thereby extending their half-life.

Notably, we revealed that antibiotic enhancement not only results in

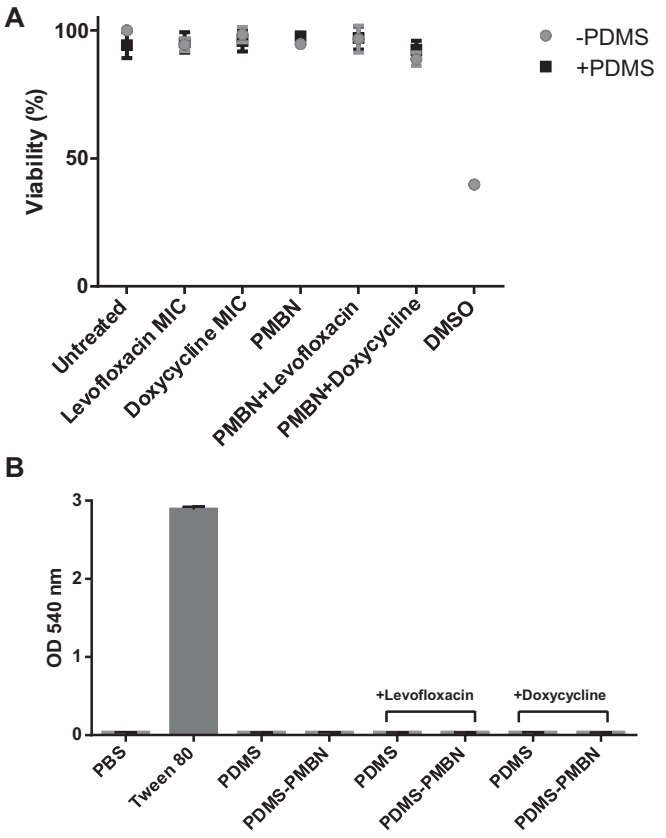

Fig. 6. Cytotoxicity and hemolytic activity of PMBN-coated coupons in the presence of antibiotics.

A. Cytotoxicity of PMBN-coated coupons was determined on the 3T3 fibroblast cell line in the presence of antibiotics (2 μg/mL of either Levofloxacin or Doxycycline). Coupons were added to the cells such that the functionalized surfaces were in close proximity (1 mm, approximately) to the cell layer. After 24 h of incubation at 37 °C, cell viability was assessed by the MTS assay and expressed with respect to that of an unexposed control (100%). A positive control of cytotoxicity was carried out by exposing the cells to 2% DMSO (Dimethyl sulfoxide). Presented data are the means ± S.D. of 3 independent experiments performed in triplicate.

B. Fresh human red blood cells were incubated in the presence of PMBN-coated coupons and antibiotics (2 μg/mL of either Levofloxacin or Doxycycline) for 1 h at 37 °C with shaking. Then, suspensions were centrifuged and the amount of hemoglobin present in the supernatant was quantified by spectrometry at 540 nm. Negative and positive controls of hemolysis were prepared by suspending the hRBCs solution in PBS or 1% Tween 80, respectively. Presented data are the means ± S.D. of 3 independent experiments performed in triplicate.

biofilm inhibition but is rapidly bactericidal. This makes our experimental approach very attractive from a therapeutic point of view because it reduces the possibility of mutant emergence during antibiotic treatment. In addition, the observed bactericidal activity demonstrates that, after immobilization, PMBN retains the molecular conformation and the mobility necessary to disturb the outer membrane of *P. aeruginosa*. By contrast, other molecules frequently require complex immobilization procedures to ensure the proper flexibility, orientation and exposure required for antimicrobial activity [25].

Our data suggest that PMBN is stably bound to the surface and that the nonapeptide does not get released to the lumen in significant amounts. In support of this, the killing activity displayed by the coating in the presence of antibiotics was very potent and rapid (i.e. 6-log reduction of viability in 4 h). If such activity resulted from PMBN release, shedding of PMBN should also occur very quickly. To the contrary, the anti-biofilm activity of the PMBN-coated silicone subjected to 3-day incubation in water was indistinguishable from that of the pre-incubated surface.

