Polymeric micelles for the dispersal of infectious biofilms

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Chapter 5

General discussion

1 Comparison of Different Micellar Dispersants Applied to Biofilms of the ESKAPE-panel Pathogens

Abstract

Infectious biofilms are glued together by a self-produced matrix composed of extracellular-polymeric-substances. Biofilm dispersal for infection control bears the risk of sepsis due to a suddenly increasing bacterial concentration in blood. Therefore, dispersal must be accompanied by antibiotic therapy or be sufficiently balanced for the host immune system to handle. However, a generally applicable biofilm-dispersal-index (BDI) for comparison of the strength of different dispersants does not exist. Here, we propose a BDI, based on reductions in biomass or number of colony forming units in biofilm remaining after dispersant exposure. The BDI proposed is applied for the comparison of three micellar dispersants. Across all ESKAPE pathogens, zwitterionic PEG/PQAE micelles aiming to interact with matrix eDNA and amyloid proteinaceous fibers to break biofilm integrity, had the lowest BDI (< 0.1), while EGCG-loaded PEG/PAE micelles aiming to disassemble amyloid fibers had an intermediate BDI (0.1 – 0.3). DNase-loaded PEG/PAE micelles aiming to degrade matrix eDNA had the highest BDI (0.2 – 0.6). In previous experiments and chapters, we observed that hypothermia and death due to sepsis in mice with a *Staphylococcus aureus* pneumonia, increased compared with a PBS-control after biofilm dispersal by PEG/PAE-DNase micelles. Hypothermia decreased for an intra-abdominal and implant associated biofilm after dispersal by PEG/PQAE or PEG/PAE-EGCG micelles. This suggests that PEG/PAE-DNase micelles may yield an overly, non-balanced dispersal of biofilms that the murine immune system cannot handle. Concluding, the BDI proposed allows quantitative comparison of different dispersants and will be important for selecting dispersants that perform optimally with respect to specific pathogens in absence of accompanying antibiotics.

1.1 Introduction

Bacteria in an infectious biofilm are glued together by their self-produced matrix of extracellular polymeric substances (EPS).[1] Main components of the EPS matrix are eDNA, proteins and polysaccharides.[2] In an infection control strategy based on dispersing biofilm bacteria, each of these main components of the EPS matrix can be the target of degradation by a dispersant.[3–5] The potential success of biofilm dispersants as a clinically applicable strategy to control infectious biofilms, critically depends on the host immune response to a sudden increase in the concentration of dispersed bacteria in blood.[6,7] Dispersal of motile, infectious bacteria from infected wounds using glycoside hydrolases for instance, caused lethal sepsis in mice when not used in combination with a suitable antibiotic therapy.[8] However, considering the rise in the number of antibiotic-resistant bacteria,[9] dispersants yielding a balanced increase in the concentration of dispersed bacteria in blood that can be handled by the host immune system without the need for additional antibiotic therapy may be preferable.

Lately, a variety of polymeric micelles has been described for the protective encapsulation of dispersants in order to allow transportation through the blood circulation without enzymatic inactivation.[10,11] Polymeric micelles provide a relatively versatile means of encapsulating dispersants, that are often too hydrophobic to be encapsulated in liposomes.[12] Moreover, micelles are easy to be functionalized with stimuli-responsive functionalities, that respond to the acidic pH or low oxygen saturation levels in or near infectious biofilms.[13] In or near a biofilm, stimuli-responsive micelles change their charge properties to become electrostatically attracted to negatively charged bacteria in the biofilm ("self-targeting"), where they release their cargo and interact with the biofilm matrix and inhabiting bacteria.[14] Self-targeting zwitterionic micelles (see **Table 1**) interacted with matrix eDNA and proteinaceous amyloid fibers to break biofilm integrity, therewith dispersing a *Staphylococcus aureus* biofilm in mice. Other self-targeting micelles have been loaded with DNase I or EGCG to degrade matrix eDNA or disassemble proteinaceous amyloid fibers, respectively in *S. aureus* biofilms (see also **Table 1**).

Most methods to evaluate biofilm dispersal are based on qualitative micrographs, or more quantitative outcome parameters such as reductions in

biomass or the number of colony forming units (CFU) in a biofilm.[18] However, neither qualitative images nor quantitative outcome parameters can be directly used for comparison without accounting for differences in exposure time and dispersant concentration. Yet, with the increasing interest in biofilm dispersal as an infection control strategy and the accompanying increase in the number of dispersants forwarded in the literature, the need for a quantitative biofilm dispersal index is increasing as well.

Table 1. Micellar dispersants used in this study, their composing polymers and matrix components to be degraded.

