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Antimicrobial dendrimer active against *Escherichia coli* biofilms

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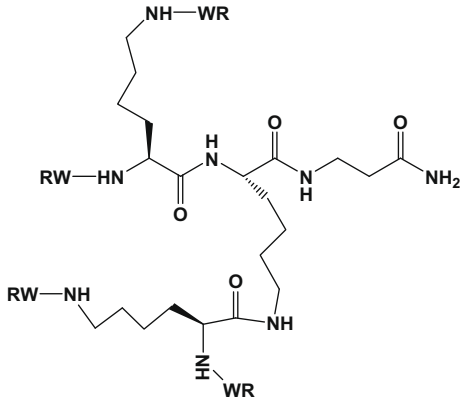
ABSTRACT

We have investigated the ability of a previously reported antimicrobial peptide dendrimer $(RW)_{4D}$ to inactivate *Escherichia coli* RP437 in planktonic culture and in biofilms. The results show that the dendrimer inhibits bacterial growth in both planktonic and biofilm states. Live/Dead staining assays reveal that most bacteria in a preformed biofilm lose viability after treatment with this peptide. This result is in marked contrast to most existing reports that antimicrobial peptides are ineffective against mature bacterial biofilms.

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces in contrast to free swimming planktonic bacterial cells. Biofilms are generally more resistant to biocidal agents because of the intrinsic barrier of the external matrix, their inert physiological state, and the presence of subpopulations of resistant phenotypes within the film.[1] Bacterial biofilms are implicated in an increasing number of bacterial infections including those associated with implanted devices.[2] Killing bacteria in biofilms remains an important target for new therapeutic agents. Many studies have shown that biofilms are much more resistant to antibiotics than planktonic cells and in particular that preformed biofilms on surfaces are extremely difficult to remove.[3,4] However, growth of biofilms on new surfaces can be inhibited by many antimicrobial agents, even at sub-MICs of antibiotics.[5–7]

In this study, we examined a previously designed dendrimeric peptide $(RW)_{4D}$ (Scheme 1) based on a short cationic antimicrobial peptide sequence[8] to determine its efficacy against planktonic bacterial growth, biofilm formation, and ability to eradicate preformed biofilms. The dendrimeric peptide has been shown to be active against multi-drug resistant (MDR) strain *Staphylococcus aureus* and *Escherichia coli* (D31), the latter is resistant to ampicillin and streptomycin, as well as *Acinetobacter baumannii*. Furthermore, we assayed the viability of cells within biofilm colonies after treatment with $(RW)_{4D}$ using Live/Dead fluorescent dye staining. The results revealed that most cells remaining in the film were killed by the dendrimer.

Dendrimeric peptides were first tested for their antimicrobial activity against planktonic cells. A microplate-based assay[10] was used to investigate the antimicrobial activity of dendrimeric peptides at increasing concentrations from 0, 5, 10, 20, to 40 μM, all of which were added into the growth medium directly at the time of inoculation. Representative growth curves (mean value ± standard deviation) are shown in Figure 1A. The results show that



Scheme 1. Structure of $(RW)_{4D}$. $(RW)_{4D}$ was synthesized according to Tam.[9] Peptide was purified on HPLC and the molecular weight was verified by mass spectrometry using a Bruker MALDI-TOF spectrometer.

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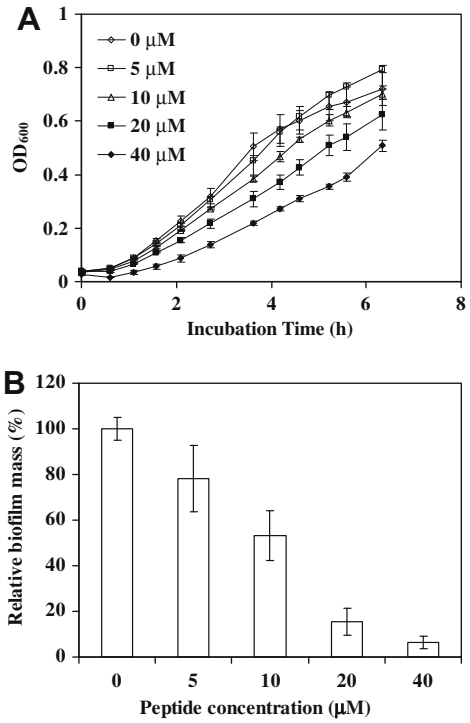

Figure 1. (A) Planktonic growth of *E. coli* RP437[11–13] in the presence of increasing concentrations of $(RW)_{4D}$. (B) Microplate assays of biofilm formation. Biofilms were formed in 96-well plates and quantified by staining with 0.1% crystal violet. The biofilm mass without peptide was normalized as 100%.

planktonic growth of *E. coli* RP437 was inhibited by dendrimeric peptides in a dose-dependent manner. Compared to the blank control, the specific growth rates of *E. coli* in the presence of dendrimeric peptides at 5, 10, 20, and 40 μM were reduced by 3.2%, 15.3%, 23.6%, and 33.5%, respectively.

