Maggot therapy´s modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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CHAPTER TWO

Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Objectives: Lucilia sericata maggots are successfully used for treating chronic wounds. As the healing process in these wounds is complicated by bacteria, particularly when residing in biofilms which protect them from antibiotics and the immune system, we assessed the effects of maggot excretions/secretions (ES) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms, the clinically most relevant species.

Methods: We assessed the effects of ES on biofilms using microtiter plate assays, on bacterial viability using *in vitro* killing and radial diffusion assays, and on quorum sensing systems using specific reporter bacteria.

Results: As little as 0.2 µg of ES prevented *S. aureus* biofilm formation and 2 µg of ES rapidly degraded biofilms. In contrast, ES initially promoted *P. aeruginosa* biofilm formation, but after 10 h the biofilms collapsed. Degradation of *P. aeruginosa* biofilms started after 10 h and required 10-fold more ES than *S. aureus* biofilms. Boiling of ES abrogated their effects on *S. aureus*, but not *P. aeruginosa* biofilms, indicating that different molecules within ES are responsible for the observed effects. Modulation of biofilms by ES did not involve bacterial killing or effects on quorum sensing systems.

Conclusion: Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Introduction

Chronic wounds cause considerable morbidity and present the health care system with significant costs[1]. Such wounds are common in patients suffering from acute, extended trauma as well as patients with vascular insufficiencies and underlying chronic conditions like diabetes mellitus[2,3] in which even minor wounds become infected and show little tendency to heal. The healing process is often complicated by bacterial infections of the wound surface[4-6]. Bacteria within chronic wounds often reside in biofilms[7] and these bacteria exhibit altered growth characteristics and gene expression profiles as compared with planktonic bacteria[8]. Biofilm formation has been associated with a number of diseases, such as endocarditis[9], cystic fibrosis[10] and osteomyelitis[11]. An important practical consequence of biofilm formation is that the bacteria are protected against the actions of antibiotics[12,13] and cells and effecter molecules of the immune system[6,14]. Moreover, bacterial fragments/ products released from biofilms will continuously attract host immune cells, like neutrophils, to the wound. As these cells cannot remove the infectious cause of inflammation, this will eventually lead to tissue destruction through the actions of bioactive products like reactive oxygen species and proteases released by activated phagocytes[15].

Nowadays, the use of sterile larvae of the green bottle blowfly *Lucilia sericata* in the management of sores, ulcers, and other chronic wounds is becoming increasingly widespread[16-18]. Especially in trauma surgery these maggots can prevent or at least reduce major disabling amputations. Maggots may contribute to wound healing by removing cell debris and non-viable tissue[19], inhibiting the pro-inflammatory responses of phagocytes[20] and promoting tissue remodelling[21]. The molecules involved in these actions are believed to be contained in the excretions/secretions (ES) of the maggots. Interestingly, clinical observations indicated that maggot therapy is more effective in patients with wounds infected with Gram-positive bacteria, like *Staphylococcus aureus,* than those infected with Gram-negative bacteria, like *Pseudomonas aeruginosa*. Additionally, more maggots are needed to accomplish healing of wounds infected with the latter bacterium[22]. Since modulation of bacterial biofilms will have a major impact on the healing process of chronically infected wounds the aim of this study was to investigate the effects of ES on the formation of *S. aureus* and *P. aeruginosa* biofilms and on established biofilms.

Materials and methods

Maggots and maggot excretions/secretions

ES of sterile second- and third-instar larvae of *Lucilia sericata* (a kind gift from BioMonde GmbH, Barsbüttel, Germany) were collected as described[20]. In short, larvae were incubated in water for 60 min. Next, collected ES preparations were checked for sterility and stored at

-20°C. For comparison, we also collected ES according to the method described by Kerridge *et al*[23].

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 29213 (Manassas, VA, USA) were grown in Tryptone Soya Broth (TSB) at 37°C and *Pseudomonas aeruginosa* PAO1[24] in Luria Bertani (LB) medium at 28°C, both under vigorous shaking. The reporter bacteria *Chromobacterium violaceum* CVO26[25] and *Escherichia coli* DH5α strains pAK211[26] and pSB1075[27] were grown in LB medium at 28°C.