Our strategy has limitations that are common to other anti-biofilm approaches based on surface-bound active molecules. On the one hand, the implantation of a foreign device inside the body induces the deposition of host proteins on its surface. If this happens on a functionalized surface like ours, it can compromise its antimicrobial activity. In addition, even after its immobilization, PMBN may be susceptible to degradation by host proteases. Although the stability of our functionalized surface in the presence of human proteases needs to be determined, it is known that polymyxins' cyclical structure helps protect them from proteolytic peptidases, thereby explaining why half-life of these compounds is longer than that of many peptides [55].

In summary, we successfully immobilized an antibiotic enhancer on a silicone surface without affecting its functionality and the resultant coating exhibited excellent stability and biocompatibility. The experimental surface displayed potent bactericidal and biofilm preventive activity when combined with antibiotics both exogenously added and incorporated into the silicone matrix. Although our in vitro data are promising, experimentation in animal models of infection associated with implanted devices is needed to determine the potential utility of PMBN-coated devices.

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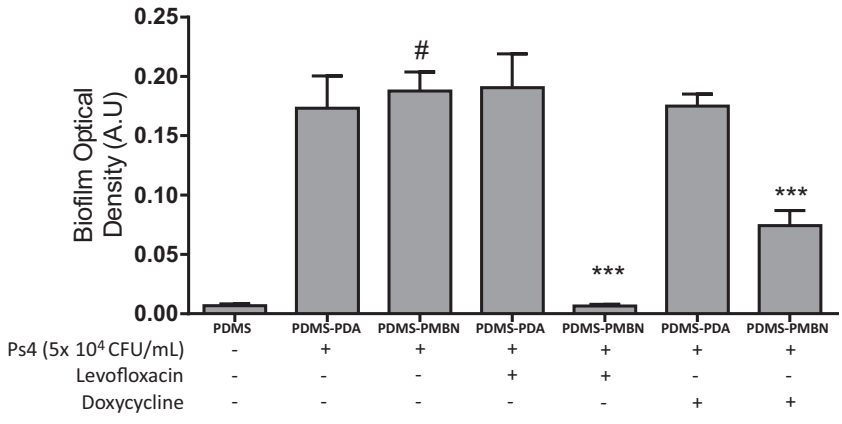

Fig. 7. Biofilm preventive capacity of a silicone surface integrating antibiotics and immobilized PMBN. PMBN-coated coupons swollen with either Levofloxacin or Doxycycline were incubated for 24 h at 37 °C in MHCA inoculated with *Pseudomonas aeruginosa* CUN 4158–02 Ps4. Then, the biofilm attached to the coupons was first stained with safranin and then suspended in water and quantified by spectrophotometry at 490 nm. Coupons soaked in acetone/water 9:1 ratio were used as controls. Results are the means ± S.D. of 3 independent experiments performed in triplicate. Obtained results were analyzed using student t-test and statistical differences were significant (***; $p < 0.001$) compared to the untreated control (#).

Author statement

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2021.111876.

References

[1] H. Shahrour, R. Ferrer-Espada, I. Dandache, S. Bárcena-Varela, S. Sánchez-Gómez, A. Chokr, G. Martinez-de-Tejada, AMPs as anti-biofilm agents for human therapy and prophylaxis, Adv. Exp. Med. Biol. 1117 (2019) 257–279, https://doi.org/10.1007/978-981-13-3588-4_14.

[2] T. Bjarnsholt, The role of bacterial biofilms in chronic infections, APMIS. Suppl. (2013) 1–51, https://doi.org/10.1111/apm.12099.

[3] H.H. Al-Mousa, A.A. Omar, V.D. Rosenthal, M.F. Salama, N.Y. Aly, M. El-Dossoky Noweir, F.M. Rebello, D.M. Narciso, A.F. Sayed, A. Kurian, S.M. George, A. M. Mohamed, R.J. Ramapurath, S.T. Varghese, Device-associated infection rates, bacterial resistance, length of stay, and mortality in Kuwait: International Nosocomial Infection Consortium findings, Am. J. Infect. Control 44 (2016) 444–449, https://doi.org/10.1016/j.ajic.2015.10.031.