To investigate the antimicrobial activity of dendrimeric peptides on *E. coli* biofilm formation, a microplate-based assay was used to quantify biofilm formation on polystyrene surfaces.[14,15] Biofilms were incubated in the microplate for 24 h and then stained with 0.1% crystal violet. Dendrimeric peptides at a series of concentrations from 0 to 40 μM were added to each well before incubation. The OD_{540} readings of bottom biofilms were used to quantify biofilm mass. The relative biofilm mass was calculated by normalizing the data to the 0 μM control as shown in Figure 1B. The data indicate that the inhibition of biofilm formation on the polystyrene surfaces by dendrimeric peptides was also dose-dependent: in the presence of 5, 10, 20, and 40 μM dendrimeric peptide, biofilm formation of *E. coli* RP437 in 96-well plates was reduced by 21.8 ± 4.9%, 47.1 ± 10.9%, 84.4 ± 5.9%, 93.5 ± 2.7%, respectively, as compared to the peptide-free control. Further tests of the viability of biofilm cells on surfaces were conducted using Live/Dead staining as detailed below.

To gain further insight into the antimicrobial effects of dendrimeric peptides on biofilm formation, we used a fluorescent staining assay to visualize biofilm cells with fluorescence microscopy. Stainless steel coupons were used to form *E. coli* RP437 biofilms, treated with and without the peptide, and then stained with Live/Dead BacLight™ bacterial viability kit. A Carl Zeiss Axio Imager M1 Microscope was used to record fluorescence images of biofilms after 24 h incubation in the presence of dendrimeric peptides at concentrations of 0, 5, 10, 20, and 40 μM, respectively. Representa-