Biofilm assay

Biofilm formation of *S. aureus* and *P. aeruginosa* in 96-wells polyvinyl chloride (PVC) plates was conducted as described[28]. In short, bacteria from overnight cultures were diluted with medium 1:1,000 for *S. aureus* and 1:100 for *P. aeruginosa* and 5 µL of these bacterial suspensions were added to each well containing 100 µL of the medium with or without ES (range 0.2-20 µg): the medium for *S. aureus* was 0.5x TSB supplemented with 0.2% (w/v) glucose and for *P. aeruginosa* 0.7x M63. At the indicated intervals, planktonic cells were removed and the wells were washed with tap water. Subsequently, biofilms were exposed to a 1% (w/v) crystal violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the crystal violet retained by the cells. Next, this solution was transferred to 96-wells plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) and used to quantify the amount of biofilm by measuring at OD590 nm. In addition, at various intervals after the start of the experiment, the planktonic cells were harvested and then the bacteria residing in these biofilms were recovered by sonicating three times for 15 s on ice with 30 s between each sonication step. Next, the number of viable bacteria in the suspensions of planktonic cells and of bacteria dispersed from the biofilms was determined microbiologically using serial dilutions of these suspensions plated in six-fold onto COS blood agar plates.

To investigate the effects of ES on established biofilms, we first formed biofilms for 24 h, then the planktonic cells were removed and 100 µL of medium with or without ES (range 0.2-20 µg) were added to the wells.

In vitro killing assay

To further determine the bactericidal effect of ES on planktonic cells, *in vitro* killing assays were conducted as described[29] with minor modifications. Bacteria in mid-log phase were centrifuged at 2,000xg for 10 min, washed with PBS and suspended in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 1% (v/v) TSB to a concentration of $1x10^6$ cells/mL. Subsequently, 200 µL of the bacterial suspension were transferred to Eppendorf

tubes containing vacuum dried ES (range 2-400 µg). After 1 h and 3 h, the number of surviving bacteria was determined microbiologically as described above.

Radial Diffusion Assay (RDA)

To further investigate the antibacterial activity of ES, we used the more sensitive RDA as described[30] with minor modifications. In short, bacteria in mid-log phase were centrifuged at 2,000xg for 10 min and washed with PBS. Next, $1x10^5$ bacteria/mL were dispersed in agar consisting of 1% (w/v) agarose (Sigma-Aldrich, St. Louis, MO, USA) and 1% (w/v) TSB in 10 mM sodium phosphate buffer at 42°C. Subsequently, the agar was poured into petridishes (Greiner Bio-One) and solidified. Next, wells of 3 mm in diameter were made in this agar and 5 µL of vacuum dried ES (range 2-400 µg) solubilised in 0.01% (v/v) acetic acid were transferred to the wells. After 3 h incubation, an overlay agar was poured on top of the bacterial agar. The following day, the diameters of the growth inhibition zones were measured. We validated the assay using 50 µg/mL of human neutrophil peptide 1-3 (hnp1-3) and human lactoferrin-derived peptide (hLF1-11).

Detection of autoinducer activity

Autoinducer activity was measured using the reporter strains *C. violaceum* CVO26 and *E. coli* DH5α containing pAK211 or pSB1075 as described[31]. In short, bacteria were grown overnight in LB medium supplemented with respectively kanamycin (25 µg/mL), chloramphenicol (20 µg/mL) or carbomycin (200 µg/mL). Subsequently, plates were overlaid with top agar existing of LB medium containing 0.8% (w/v) agar (Bacto™agar, BD, Sparks, MD, USA) and 10 µL of the bacterial suspension per mL. Next, 5 µL of vacuum dried ES (range 2-400 µg) solubilised in water or, as a negative control, only water were transferred to the agar and incubated at 28°C for 16 h. As a positive control 0.5 µg of synthetic acyl homoserine lactone autoinducers (kindly provided by Prof. P. Williams, University of Nottingham, UK) was used. Autoinducer activity was detected by the production of a purple pigment (violacein) by *C. violaceum* and by the emission of light when using *E. coli* after applying a Fuji medical X-Ray (Fuji Photo Film Co., Ltd., Tokyo, Japan) on the plates.