Abbreviation	**Polymeric components***	**Dispersants and DLE (%)**	**Acting on**	**Ref.**
PEG/PQAE	PEG-*b*-PCL /PCL-*b*-PQAE	PQAE (100**)	eDNA, amyloid fibers	15, Chapter 2
PEG/PAE-EGCG	PEG-*b*-PCL /PCL-*b*-PAE	EGCG (75)	Amyloid fibers	16, Chapter 4
PEG/PAE-DNase	PEG-*b*-PCL /PCL-*b*-PAE	DNase I (88)	eDNA	17, Chapter 3

*Abbreviations: PEG-*b*-PCL, Poly(ethylene glycol)-*b*-poly(ε-caprolactone); PCL-*b*-PQAE, PCL-*b*-poly(quaternary amino ester); PCL-*b*-PAE, PCL-*b*-poly(amino ester); EGCG, (-)-Epigallocatechin gallate; DLE, drug loading efficiency.
**implying that micelles themselves are the active dispersants, without any additional loading.

The aim of this article is to propose a biofilm dispersal index (BDI), based either on a reduction in biomass or CFUs of biofilm remaining after exposure to a dispersant. The BDI proposed is employed for a quantitative comparison of different self-targeting micellar dispersal systems. Uniquely, the comparison is carried out for the complete set of bacterial strains with the ability to "escape" current antibiotic treatment (ESKAPE pathogens,[19] i.e. *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, and *Enterobacter spp*). The relevance of the BDI proposed for estimating the risk of sepsis when applied *in vivo*, will be addressed by relating BDI values with septic symptoms observed in previous experiments and chapters of these micellar dispersants in murine infection models.

1.2 Materials and Methods

1.2.1 Micellar dispersants used

The micellar dispersants summarized in **Table 1** were all prepared as previously reported[15–17] without any additional antimicrobial core-loading. In essence, all micelles were prepared through self-assembly of the composing surfactants in a polar solvent (dimethyl sulfoxide) and have been demonstrated to be self-targeting and accumulate in an infectious biofilm upon tail-vein injection in mice. Zwitterionic PEG/PQAE micelles were used as a dispersant and shown to interact with eDNA and amyloid proteinaceous fibers. DNase I in PEG/PAE micelles was conjugated to PAE to become part of the micellar shell where it was shown to be protected against inactivation by blood-borne enzymes. In the acidic environment of an infectious biofilm, PAE stretches to expose the conjugated DNase I and allows it to become active in degrading eDNA. EGCG was also included in the shell of PEG/PAE micelles in order to be protected against inactivation at physiological pH. EGCG was cross-linked with PAE through pH-reversible boronic-ester binding, allowing release of EGCG inside an infectious biofilm to disassemble amyloid protein fibers.

1.2.2 Bacterial culturing and harvesting

E. faecium ATCC 35667, *S. aureus* ATCC 12600, *K. pneumoniae*-1, *A. baumannii* ATCC 19606, *P. aeruginosa* PAO1 and *E. cloacae* BS 1037 were grown from frozen stock on blood agar plates at 37°C for 24 h. For preculture, a single bacterial colony was transferred into 10 mL of tryptone soy broth (TSB, OXOID, Basingstoke, UK) and incubated 24 h at 37°C. For main cultures, the preculture was transferred into 200 mL of TSB and incubated for 16 h at 37°C. Then, bacteria were harvested by centrifugation (5000g, 5 min, 10°C) followed by washing twice in sterile phosphate buffered saline (PBS, 5 mM K_2HPO_4, 5 mM KH_2PO_4, and 150 mM NaCl, pH 7.0). The bacterial suspension was sonicated three times each for 10 s with 30 s

intervals between each cycle on ice to obtain a suspension with single bacteria. The final bacterial concentrations of the suspension were determined using a Bürker–Türk counting chamber and fixed at 1×10^9 bacteria mL^{-1} for further experiments.

1.2.3 Biofilm formation

For biofilm formation, bacterial suspensions in PBS (0.5 mL) were put into sterile polystyrene 48-well plates for 2 h at room temperature to allow bacterial adhesion. Next, the suspensions were removed, wells were washed three times with sterile PBS, filled with fresh TSB (1 mL) and incubated for 48 h at 37°C.