[4] V.D. Rosenthal, D.G. Maki, N. Graves, The International Nosocomial Infection Control Consortium (INICC): goals and objectives, description of surveillance methods, and operational activities, Am. J. Infect. Control 36 (2008) e1–e12, https://doi.org/10.1016/j.ajic.2008.06.003.

[5] E. Alp, N. Damani, Healthcare-associated infections in intensive care units: epidemiology and infection control in low-to-middle income countries, J. Infect. Dev. Ctries. 9 (2015) 1040–1045, https://doi.org/10.3855/jidc.6832.

[6] F. Hahn, R. Zbinden, K. Min, Late implant infections caused by *Propionibacterium acnes* in scoliosis surgery, Eur. Spine J. 14 (2005) 783–788, https://doi.org/10.1007/s00586-004-0854-6.

[7] I. Collins, J. Wilson-MacDonald, G. Chami, W. Burgoyne, P. Vineyakam, T. Berendt, J. Fairbank, The diagnosis and management of infection following instrumented spinal fusion, Eur. Spine J. 17 (2008) 445–450, https://doi.org/10.1007/s00586-007-0559-8.

[8] T.-F. Mah, Biofilm-specific antibiotic resistance, Future Microbiol. 7 (2012) 1061–1072, https://doi.org/10.2217/fmb.12.76.

[9] D. Lebeaux, J.-M. Ghigo, C. Beloin, Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics, Microbiol. Mol. Biol. Rev. 78 (2014) 510–543, https://doi.org/10.1128/MMBR.00013-14.

[10] S. Sánchez-Gómez, R. Ferrer-Espada, P.S. Stewart, B. Pitts, K. Lohner, G. Martínez de Tejada, Antimicrobial activity of synthetic cationic peptides and lipopeptides derived from human lactoferricin against *Pseudomonas aeruginosa* planktonic cultures and biofilms, BMC Microbiol. 15 (2015) 137, https://doi.org/10.1186/s12866-015-0473-x.

[11] P.Y. Chung, R. Khanum, Antimicrobial peptides as potential anti-biofilm agents against multidrug- resistant bacteria, J. Microbiol. Immunol. Infect. 50 (2017) 405–410, https://doi.org/10.1016/j.jmii.2016.12.005.

[12] A.A. Bahar, Z. Liu, M. Garafalo, N. Kallenbach, D. Ren, Controlling persister and biofilm cells of gram-negative bacteria with a new 1,3,5-triazine derivative, Pharmaceuticals. 8 (2015) 696–710, https://doi.org/10.3390/ph8040696.

[13] D. Pletzer, S.R. Coleman, R.E. Hancock, Anti-biofilm peptides as a new weapon in antimicrobial warfare, Curr. Opin. Microbiol. 33 (2016) 35–40, https://doi.org/10.1016/j.mib.2016.05.016.

[14] T.-H. Lee, K.N. Hall, M.-I. Aguilar, Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure, Curr. Top. Med. Chem. 16 (2016) 25–39, https://doi.org/10.2174/1568026615666150703121700.

[15] G. Wang, B. Mishra, K. Lau, T. Lushnikova, R. Golla, X. Wang, Antimicrobial peptides in 2014, Pharmaceuticals 8 (2015) 123–150, https://doi.org/10.3390/ph8010123.

[16] J.C. Wenke, B.D. Owens, S.J. Svoboda, D.E. Brooks, Effectiveness of commercially-available antibiotic-impregnated implants, J. Bone Jt. Surg. - Ser. B. 88 (2006) 1102–1104, https://doi.org/10.1302/0301-620X.88B8.17368.