Statistical analysis

Results are means ± SEM of at least three experiments using in each experiment two different batches of ES. Differences between the values for ES-exposed and non-exposed bacteria were analyzed using a one-way ANOVA with Dunnett's post-test for multiple comparisons. The level of significance was set at $p<0.05$.

Results

Effect of ES on biofilm formation

To find out if ES can prevent biofilm formation, we determined the amount of biofilm at various intervals after addition of 0-20 µg of ES. The results revealed that after a lag time of 8 h, *S. aureus* started to form a detectable biofilm and that the biofilm formation levelled off after 14 h (Figure 1A). In addition, as little as 0.2 µg of ES completely blocked *S. aureus* biofilm formation. The kinetics of *P. aeruginosa* biofilm formation during the first 24 h were similar to those found for *S. aureus,* but thereafter *P. aeruginosa* biofilms became unstable in several experiments (Figure 1B). Furthermore, enhanced *P. aeruginosa* biofilm formation was seen at 8-10 h after addition of 2 and 20 µg of ES, but thereafter the biofilms formed in the presence of 20 µg of ES, but not 2 µg of ES, collapsed. In agreement, we observed that the number of bacteria in the biofilms exposed to ES for 8-10 h was almost ten-fold higher than in unexposed biofilms (Table 1). Further experiments with higher doses of ES (up to100 µg) revealed that the start of the *P. aeruginosa* biofilm breakdown was dose-dependently enhanced by ES, yet all these biofilms were broken down within 48 h (data not shown). In addition, replacing the medium of biofilms developed in the presence of 20 µg of ES for 8 h with fresh ES-containing medium resulted after 24 h in the breakdown of *P. aeruginosa* biofilms, whereas no breakdown was seen in the wells reincubated with medium alone, indicating that components in ES degraded the biofilms. Of note, *S. aureus* formed biofilms mostly on the bottom of the wells while *P. aeruginosa* formed biofilms on the wall of the

Table 1 The number of bacteria present in the wells of the biofilm formation experiments at 8 and 24 h after starting the experiments.

	Biofilm		Planktonic cells	
ES (µg/mL)	0	20	0	20
S. aureus				
t = 8 h	3.2 ± 1.7 (x 10^6)	no	4.2 ± 0.6 (x10^7)	3.8 ± 0.8 (x10^7)
t = 24h	6.7 ± 1.1 (x 10^6)	no	3.8 ± 0.8 (x10^7)	5.1 ± 0.5 (x10^7)
P. aeruginosa				
t = 8 h	7.0 ± 1.2 (x 10^5)	5.4 ± 2.6 (x 10^6)*	1.6 ± 0.7 (x 10^7)	1.9 ± 1.1 (x 10^7)
t = 24h	2.9 ± 1.0 (x 10^7)	no	4.0 ± 2.4 (x 10^8)	4.4 ± 2.0 (x 10^8)

Results are means ± SEM of 4-6 experiments. 'no' indicates that no biofilm was detectable. *Significant ($p<0.05$) differences between the values for bacteria exposed to ES and those for non-exposed bacteria.