1.2.4 Biofilm dispersal

For measuring biofilm dispersal, biofilms grown as described above, were exposed to 0.5 mL of a micellar suspension (200 μg mL^{-1}) in PBS for 120 min after removal of TSB. Note, that micellar concentrations can be easily transformed into dispersant concentrations based on the loading efficiencies of the micelles (see **Table 1**). After exposure, the suspension above the biofilm was removed and the remaining biofilm was exposed to a crystal violet solution (1%, w/v) which was added to each well. After 20 min, the crystal violet solution was removed and wells were gently rinsed three times with PBS. After rinsing, 500 μL of 33% acetic acid was added to resuspend stained biofilms for 15 min and absorbance in each well was read on a microplate reader (Thermo Fisher Scientific Inc., Waltham, USA) at 595 nm as a measure for the amount of biomass in a biofilm. Alternatively, the remaining biofilm after rinsing with PBS was removed by scraping and bacteria were suspended in PBS. Subsequently, the resulting suspension was serially diluted, plated on TSB agar and incubated at 37°C. After 24 h, the number of CFUs in the biofilm remaining after dispersal was enumerated and expressed in log-units per cm^2.

For *S. aureus* ATCC 12600, biofilms on glass slides in 48-well plates grown as described above and after exposure to dispersants, were imaged with scanning electron microscopy (SEM). After exposure to a dispersant, biofilms were fixed with 2.5% glutaraldehyde for 2 h at 4°C, dehydrated with a series of ethanol solutions and gold sprayed for SEM (Quanta 200, FEI, Hillsboro, USA) at an acceleration voltage of 15 kV.

1.2.5 Statistical analysis

Differences between two groups were examined for statistical significance using a two-tailed, paired Student's *t* test. Multivariate parametric data were analyzed using analysis of variance (ANOVA) with Tukey's post hoc test. Statistical significance between groups was accepted at $P < 0.05$.

1.3 Results

Biofilms of all ESKAPE-panel pathogens were grown in TSB at 37°C for 48 h. After growth, biofilms were either stained with crystal violet and absorbances measured (**Figure 1a**) or the number of CFUs were enumerated after scraping off the remaining biofilm and agar plating (**Figure 1b**). All bacterial strains were capable of strong biofilm formation. Although biomass, as derived from absorbance measurements varied somewhat across the different ESKAPE strains, the number of CFUs per cm^2 varied within a very narrow limit. Likely, the variation in biomass reflects that the amount of EPS produced varied across the different strains.

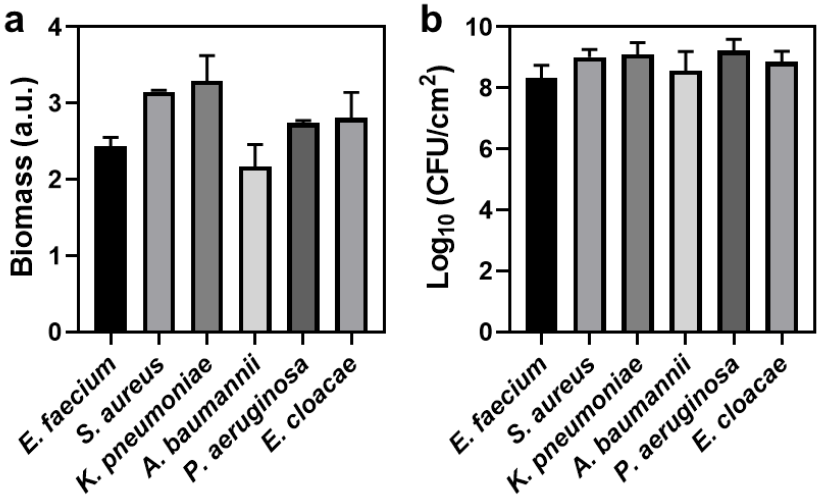

Figure 1. Biofilm formation by different ESKAPE pathogens in TSB after 48 h growth at 37°C. (a) Biomass of biofilms, derived from absorbance (595 nm) measurements after crystal violet staining of biofilms. **(b)** The number of CFUs retrieved per cm^2 substratum surface from biofilms determined by plate counting. All error bars denote standard deviations over three experiments with separately grown bacterial cultures.

In a separate experiment, biofilms were exposed for a standardized period of time (120 min) to a micellar suspension (200 µg mL^{-1}). In order to create a generally applicable Biofilm Dispersal Index (BDI) that can be employed for comparison with other studies in which different exposure times and dispersant concentrations are used, biofilm dispersal must be normalized with respect to time and dispersant concentration. For normalization, we here propose to express exposure time in minutes and normalize to an exposure time of 120 min. For dispersal concentrations, we propose to normalize to a dispersant equivalent concentration of 20 µg mL^{-1}. Dispersant equivalent concentrations can be calculated based on the loading efficiency of a dispersant in a micellar carrier. Accordingly, assuming a linear increase in outcome parameter with time, a normalized change in outcome parameters after exposure to dispersants, can be calculated according to