[17] M. Chohfi, F. Langlais, J. Fourastier, J. Minet, H. Thomazeau, M. Cormier, Pharmacokinetics, uses, and limitations of vancomycin-loaded bone cement, Int. Orthop. 22 (1998) 171–177, https://doi.org/10.1007/s002640050235.

[18] Q. Zeng, Y. Zhu, B. Yu, Y. Sun, X. Ding, C. Xu, Y.-W. Wu, Z. Tang, F.-J. Xu, Antimicrobial and antifouling polymeric agents for surface functionalization of medical implants, Biomacromolecules 19 (2018) 2805–2811, https://doi.org/10.1021/acs.biomac.8b00399.

[19] S. Atefyekta, M. Pihl, C. Lindsay, S.C. Heilshorn, M. Andersson, Antibiofilm elastin-like polypeptide coatings: functionality, stability, and selectivity, Acta Biomater. 83 (2019) 245–256, https://doi.org/10.1016/j.actbio.2018.10.039.

[20] M. Kazemzadeh-Narbat, J. Kindrachuk, K. Duan, H. Jenssen, R.E.W. Hancock, R. Wang, Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections, Biomaterials. 31 (2010) 9519–9526, https://doi.org/10.1016/j.biomaterials.2010.08.035.

[21] B. Mishra, A. Basu, R.R.Y. Chua, R. Saravanan, P.A. Tambyah, B. Ho, M.W. Chang, S.S.J. Leong, Site specific immobilization of a potent antimicrobial peptide onto silicone catheters: evaluation against urinary tract infection pathogens, J. Mater. Chem. B 2 (2014) 1706, https://doi.org/10.1039/c3tb21300e.

[22] R. Gopal, Y.G. Kim, J.H. Lee, S.K. Lee, J.D. Chae, B.K. Son, C.H. Seo, Y. Park, Synergistic effects and antibiofilm properties of chimeric peptides against multidrug-resistant acinetobacter baumannii strains, Antimicrob. Agents Chemother. 58 (2014) 1622–1629, https://doi.org/10.1128/AAC.02473-13.

[23] P. Parreira, C. Monteiro, V. Graça, J. Gomes, S. Maia, P. Gomes, I.C. Gonçalves, M. C.L. Martins, Surface grafted MSI-78A antimicrobial peptide has high potential for gastric infection management, Sci. Rep. 9 (2019), https://doi.org/10.1038/s41598-019-53918-4.

[24] J. Chen, Y. Zhu, Y. Song, L. Wang, J. Zhan, J. He, J. Zheng, C. Zhong, X. Shi, S. Liu, L. Ren, Y. Wang, Preparation of an antimicrobial surface by direct assembly of antimicrobial peptide with its surface binding activity, J. Mater. Chem. B 5 (2017) 2407–2415, https://doi.org/10.1039/c6tb03337g.

[25] F. Costa, I.F. Carvalho, R.C. Montelaro, P. Gomes, M.C.L. Martins, Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces, Acta Biomater. 7 (2011) 1431–1440, https://doi.org/10.1016/j.actbio.2010.11.005.

[26] M.E. Evans, D.J. Feola, R.P. Rapp, Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria, Ann. Pharmacother. (1999), https://doi.org/10.1345/aph.18426.

[27] M. Beganovic, M.K. Luther, K.E. Daffinee, K.L. LaPlante, Biofilm prevention concentrations (BPC) of minocycline compared to polymyxin B, meropenem, and amikacin against *Acinetobacter baumannii*, Diagn. Microbiol. Infect. Dis. 94 (2019) 223–226, https://doi.org/10.1016/j.diagmicrobio.2019.01.016.

[28] K.M.S. Herrera, F.K. da Silva, M.E. de Oliveira, M.C. de Paiva, A.C. Soares, J. M. Siqueira Ferreira, First report of polymyxin B activity in Klebsiella pneumoniae biofilm, J. Chemother. 31 (2019) 127–131, https://doi.org/10.1080/1120009X.2018.1558751.