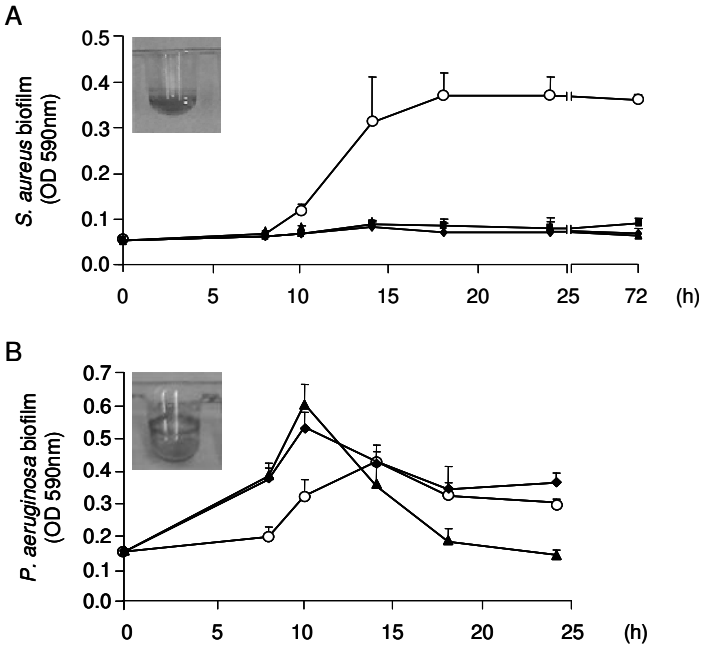

Figure 1 Effect of maggot excretions/secretions on biofilm formation by *S. aureus* (A) and *P. aeruginosa* (B). Results are means ± SEM of 4-5 experiments. Open circles = no ES; filled squares= 0.2 µg of ES; filled diamonds = 2 µg of ES; filled triangles = 20 µg of ES.
A: From 10 h on, all values are significantly ($p<0.05$) different from those for biofilms without ES. *S. aureus* mainly formed biofilms at the bottom of the wells (insert).
B: Values for 20 µg of ES are significantly higher at 8 h and 10 h, and significantly lower at 18 h and 24 h than those for biofilms without ES. *P. aeruginosa* formed a ring on the wall of the wells at the air-liquid interphase (insert).

Table 2 Effect of heat-treatment on the activity of 20 µg of ES against biofilms.

	Treatment		
	no ES	native ES	boiled ES
S. aureus			
Biofilm formation	0.37 ± 0.04	0.09 ± 0.01	0.29 ± 0.03*
Biofilm breakdown	0.38 ± 0.07	0.10 ± 0.06	0.46 ± 0.07*
P. aeruginosa			
Biofilm formation	0.29 ± 0.01	0.15 ± 0.02	0.12 ± 0.02
Biofilm breakdown	0.42 ± 0.03	0.22 ± 0.06	0.16 ± 0.01

Results are means ± SEM of 3-5 experiments. *Significant ($p<0.05$) differences between the values for biofilms exposed to boiled ES and those to native ES.

wells at the air-liquid interphase (Figure 1 A,B inserts). Interestingly, treatment of 20 µg of ES for 2 h at 100°C completely abrogated the effects on *S. aureus* biofilm formation, but not on *P. aeruginosa* biofilm formation (Table 2), indicating that different molecules within ES modulate *S. aureus* and *P. aeruginosa* biofilm formation.

Effect of ES on established biofilms

Next, we determined the effects of ES on established biofilms. The results showed that within 2 h after addition of ES the amount of *S. aureus* biofilm was dose-dependently reduced and a complete breakdown was seen with 2 and 20 µg of ES (Figure 2A). Furthermore, 0.2 µg of ES gradually reduced the amount of biofilm within the first 6 h and thereafter the amount of biofilm remained constant. Established *P. aeruginosa* biofilms were initially stimulated by ES and after 10 h gradually broken down by 20 µg of ES, while 2 µg of ES did not cause an effect (Figure 2B). Heat treatment of ES completely abrogated their effects on established *S. aureus* biofilms, but not on established *P. aeruginosa* biofilms (Table 2).