$$\begin{aligned} &Normalized\ Outcome\ Change \\ &= \\ &\big(Outcome\ (PBS\ control) - Outcome\ (dispersant)\big) \\ &\times \frac{120}{exposure\ time} \times \frac{20}{concentration\ applied} \end{aligned} \tag{1}$$

in which *Outcome (PBS control)* is the experimental outcome for a biofilm that has not been exposed to a dispersant but solely to PBS as a negative control and *Outcome (dispersant)* is the experimental outcome for a biofilm that has been exposed to a dispersant for a specified *exposure time* (min) and *concentration* (µg mL^{-1}). Subsequently, a BDI can be calculated either using biomass obtained from absorbance after crystal violet staining (BDI_{CV}) or using the number of CFU (BDI_{CFU})

$$BDI = \frac{Normalized\ Outcome\ Change}{Outcome\ (PBS\ control)} \tag{2}$$

A resulting BDI equal to 0 represents absence of biofilm dispersal, while 1 indicates full dispersal of a biofilm.

Thus calculated BDIs are summarized in **Table 2** for biofilms grown from different ESKAPE-panel strains, exposed to each of the three micellar dispersants in **Table 1**. As an important conclusion from **Table 2**, biofilm dispersal indices derived from biomass and CFU enumeration are similar ($P > 0.05$, two-tailed, paired Student's t test), contributing to the general

applicability of the proposed index. PEG/PQAE micelles yielded the lowest dispersal index across all six ESKAPE pathogens, while PEG/PAE-DNase micelles yielded the highest dispersal index. Interestingly, PEG/PAE-EGCG micelles previously suggested to yield a balanced dispersal, had intermediate biofilm dispersal indices. PEG/PAE-DNase micelles yielded most dispersal of *S. aureus*, *K. pneumonia*, *A. baumannii* and *P. aeruginosa* biofilms, while performing least against biofilms of *E. faecium* and *E. cloacae*.

Table 2. Biofilm dispersal index (BDI) of ESKAPE pathogens by different micellar dispersants (see **Table 1** for details). Data represent averages with standard deviations over three separate bacterial cultures and separately prepared micelles.

Strains	BDI_{CV}			BDI_{CFU}		
	PEG/ PQAE	PEG/PAE -EGCG	PEG/PAE -DNase	PEG/ PQAE	PEG/PAE -EGCG	PEG/PAE -DNase
E. faecium	0.03±0.01	0.16±0.01	0.21±0.01	0.03±0.01	0.15±0.01	0.24±0.03
S. aureus	0.08±0.01	0.19±0.01	0.61±0.04	0.07±0.01	0.19±0.01	0.58±0.02
K. pneumoniae	0.06±0.01	0.14±0.01	0.50±0.01	0.06±0.01	0.12±0.01	0.53±0.02
A. baumannii	0.06±0.01	0.20±0.01	0.56±0.02	0.07±0.01	0.18±0.01	0.54±0.04
P. aeruginosa	0.08±0.01	0.22±0.01	0.58±0.02	0.08±0.01	0.22±0.01	0.53±0.01
E. cloacae	0.04±0.01	0.12±0.01	0.29±0.01	0.04±0.01	0.14±0.01	0.27±0.01

Imaging of *S. aureus* biofilms prior to dispersal using SEM (**Figure 2**) showed a dense biofilm comprised of large aggregates that are all connected with visible EPS after exposure to PBS. Dispersal by exposure to the different

micellar dispersants becomes evident from absence of visible EPS and increasing occurrence of small aggregates and less dense biofilm with more open structure. Biofilm density as judged from these images, decreases from exposure to PBS as the negative control (BDI = 0), to PEG/PQAE (BDI = 0.08), PEG-PAE-EGCG (BDI = 0.19) to PEG-PAE-DNase micelles (BDI = 0.61), which constitutes a similar ranking as can be obtained based on our proposed dispersal index.

1.4 Discussion

We proposed a quantitative biofilm dispersal index (BDI) for the comparison of biofilm dispersants that can be applied to compare *in vitro* data of various types of dispersants. The index accounts for the use of different concentrations and exposure times applied and as presented here, requires biomass or CFUs of biofilms prior to and remaining after dispersal. BDI_{CV} and BDI_{CFU} values obtained for the same dispersant and the same bacterial strain were statistically identical (**Table 2**) when derived from biomass obtained using crystal violet staining or from CFU enumeration of biofilms after agar plating. Accordingly, it is anticipated that biofilm dispersal evaluated based on e.g. biofilm thickness as an output parameter will also yield identical BDI values.