[29] K. Abdelraouf, K.H. Braggs, T. Yin, L.D. Truong, M. Hu, V.H. Tam, Characterization of polymyxin B-induced nephrotoxicity: implications for dosing regimen design, Antimicrob. Agents Chemother. 56 (2012) 4625–4629, https://doi.org/10.1128/AAC.00280-12.

[30] M.E. Falagas, S.K. Kasiakou, Toxicity of polymyxins: a systematic review of the evidence from old and recent studies, Crit. Care. 10 (2006), https://doi.org/10.1186/cc3995.

[31] A.P. Zavascki, R.L. Nation, Nephrotoxicity of polymyxins: is there any difference between colistimethate and polymyxin B? Antimicrob. Agents Chemother. 61 (2017) https://doi.org/10.1128/AAC.02319-16.

[32] H. Tsubery, H. Yaakov, S. Cohen, T. Giterman, A. Matityahou, M. Fridkin, I. Ofek, Neopeptide antibiotics that function as opsonins and membrane- permeabilizing agents for gram-negative bacteria, Antimicrob. Agents Chemother. 49 (2005) 3122–3128, https://doi.org/10.1128/AAC.49.8.3122-3128.2005.

[33] H. Tsubery, I. Ofek, S. Cohen, M. Fridkin, Structure-function studies of polymyxin B nonapeptide: implications to sensitization of gram-negative bacteria, J. Med. Chem. 43 (2000) 3085–3092, https://doi.org/10.1021/jm0000057.

[34] A.Z. Sahalan, R.A. Dixon, Role of the cell envelope in the antibacterial activities of polymyxin B and polymyxin B nonapeptide against *Escherichia coli*, Int. J. Antimicrob. Agents 31 (2008) 224–227, https://doi.org/10.1016/j.ijantimicag.2007.10.005.

[35] M. Vaara, T. Vaara, Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide, Nature. 303 (1983) 526–528, https://doi.org/10.1038/303526a0.

[36] R. Ferrer-Espada, H. Shahrour, B. Pitts, P.S. Stewart, S. Sánchez-Gómez, G. Martínez-de-Tejada, A permeability-increasing drug synergizes with bacterial efflux pump inhibitors and restores susceptibility to antibiotics in multi-drug resistant *Pseudomonas aeruginosa* strains, Sci. Rep. 9 (2019) 3452, https://doi.org/10.1038/s41598-019-39659-4.

[37] R. Ferrer-Espada, S. Sánchez-Gómez, B. Pitts, P.S. Stewart, G. Martínez-de-Tejada, Permeability Enhancers Sensitize β-Lactamase-expressing Enterobacteriaceae and *Pseudomonas aeruginosa* to β-Lactamase Inhibitors, Thereby Restoring Their β-Lactam Susceptibility vol. 56, 2020, https://doi.org/10.1016/j.ijantimicag.2020.105986.

[38] S. Sánchez-Gómez, M. Lamata, J. Leiva, S.E. Blondelle, R. Jerala, J. Andrä, K. Brandenburg, K. Lohner, I. Moriyón, G. Martínez-de-Tejada, Comparative analysis of selected methods for the assessment of antimicrobial and membrane-permeabilizing activity: a case study for lactoferricin derived peptides, BMC Microbiol. 8 (2008) 196, https://doi.org/10.1186/1471-2180-8-196.

