Controlled Release of Plectasin NZ2114 from a Hybrid Silicone-Hydrogel Material for Inhibition of *Staphylococcus aureus* Biofilm

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ABSTRACT *Staphylococcus aureus* is a major human pathogen in catheter-related infections. Modifying catheter material with interpenetrating polymer networks is a novel material technology that allows for impregnation with drugs and subsequent controlled release. Here, we evaluated the potential for combining this system with plectasin derivate NZ2114 in an attempt to design an *S. aureus* biofilm-resistant catheter. The material demonstrated promising antibiofilm properties, including properties against methicillin-resistant *S. aureus*, thus suggesting a novel application of this antimicrobial peptide.

KEYWORDS *Staphylococcus aureus*, antimicrobial peptides, plectasin, biofilm formation, catheter infections, biofilms, catheter, hydrogel

A decade ago, antimicrobial peptides (AMPs) were considered to be one of the most important new classes of drugs in the fight against antimicrobial resistance. Today, however, most AMPs have been removed from development pipelines, with only a few having reached actual clinical use (1).

Despite their obvious potential, the systemic use of AMPs is hampered by their intrinsic immunogenicity and low *in vivo* stability (2), which together with regulatory requirements of superior efficiency to current treatment have been major obstacles for obtaining approval (1). The use of AMPs in combination with medical devices to prevent device-associated infections is, however, an enticing alternative application that to a lesser extent is restricted by the above problems (3, 4, 5).

Device-associated and, specifically, catheter-related bloodstream infections (CRBSI) constitute a major problem in modern health care. Methicillin-sensitive *Staphylococcus aureus* (MSSA) and, in particular, methicillin-resistant *S. aureus* (MRSA) are among the most important pathogens in CRBSI. The plectasin derivate NZ2114 is a promising AMP that has been potentiated toward MSSA, MRSA, and vancomycin-resistant *S. aureus* with proven *in vivo* efficiency (6, 7) and, therefore, holds potential for use against device-associated infections, including CRBSI.

Here, we evaluated the properties of a novel hybrid catheter material loaded with plectasin NZ2114 in order to inhibit MRSA biofilms. The material consists of an interpenetrating polymer network (IPN) based on silicone elastomer (polydimethylsiloxane) as the host polymer and poly(2-hydroxyethyl methacrylate)-co-poly(ethylene glycol) methyl ether acrylate (PHEMA-co-PEGMEA) hydrogel as the guest polymer. This IPN material exhibits unique drug loading and release properties through adsorption of drugs into the hydrogel component in the bulk of the material and subsequent release upon exposure to aqueous solutions, such as bodily fluids (8, 9,

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FIG 1 (A) Mean accumulated release from catheter specimens measured in micrograms ($n = 4$). (B) Mean release per day measured in micrograms per milliliter (release volume = 1.5 ml; $n = 4$).

10, 11). Hypothetically, the catheter matrix protects the embedded drug, in this case plectasin NZ2114, from proteolytic degradation. Furthermore, the IPN matrix ensures a controlled release of the drug, potentially resulting in prolonged efficiency and fewer side effects compared to those of the burst release often associated with conventional drug coatings.

The IPN material was produced as previously described (8) with modifications. Briefly, silicone samples were punched out from 2-mm-thick sheets of silicone rubber (PE4062; Lebo Production, Skogås, Sweden) or cut from silicone tubing (outer/inner diameter, 1.65/0.76 mm; Helix Medical, VWR, Radnor, PA). The samples were placed in a high-pressure reactor with equal amounts of HEMA (2-hydroxyethyl methacrylate; Aldrich Chemistry, Germany) and EGMEA (ethylene glycol methyl ether acrylate; Aldrich, Germany) with supercritical carbon dioxide as an aiding solvent. After polymerization into PHEMA-co-PEGMEA inside the silicone material, the samples were placed in 96% ethanol for 7 days to remove residual monomer followed by drying at 50°C until final mass was reached. Two IPN sample sets with hydrogel contents of 23% and 28%, respectively, were produced and evaluated. The amount of PHEMA-co-PEGMEA hydrogel in the samples was determined by mass increase. IPN samples were drug loaded by immersion in Milli-Q water containing 10 mg/ml of plectasin NZ2114 (6) (Novozymes A/S, Bagsværd, Denmark) or 10 mg/ml of dicloxacillin (Bristol-Myers Squibb, New York City, NY) for 7 days.