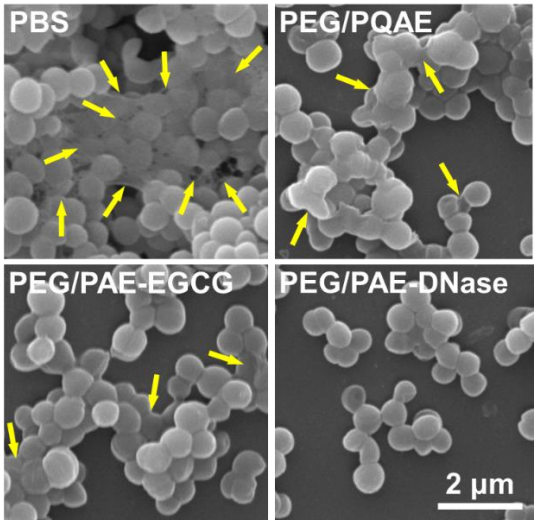

Figure 2. SEM micrographs of *S. aureus* biofilm after exposure to PBS as a negative control and different micellar dispersant systems (see **Table 1** for

details). Note abundant EPS in biofilms exposed to PBS, and absence of large bacterial aggregates after exposure to PEG/PAE-DNase. Yellow arrows indicate EPS.

The biofilm matrix is composed of many EPS components,[20] the production of which is not only strain dependent but also dependent on environmental conditions, including nutrient availability. EPS components produced by all ESKAPE-panel pathogens are summarized in **Table 3**. PEG/PQAE micelles perform poorly across all ESKAPE strains regardless of their matrix composition, probably because breaking biofilm integrity through electrostatic interactions with EPS matrix is slower than enzymatic degradation of DNase or chemical disassembly by EGCG. Proteinaceous amyloid or curli fibers occur in all ESKAPE strains together with other matrix components. This explains why PEG/PAE-EGCG micelles that are especially able to disassemble proteinaceous structures perform better than PEG/PQAE micelles across all ESKAPE-panel strains. eDNA is a relatively long molecule, making it a glue that can act over relatively long distances.[21] It occurs abundantly in all ESKAPE biofilms with the exception of *E. faecium*[22] and *E. cloacae*.[23] Accordingly, PEG/PAE-DNase micelles have high BDI values across all ESKAPE strains with the exception of *E. faecium* and *E. cloacae*. Another possible reason of PEG/PAE-DNase failing to disperse *E. faecium* and *E. cloacae* biofilms is that eDNA exists as a complex bound to other EPS components as e.g. alginate and curli fimbriae during biofilm maturation, making it more difficult to be degraded.[4,24]

Table 3. Major EPS components of ESKAPE-panel pathogens

Strain	**EPS component**	**Growth medium**	**Ref.**
E. faecium	eDNA	TSB with glucose	25
	Secreted antigen A, Surface protein Esp	TSB with glucose, BHI, TSB	25,26
	Alginate	MHB, BHI	22

S. aureus	eDNA	TSB with glucose	27
	PSM amyloid fibers, Surface proteins	BHI, TSB with glucose	28–30
	PNAG	TSB with yeast extract and glucose	31
K. pneumoniae	eDNA	LB	32
	Proteins	LB	32,33
	PNAG	LB with glucose	34
A. baumannii	eDNA	LB	35
	Bap, CSu pili	TSB, LB	36,37
	PNAG, lipooligosaccharide, capsular polysaccharides	TSB	38,39
P. aeruginosa	eDNA	LB, minimal glucose medium	40,41
	Chitin-binding protein, FapC amyloid fibers	M9 broth, LB, MHB	42–44
	Psl, Pel, alginate	LB	45–47
E. cloacae	eDNA	TSB	48,49

	Curli fimbriae	TSB	23,48,50
	Polysaccharide, cellulose	LB	50,51

Abbreviations: MHB, Mueller Hinton broth; BHI, brain heart infusion; TSB, tryptone soy broth; LB, Luria Bertani broth; PSM, phenol soluble modulin; PNAG, Poly-*N*-Acetylglucosamine; Bap, Biofilm-associated protein.

Herewith, our BDI has become an *in vitro* characteristic of a dispersant, expressing the strength of dispersal in quantitative terms and relating with known components of biofilm matrices as produced by different strains.