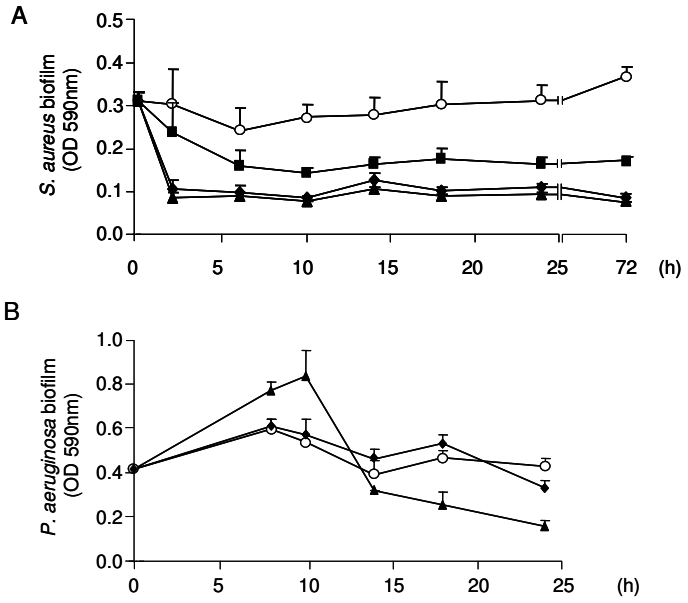

Figure 2 Effect of maggot excretions/secretions on established biofilms of *S. aureus* (A) and *P. aeruginosa* (B).
Results are means ± SEM of 5-6 experiments. Open circles = no ES; filled squares = 0.2 µg of ES; filled diamonds = 2 µg of ES; filled triangles = 20 µg of ES.
A: All values of 2 and 20 µg ES are significantly ($p<0.05$) different from those for biofilms without ES. From 10 h on, 0.2 µg of ES are significantly different from those for biofilms without ES.
B: Values for 20 µg of ES are significantly higher at 8 h and 10 h, and significantly lower at 18 h and 24 h compared to biofilms without ES.

Effect of ES on bacterial viability

Since ES may have bactericidal activities against Gram-positive and Gram-negative bacteria,[23,32] we determined the effect of ES on the number of viable biofilm-associated and planktonic *S. aureus* and *P. aeruginosa* in our biofilm experiments. The results revealed that at the current doses and conditions ES did not kill planktonic bacteria (Table 1). In addition, the total number of bacteria in the wells was not significantly altered indicating that ES did not disrupt biofilms simply by killing bacteria. Furthermore, 20 µg of ES were not bactericidal against *S. aureus* and *P. aeruginosa* in *in vitro* killing and radial diffusion assays. *In vitro* killing experiments revealed that only the largest dose of ES studied (400 µg) reduced the number of viable *S. aureus* after 3 h by 73 ± 10%, but not after 1 h, as compared with the control (n = 7). Using RDAs we found that ES killed *S. aureus* in a dose-dependent fashion with as little as 40 µg of ES being effective (Figure 3). Heat-treatment abolished the bactericidal effects of ES on *S. aureus* in the *in vitro* killing assays and it reduced the effects in the RDAs by 79 ± 16% (n = 4). In contrast, ES (up to 800 µg) did not reduce the number of viable *P. aeruginosa*. Finally, no differences in the antibacterial activity between ES preparations obtained by the method of Kerridge *et al* [23] and our ES preparations were noted.

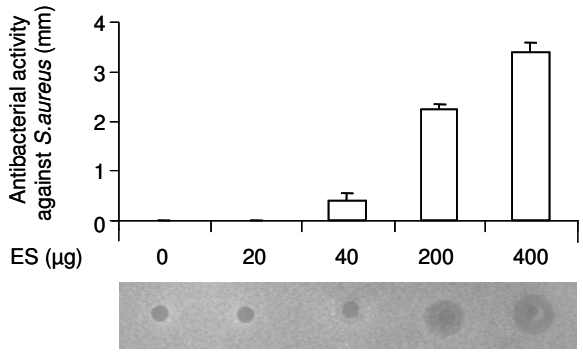

Figure 3 Antimicrobial activity of maggot excretions/secretions against *S. aureus* using a radial diffusion assay. Results are means ± SEM of 6 experiments. The diameter of the clearance zone was corrected for the diameter of the well.

Effect of ES on quorum sensing systems of Gram negative bacteria

As quorum sensing systems control bacterial functions, such as biofilm formation[33], interference with these bacterial systems could explain the effects of ES on biofilms. Therefore, we determined the ability of ES to mimic or antagonize the actions of various *N*-acyl homoserine lactones (AHLs) using specific reporter bacteria. The results showed that

ES (0.2-200 µg) had neither mimicking nor antagonizing effects on quorum sensing systems detecting short chain (C6/C8) AHLs, as assayed with the reporter bacteria *C. violaceum* CVO26 and *E. coli* DH5α containing pAK211. The positive control (synthetic C6 AHLs) showed zones of approximately 5 cm in both systems (n = 3). Furthermore, ES had no effect on quorum sensing systems responding to long chain (C10/C12) AHLs assayed in *E. coli* DH5α containing pSB1075; the positive control (synthetic C10 AHLs) caused a zone of 5 ± 0.4 cm (n = 3).