To evaluate release from the catheter material, release sampling was performed daily over 14 days from catheter tubing, 23 mm in length, placed in phosphate-buffered saline (PBS) with a pH of 7.4. The plectasin NZ2114 concentration was quantified by ultraperformance liquid chromatography (UPLC) (Fig. 1). Mean totals of $46.1 \mu\text{g} \pm 21.3$ (mean $\pm$ standard deviation) and $42.9 \mu\text{g} \pm 20.9$ for 23% and 28% PHEMA-co-PEGMEA, respectively, were released from the catheter specimens during this time period. The release correlated well with first-order kinetics ($R^2 = 0.97$ and 0.92 for 23% and 28% PHEMA-co-PEGMEA, respectively), with an approximate release of 40.7% and 31.9% of remaining loaded drug each day for the 23% and 28% samples, respectively, indicating a better long-term slow release profile for catheters with the higher IPN content.

Antimicrobial activity of plectasin NZ2114 and dicloxacillin against MSSA and MRSA was determined as MIC and minimum bactericidal concentration (MBC) by broth dilution according to ISO 20776-1 standards (Table 1).

The loaded catheter tubing was tested for bactericidal effect in a static setup. The catheter tubing was placed in test tubes containing 10% heparinized human plasma in PBS, inoculated with approximately 5.0×10^4 CFU/ml of MRSA strain ATCC 33591, and incubated overnight. The CFU count in the suspension was then estimated, and the catheter tubing was transferred to a new test tube containing inoculated plasma and the procedure repeated for 11 days (Fig. 2).

TABLE 1 MICs and MBCs measured by broth dilution assay according to ISO standard 20776-1

Strain	MIC ($\mu\text{g/ml}$)		MBC ($\mu\text{g/ml}$)	
	Dicloxacin	Plectasin NZ2114	Dicloxacin	Plectasin NZ2114
MSSA ATCC 29213	1	0.5	8	8
MRSA ATCC 33591	32	1	>1,024	16

As it appears in Fig. 2, some fluctuations occur in this type of experiment, a phenomenon we have observed in an earlier study as well (11). To evaluate whether this is due to plectasin NZ2114 destabilization during loading, storage in, and release from the IPN hydrogel, the activity of the compound after release in buffer was tested using MIC determination, and dilutions were matched to the high-pressure liquid chromatography (HPLC) release quantification data (Fig. 1). Comparing these data to the MIC values for the stock showed no loss of activity upon loading and release (data not shown). Together with the relatively low day-to-day fluctuation observed in the direct release measurements (Fig. 1), we speculate that the fluctuations in Fig. 2 occur due to biological variation in bacterial sensitivity, where subpopulations may enter biofilm or an otherwise more persistent growth mode differently from day to day.

Assuming that the catheters remain effective as long as the mean CFU counts are below the initial inoculum (i.e., the 100% point on the y axis in Fig. 2), the dicloxacin-loaded tubing lasts for 2 days whereas the plectasin NZ2114-loaded specimens last until day 10 (Fig. 2).

In an *in vivo* setting, a considerable part of venous catheters is exposed to blood flow, placing high demands on this part of the material with respect to maintaining an effective release of drug. Bacteria spreading to catheters may, furthermore, originate from biofilms growing on skin/wound sites; i.e., they are already more resilient than traditional broth-cultured bacteria when reaching the catheter material. To account for these conditions, plectasin NZ2114-loaded IPN discs (hydrogel content of 24%) were challenged with MRSA ATCC 33591 in a flow chamber model as previously described (11, 12) using an initial bacterial seeding inoculum of optical density at 600 nm (OD_{600}) of 0.100 in PBS for 30 min at 30 $\mu\text{l/min}$. This was followed by a growth phase in 10% heparinized human plasma in PBS at 30 $\mu\text{l/min}$, which leads to a continuous seeding over the catheter surface with resilient biofilm emboli (11). The plectasin NZ2114-

FIG 2 Functional release assay ($n = 3$) using PEGMEA-co-PHEMA IPN material impregnated with dicloxacin or plectasin for inhibition of MRSA ATCC 33591. The CFU values in vials containing test catheters were measured after daily challenge with 5×10^4 CFU per ml plasma medium and are shown in the graph as a percentage of this daily inoculum. In control vials containing pristine silicone and unloaded IPN samples, CFU reached >1,000% of the inoculum added on day 1 (data not shown).

FIG 3 Microscopy of biofilms formed by MRSA ATCC 33591 on IPN material either as unloaded control (A, B, C) or loaded with plectasin NZ2114 (D, E, F). (A, B, D, and E) 0.64 mm by 0.64 mm CLSM images obtained with an Olympus FV1000MPE. (C and F) Full fluorescence microscopy scans of the flow chamber test surface performed using an Olympus BX51 microscope with motorized stage and image processing using Olympus cellSens software (only the green/GFP channel is shown). Over the 24-h experiment, the surface was continuously seeded with biofilm emboli (11).

loaded disks showed effective inhibition of bacterial surface colonization compared to unloaded IPN disks as visualized with the LIVE/DEAD BacLight bacterial viability kit (L7012; Molecular Probes, Eugene, OR) using fluorescence and confocal laser scanning microscopy (CLSM) (Fig. 3).