Finally, we discuss the question whether our BDI has any value for estimating the risk of sepsis when applied *in vivo*. This discussion is hampered by the fact that different strains have a different virulence.[52,53] Also the natural tendency of biofilms towards shedding of its inhabitants and cause sepsis differs amongst different strains.[54–56] Accordingly, the relevance of the BDI can only be discussed with respect to a given bacterial strain. In previous chapters 2 to 4, we evaluated eradication of *S. aureus* biofilms in different murine infection models[15–17] during the development of the micellar dispersants included here, while paying little or no attention to septic symptoms. Opposite to humans, septic symptoms in mice are manifest from a decrease in body temperature. In **Figure 3**, the body temperatures of mice with an intra-abdominal (**Figure 3a**), implant-associated (**Figure 3b**) or pneumonia (**Figure 3c**) infection are summarized (see Supporting Information for details about the *in vivo* biofilm models). Septic symptoms were evident within two days after initiating infection, with hyperthermia being most severe for pneumonia infection, followed by the implant-associated infection. The intra-abdominal infections only yielded a minor temperature drop. The immune system of the mice was not able to cure the infection and septic symptoms persisted at a high level during treatment with a PBS control, leading to the death of mice within 8 days. Treatment with PEG/PAE-DNase micelles possessing the highest BDI of the three micellar dispersants included, made things worse despite the low dose applied: the temperature drop became more severe and all mice died within a shorter period of 6 days. PEG/PQAE and PEG/PAE-EGCG micellar dispersant

clearly aided the immune system in handling sepsis. All mice survived the experiments. Thus, within the limitation of different doses used, it is concluded that in order to reduce the risk of sepsis when using a dispersal strategy (of *S. aureus* infections) that relies on the host immune system for killing of dispersed bacteria, the BDI should be smaller than 0.6. Use of dispersants with higher BDI values should be accompanied by antibiotic administration, which may not always be possible in an era of increasing antibiotic resistance.

1.5 Conclusion

In a brilliant metaphor, Wille and Coenye compared biofilm dispersal strategies with the opening of Pandora's box.[57] In the metaphor, Pandora's box represents the biofilm in which bacteria are safely tucked away. Upon opening the box, disasters are released and disperse over the world, i.e. upon biofilm dispersal dispersed bacteria may cause serious health problems. In this article, a biofilm dispersal index is proposed to assessed how far Pandora's box can be safely opened by different dispersants. The biofilm dispersal index proposed accounts for differences in dispersant concentration and exposure time and has been measured for six different members of the ESKAPE-panel. The BDI value of different micellar dispersants could be explained based on the EPS components they were designed to act against and their prevalence in biofilms of the different ESKAPE strains. For the dispersal of *S. aureus* biofilms in mice, while relying solely on killing of dispersed bacteria by the host immune system, it is suggested that dispersants should be used with a moderate BDI of less than 0.61 in order to prevent septic complications. Although balanced dispersal is also required in humans in order to prevent sepsis, this conclusion may not be extrapolated towards the human clinical situation, because different dispersant doses are involved while sepsis in humans proceeds along different pathways with different symptoms than in rodents.