Discussion

The main conclusion from the present study is that maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This conclusion is based on the following observations. First, *S. aureus* biofilm formation was blocked by as little as 0.2 µg of ES per well, whereas 2 µg of ES per well was sufficient to degrade established biofilms within 2 h. Secondly, *P. aeruginosa* biofilm formation was initially enhanced by ES and after 10 h biofilms treated with 20 µg of ES, but not 2 µg of ES, degraded and during the remaining period of the analysis no biofilms could be detected. Interestingly, others reported similar effects of the prokaryotic predator *Micavibrio aeruginosavorus* on *P. aeruginosa* biofilm formation and suggested that increased cell-cell interactions may explain the initial enhancement of biofilms[34]. Thirdly, the doses of ES used in this study were within the therapeutic range, i.e., those present at the surface of maggot-treated wounds[35,36]. For instance, 20 µg of ES were obtained after incubating approximately 10 maggots in distilled water for 1 h. It should be realized that in our *in vitro* experiments ES were added only once to the bacteria and/or bacterial biofilms, whereas in wounds, maggots are continuously present. Furthermore, ES were obtained from sterile maggots. Since it is likely that ES of maggots exposed to bacteria in a wound have an altered composition, it is of interest that ES obtained from bacteria-exposed maggots were as effective against bacterial biofilms as sterile ES (MJA van der Plas *et al*, unpublished observations).

The second conclusion pertains to the mechanism(s) underlying the prevention of biofilm formation and the breakdown of bacterial biofilms by ES. The possibility that ES modulate biofilms simply by killing the bacteria is highly unlikely since in our biofilm experiments ES did not affect the number of viable bacteria in the wells. However, it is reported by several groups[23,32,37] that ES have bactericidal properties against planktonic bacteria, although the used amounts are not within the therapeutic range or not mentioned at all. Therefore, we decided to investigate the bactericidal activity further by using two different methods described in these reports; the RDA being the most sensitive assay but the *in vitro* killing assay resembling the biofilm experiments more. In agreement with our biofilm data, *S.*

aureus were not killed at the biofilm-effective amounts of ES while *P. aeruginosa* was not killed at all. Investigation into the effects of ES on quorum sensing signalling pathways in several Gram-negative reporter strains showed that ES do not mimic or antagonize short and long chain *N*-acyl homoserine lactones. However, these data do not exclude the possibility that ES interfere with quorum sensing signalling of bacteria in the wound. Although no definitive explanation for the differences in effects of ES on *S. aureus* and *P. aeruginosa* biofilms can be offered on the basis of our data, we concluded that the observed effects are mediated by different molecules and mechanisms, since heat-treatment completely abrogated the effects of ES on *S. aureus*, but not on *P. aeruginosa*, biofilms. This suggests that proteins or heat sensitive peptides within ES may be responsible for the breakdown of *S. aureus*, but not of *P. aeruginosa* biofilms. More research, including purification of these compounds, is needed to gain a detailed understanding of the mechanisms involved in the modulatory effects of ES on biofilms.

We are the first to report that ES disrupt bacterial biofilms. It should be kept in mind that we required more ES to disrupt *P. aeruginosa* biofilms than *S. aureus* biofilms and that low doses of ES can result in enhancement of *P. aeruginosa* biofilms. In addition, it has been shown *in vitro* that *P. aeruginosa,* but not *S. aureus,* impairs maggot survival[38]. Together, these data are in agreement with clinical findings[22] indicating that more maggots should be used for wounds infected with *P. aeruginosa* (compared to *S. aureus*). Furthermore, as a result of biofilm breakdown, the bacteria become susceptible to actions of antibiotics and the immune system as well as to actions of maggots[39]. Therefore, ES (especially in combination with antibiotics) are a very promising source of candidates for the development of new treatments for biofilm-associated diseases, including cystic fibrosis, infected medical devices, like catheters and prosthesis, and chronic wounds.

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