To quantitatively assess whether the catheter tubing exhibited adequate drug-release properties for biofilm inhibition despite its limited wall thickness, catheter samples loaded with plectasin NZ2114 were tested in a flow system seeded as the previous flow chamber assay with MRSA and subsequently grown in a flow of 10% heparinized plasma. CFU in the resulting biofilm inside the tubing were quantified by pipetting 0.1% Triton X-100 in PBS through the tubing followed by plating. This experiment demonstrated effective prevention of growth in the plectasin NZ2114-loaded catheters compared to unloaded and dicloxacillin-loaded catheters (Fig. 4), displaying a significant 3-log reduction compared to unloaded IPN (Table 2). Furthermore, plectasin NZ2114-loaded catheters exposed to prerelease in PBS for 6 days prior to testing still exhibited a significant 2-log reduction compared to the unloaded IPN, indicating a clinically relevant release after 1 week of catheter placement (Table 2).

Induction of antibiotic resistance is a relevant concern for medical devices that

FIG 4 Quantitative analysis of MRSA colonization in a catheter flow model using heparinized plasma as growth medium.

passively release antimicrobials due to an unavoidable period of time near drug depletion when the release of drug becomes less than the MIC. To assess to what extent sub-MICs of plectasin NZ2114 may induce resistance in *S. aureus*, we applied a serial passage of increasing drug concentration modified from Hammer et al. (13). In brief, $\sim 10^5$ bacteria were inoculated in 5 ml tryptic soy broth (TSB) overnight at 37°C on a shaker. The following day, 100 μ l of the culture was transferred to new test tubes containing 4.9 ml TSB with either ciprofloxacin or plectasin NZ2114 at 25% of MIC and incubated overnight. Then, 100 μ l was transferred to new TSB tubes in which drug concentrations were increased by 100%. This procedure continued until growth inhibition was observed compared to positive controls without antimicrobials (21 days in this experiment). At this point, 100 μ l of the bacterial suspension was plated and MIC determined as described above. Results for MSSA ATCC 29213 and MRSA ATCC 33591 (Table 3) showed that plectasin NZ2114 induced drug resistance only to a minor extent compared to the control antibiotic ciprofloxacin.

Lastly, to assess possible immune synergism of plectasin NZ2114, we determined the MBC for MSSA ATCC 29213 and MRSA ATCC 33591, respectively, in heat-inactivated and untreated 10% pooled human serum in PBS. Here, identical MBC values were measured in untreated and heat-inactivated serum (data not shown), indicating no synergistic effects with complement factors, in contrast to what has been reported for other antimicrobial peptides (14, 15).

Plectasin NZ2114 has previously shown promising results in the treatment of various *S. aureus* diseases *in vivo* (6, 7). Using this antimicrobial peptide as an active loading agent in a novel IPN-based device material showed promising results in a comprehensive *in vitro* test procedure, accounting for several relevant factors that influence the performance of the catheter *in vivo*. This suggests that the material-drug combination may be suitable for venous catheters for more effective prevention of colonization by staphylococci and other Gram-positive pathogens.

TABLE 2 Statistical analysis of the data shown in Fig. 4^a

Vs	Dicloxacillin	Plectasin 1d	Plectasin 6d + 1d	Silicone
Plectasin 1d	<0.01			
Plectasin 6d + 1d	NS	<0.01		
Silicone	NS	<0.01	0.05	
Unloaded IPN	NS	<0.01	<0.01	NS

^aOne-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test. $n = 4$ for all 5 groups. Before testing, data were transformed by natural logarithm to obtain normal distributions. *P* values are shown. NS = not significant.

TABLE 3 MIC before and after serial passing with increasing antimicrobial concentrations^a

Strain	MIC before serial passing		MIC after serial passing	
	Ciprofloxacin	Plectasin NZ2114	Ciprofloxacin	Plectasin NZ2114
MSSA ATCC 29213	0.25 (0.25)	0.5 (0.5)	32 (32)	4 (4–8)
MRSA ATCC 33591	0.25 (0.25)	1 (1.0)	32 (32–64)	8 (4–8)

^aMIC values were determined according to ISO standard 20776-1. All numbers are in $\mu\text{g/ml}$ and presented as median with range in parentheses ($n = 3$).

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