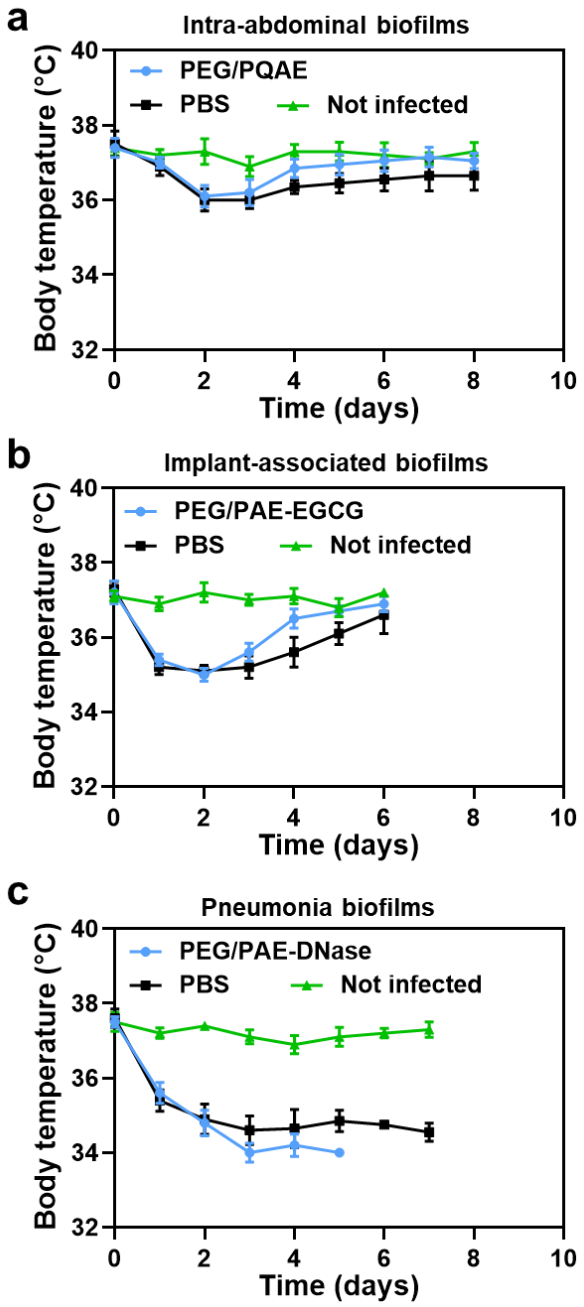

Figure 3. Murine body temperature of mice with different types of *S. aureus* infection as a function of time during treatment of with different micellar dispersant systems. Infections were initiated at day 0 and micelle treatment was initiated at day 2 (see Figure S1 for scheme). (a) Intra-abdominal biofilms treated with PEG/PQAE micelles (200 µg mL^{-1}, 200 µL yielding 2 mg micelles per kg body weight per day). Data are unpublished but were obtained during the course of a previously published study.[15] Error bars denote SDs over six mice. **(b)** Implant-associated biofilms treated with PEG/PAE-EGCG micelles (EGCG equivalent concentration 300 µg mL^{-1}, 500 µL yielding 7.5 mg EGCG per kg body weight per day). Data were taken from Ref.16 with permission from Wiley - VCH GmbH. Error bars denote SDs over six mice. **(c)** Pneumonia biofilms with treatment of PEG/PAE-DNase micelles (DNase equivalent concentration 40 µg mL^{-1}, 50 µL yielding 0.1 mg DNase per kg body weight per day). Data are unpublished but were obtained during the course of a previously published study.[17] Error bars denote SDs over surviving mice. Note that all mice treated with PBS and PEG/PAE-DNase micelles died at day 8 and day 6, respectively.

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Supporting Information

Murine study on abdominal biofilms (Chapter 2, [1])

Four- to five-week-old healthy female BALB/c nude mice were obtained from Vital River Laboratories (Beijing, China) and subjected to experiments in accordance with the Guidelines for Care and Use of Laboratory Animals of Nankai University. Experiments were approved by the Animal Ethics Committee of Nankai University (Tianjin, China). Abdominal imaging windows were implanted under anesthesia. For anesthesia, an aqueous solution of 4 wt% chloral hydrate (8.25 mL kg^{-1}) was injected in the abdominal cavity of each mouse and right flanks were disinfected with 70% (v/v) ethanol. Next, a lateral incision was made through the skin and abdominal wall and a suture was sewed along the edge of the wound. A sterilized window (circular coverslips, 12 mm diameter; Thermo Scientific, Waltham, Massachusetts, USA) in a titanium frame, was placed glass side up in the incision. The skin and the abdominal wall were placed in a slot, prepared in the side of the titanium frame. Finally, sutures were then tightened to secure the window-frame firmly in the animal. After the surgery, the mice were kept at 37°C until fully recovered. After full recovery, usually requiring 2 days, mice were anaesthetized and injected with green-fluorescent *S. aureus* $ATCC12600^{GFP}$ (10^9 bacteria mL^{-1}, 200 µL) underneath the window (but not onto the glass) in order to grow a biofilm.

To monitor the biofilm eradication *in vivo*, mice were first injected in the tail with 200 µL PBS (pH 7.4) or a suspension of zwitterionic PEG/PQAE micelles (PBS at pH 7.4; 200 µg mL^{-1}, yielding 2 mg micelles per kg body weight) in absence of any antibiotics. The consecutive injection scheme was repeated daily till the end of the experimental period (**Figure S1a**). Detailed description of the animal experiments, as well as extensive results on biofilm eradication and tissue analysis have been published previously.[1] Hyperthermia is a symptom of sepsis in mice.[2] Therefore, we here present unpublished data obtained during the above described study of the body temperature of the mice during treatment with PBS and zwitterionic PEG/PQAE micelles.