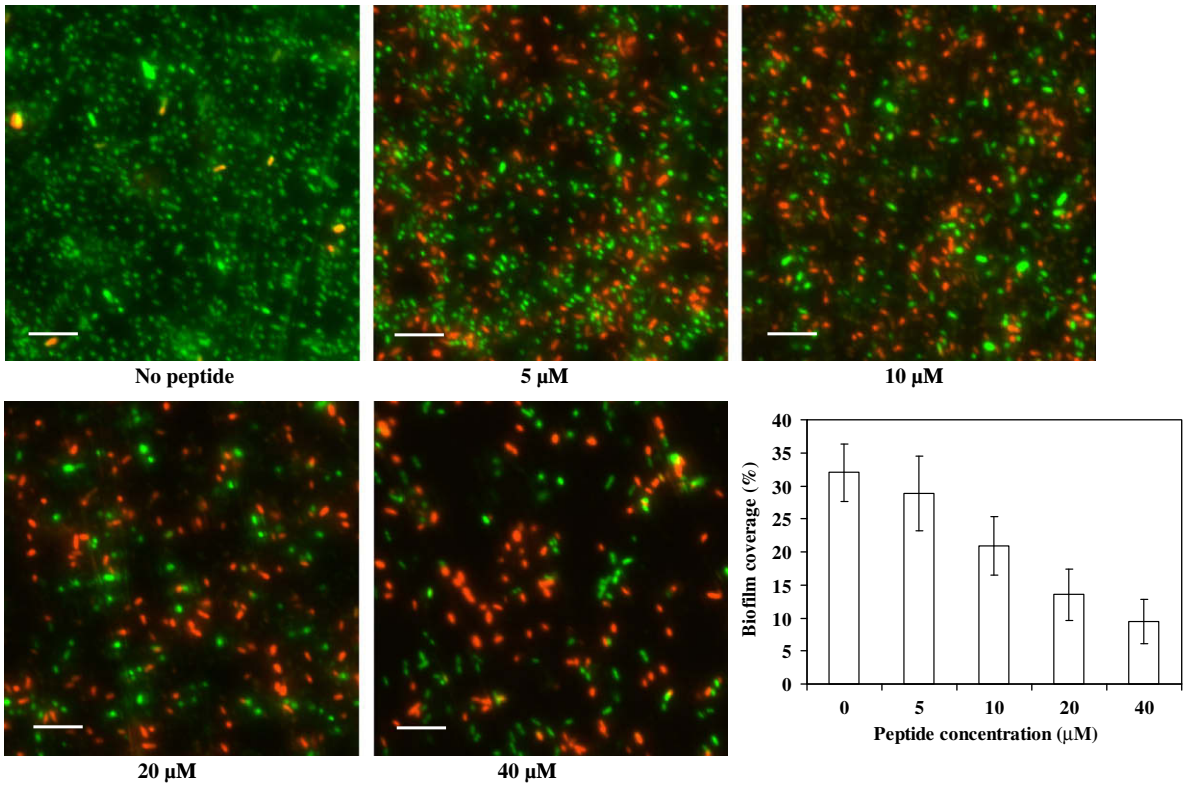

Figure 2. Live/Dead staining assay of biofilm formation. Stainless steel coupons (1/4′ long, 1/2′ wide, and 1/16′ thick) were used to form biofilms. Biofilms were stained with Live/Dead BacLight™ bacterial viability kit (Cat# L7012, Invitrogen Corporation, CA) and analyzed using fluorescence microscopy. Images were taken with an AXIO Imager M1 microscope (Carl Zeiss, Germany). Five spots on each coupon were randomly picked and analyzed. Live cells were stained green and dead cells were stained red. Bar = 10 μm (panels: 1–5). Quantification of the biofilm surface coverage. The quantification of the surface coverage for 24-h biofilms formed on stainless steel coupons in the presence of the dendrimer peptide at different concentrations was obtained using COMSTAT software (panel 6).[17]

tive fluorescence images of biofilms are shown in Figure 2. Live cells are stained green and dead cells stained red. The images indicate significant reduction of biofilm formation and cell death upon adding dendrimeric peptides. The surface coverage of biofilms was further calculated using COMSTAT software as shown in Figure 2 (panel 6). Specifically, the surface coverage of biofilms in the presence of 20 and 40 μM dendrimeric peptides was significantly reduced—by 57.7 ± 12.2% (*t*-test, $p < 0.05$) and 70.4 ± 10.7% (*t*-test, $p < 0.05$), respectively. This corroborates the results obtained in 96-well plates, that the dendrimer inhibits biofilm formation in a dose-dependent manner.

Mature biofilms are known to be more refractory to eradication relative to inhibition of *de novo* biofilm formation, which is relatively simple.[16] Thus it is important to gain insight into the antimicrobial activity of dendrimeric peptides against a mature biofilm. To assess these effects, Live/Dead staining assay was used to visualize biofilms after 3 h treatment with 0, 20, 40, 80, 120, and 160 μM peptide. Representative fluorescence images of biofilms are shown in Figure 3. Analysis indicates that the dendrimer peptide causes no apparent removal of biofilms; nevertheless, there was significant killing of biofilm cells after 3 h treatment with dendrimer peptides and the killing was dose-dependent.

The inhibitory effect of $(RW)_{4D}$ against *E. coli* RP437 planktonic cells is consistent with previous results showing that $(RW)_{4D}$ inactivates planktonic cells via a membranolytic mechanism shared by many natural antimicrobial peptides.[8] Reduced biofilm formation is observed on stainless steel coupons as well as in microplate-based assays upon applying $(RW)_{4D}$. The repressive effect of $(RW)_{4D}$ on biofilm formation likely reflects inhibition of initial adhesion of bacteria to the surface and therefore subsequent development of the biofilm.[5] Furthermore, the biofilm preformed on stainless steel surfaces was shown increasing numbers of dead cells after the treatment with increasing concentrations of $(RW)_{4D}$ (Fig. 3). This observation suggests that $(RW)_{4D}$ is capable of penetrating the exopolysaccharide (EPS) matrix and kill the biofilm cells. Both the EPS matrix and the bacterial cell wall are negatively charged, and in principle should bind $(RW)_{4D}$—a cationic peptide. As a consequence, the integrity of both the EPS matrix and the bacterial membrane might be compromised.