[39] P.A. Wayne, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, in: CLSI standards M07, 11th edClinical and Laboratory Standards Institute, 2018. http://www.clsi.org.
[40] M.C. Berenbaum, A method for testing for synergy with any number of agents, J. Infect. Dis. 137 (1978) 122–130, https://doi.org/10.1093/infdis/137.2.122.
[41] J. Meletiadis, S. Pournaras, E. Roilides, T.J. Walsh, Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against *Aspergillus fumi*, Antimicrob. Agents Chemother. 54 (2010) 602–609, https://doi.org/10.1128/AAC.00999-09.
[42] D. Alves, M.O. Pereira, Bio-inspired coating strategies for the immobilization of polymyxins to generate contact-killing surfaces, Macromol. Biosci. 16 (2016) 1450–1460, https://doi.org/10.1002/mabi.201600122.
[43] T.L. Riss, R.A. Moravec, A.L. Niles, S. Duellman, H.A. Benink, T.J. Worzella, L. Minor, Cell viability assays, eli lilly & company and the national center for advancing translational sciences. http://www.ncbi.nlm.nih.gov/pubmed/23805433, 2004. (Accessed 29 October 2020).
[44] K. Lim, R.R.Y. Chua, B. Ho, P.A. Tambyah, K. Hadinoto, S.S.J. Leong, Development of a catheter functionalized by a polydopamine peptide coating with antimicrobial and antibiofilm properties, Acta Biomater. 15 (2015) 127–138, https://doi.org/10.1016/j.actbio.2014.12.015.
[45] Z. Oren, Y. Shai, Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure-function study, Biochemistry 36 (1997) 1826–1835, https://doi.org/10.1021/bi962507l.
[46] J. Liebscher, R. Mrówczyński, H.A. Scheidt, C. Filip, N.D. Hădade, R. Turcu, A. Bende, S. Beck, Structure of polydopamine: a never-ending story? Langmuir 29 (2013) 10539–10548, https://doi.org/10.1021/la4020288.
[47] A. Kottmann, E. Mejía, T. Hémery, J. Klein, U. Kragl, Recent developments in the preparation of silicones with antimicrobial properties, Chem. - An Asian J. 12 (2017) 1168–1179, https://doi.org/10.1002/asia.201700244.
[48] P.B. Eckburg, T. Lister, S. Walpole, T. Keutzer, L. Utley, J. Tomayko, E. Kopp, N. Farinola, S. Coleman, Safety, tolerability, pharmacokinetics, and drug interaction potential of SPR741, an intravenous potentiator, after single and multiple ascending doses and when combined with β-lactam antibiotics in healthy subjects, Antimicrob. Agents Chemother. 63 (2019), https://doi.org/10.1128/AAC.00892-19.
[49] N. Shtreimer Kandiyote, G. Mohanraj, C. Mao, R. Kasher, C.J. Arnusch, Synergy on surfaces: anti-biofouling interfaces using surface-attached antimicrobial peptides PGLa and magainin-2, Langmuir 34 (2018) 11147–11155, https://doi.org/10.1021/acs.langmuir.8b01617.
[50] Z. Zhu, Z. Wang, S. Li, X. Yuan, Antimicrobial strategies for urinary catheters, J. Biomed. Mater. Res. Part A. 107 (2019) 445–467, https://doi.org/10.1002/jbm.a.36561.
[51] L. Ge, Q. Li, M. Wang, J. Ouyang, X. Li, M.M.Q. Xing, Nanosilver particles in medical applications: synthesis, performance, and toxicity, Int. J. Nanomedicine 9 (2014) 2399–2407, https://doi.org/10.2147/IJN.S55015.
[52] Y.S. Kim, J.S. Kim, H.S. Cho, D.S. Rha, J.M. Kim, J.D. Park, B.S. Choi, R. Lim, H. K. Chang, Y.H. Chung, I.H. Kwon, J. Jeong, B.S. Han, I.J. Yu, Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats, Inhal. Toxicol. 20 (2008) 575–583, https://doi.org/10.1080/08958370701874663.
[53] A.K. Duwe, C.A. Rupar, G.B. Horsman, S.I. Vas, In vitro cytotoxicity and antibiotic activity of polymyxin B nonapeptide, Antimicrob. Agents Chemother. 30 (1986) 340–341, https://doi.org/10.1128/AAC.30.2.340.
[54] Mur Nightingale, Antimicrobial Pharmacodynamics in Theory and Clinical Practice, CRC Press, 2007, https://doi.org/10.3109/9781420017137.
[55] L. Diao, B. Meibohm, Pharmacokinetics and pharmacokinetic-pharmacodynamic correlations of therapeutic peptides, Clin. Pharmacokinet. 52 (2013) 855–868, https://doi.org/10.1007/s40262-013-0079-0.