Detailed analysis of the kinetics of film formation shows that the process is not static, and that films can build up and disperse in the presence of an antibiotic.[18] Bacteria in biofilms exhibit multi-drug resistance, possibly due to elevated fractions of dormant or persister-like cells within the film. Persister phenotypes show reduced growth rates, minimizing exposure to antibiotics that target DNA, RNA, protein, or cell wall synthesis.[19] The effect does not involve genetic resistance, since persisters can rapidly revert to normal growth. Instead persistence appears to be a stochastic process in which a variable fraction of planktonic growing cells enter a dormant state.[20] The fraction of dormant cells increases in stationary phase and presumably also in the confines of a biofilm. Reported results have shown mixed effects of antimicrobial peptides on this type of dormant cell. A 14-residue peptide was found to inactivate target cells in both log and stationary phase.[21] On the other hand the antimicrobial peptide polymyxin B was less effective against cells in stationary phase by a factor of over 100.[22] The antimicrobial peptide colistin completely lost potency when tested against biofilms.[23] Evidently these discrepant results need further investigation.

In summary, we have investigated the antimicrobial effect of a dendrimeric peptide $(RW)_{4D}$ against *E. coli* RP437 planktonic cells and biofilm colonies. The results show that this bacterial strain was susceptible in both states. Hypothetically, $(RW)_{4D}$ acts against the bacterial biofilm through compromising the integrity of both

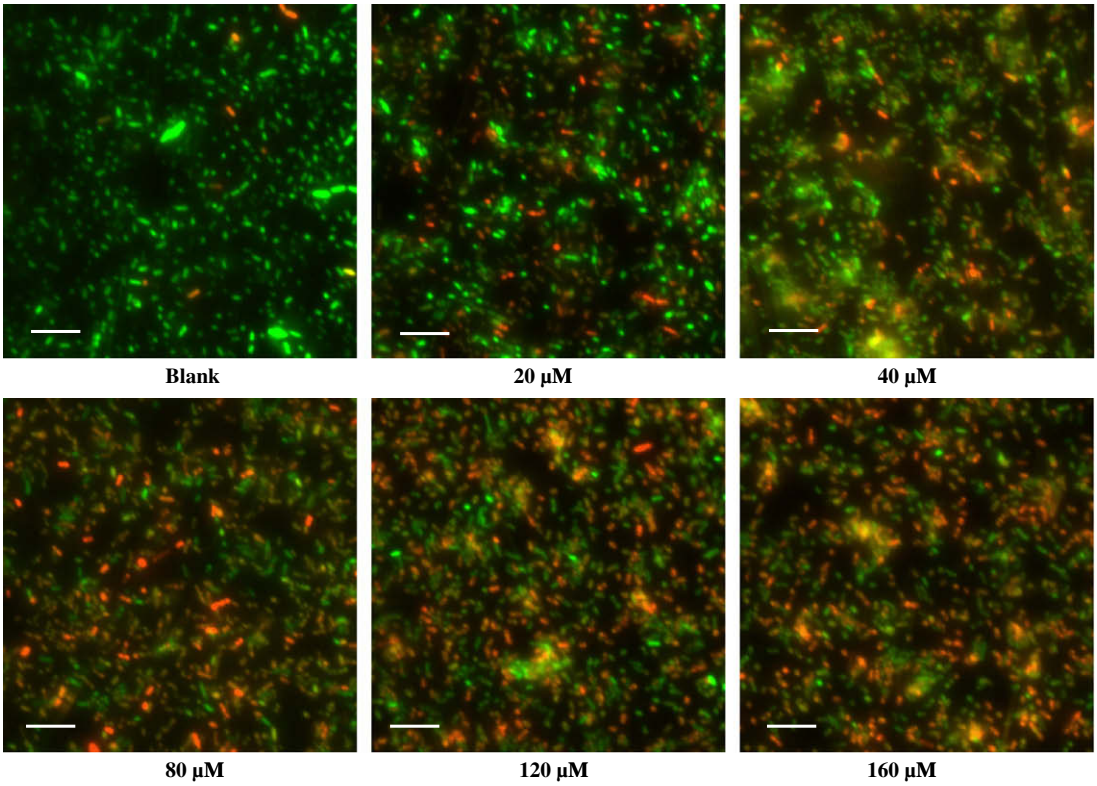

Figure 3. Assay of mature biofilms treated with and without the dendrimeric peptide. *E. coli* RP437 was used to form biofilms, and peptides were added to the preformed biofilms 24 h after inoculation, for a period of 3 h. Duplicate coupons were tested for each condition. Images were recorded with an AXIO Imager M1 microscope. Five spots on each coupon were randomly picked and analyzed. The biofilms were stained using the Live/Dead BacLight™ bacterial viability kit. The bar = 10 μm.

the EPS matrix and the bacterial membrane. Further study of the mechanism of action on biofilms will be helpful in designing new leads with broad applications.

Acknowledgments

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