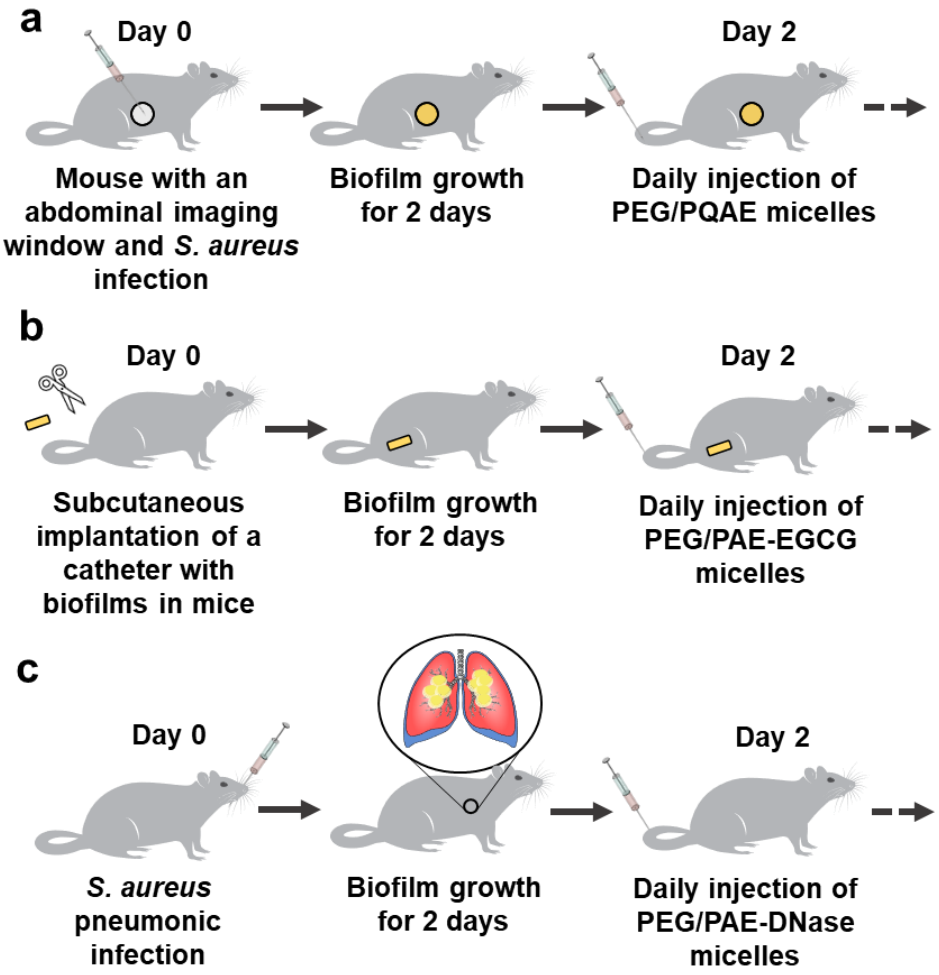

Figure S1. Experimental scheme for staphylococcal biofilm infection and treatment in mice. (a) Intra-abdominal biofilms treated with PEG/PQAE micelles. **(b)** Implant-associated biofilms treated with PEG/PAE-EGCG micelles. **(c)** Pneumonia biofilms with treatment of PEG/PAE-DNase micelles.

Murine study on implant biofilms (Chapter 4,[3])

Four- to five-week-old healthy female BALB/c mice (17 to 20 g) were obtained from HFK Bioscience Co., Ltd. (Beijing, China) and subjected to experiments in accordance with the Guidelines for Care and Use of Laboratory Animals of Nankai University. Experiments were approved (Certificate number: ACSNKU-51933006) by the Animal Ethics Committee

of Nankai University (Tianjin, China). First, *S. aureus* Xen36 biofilms were grown in commercially available, medical grade silicone rubber catheters (BD Vacutainer 178 mm length, 2.38 mm diameter; Becton-Dickinson, USA). Catheters were cut into 10 mm segments and sterilized with 75% ethanol. Catheter segments were incubated in 1 mL TSB containing *S. aureus* Xen36 (3×10^8 bacterial per mL) for 48 h. One day prior to sub-cutaneous implantation of a catheter segment, the hair on the lateral lower back of mice was removed using depilatory cream (VEET, China). For implantation, the skin after depilation was disinfected with ethanolic skin disinfectant and mice were anesthetized with isoflurane. The skin was incised over a length of around 1 cm and the upper skin layer was separated from the abdominal wall to create a pocket using a sterilized tweezer. Catheter segments with biofilms were inserted in the pockets created in the right flanks of the mice, respectively. After suturing to close the wound and disinfection of the wound area, the mice were housed in individually ventilated cages to recover from surgery for 2 days. Then, PEG/PAE-EGCG micelles (at an EGCG equivalent concentration 200 μg mL^{-1}) in absence of an antibiotic and suspended in PBS or PBS only were injected with 500 μL to reach a dose of 7.5 mg EGCG per kg body weight. The injection scheme was repeated daily till the end of the experimental period (**Figure S1b**). Detailed description of the animal experiments, as well as extensive results on biofilm eradication, tissue analysis and body temperature changes during treatment have been published previously.[3] Body temperatures reported in this paper have been taken from our previous paper.[3]

Murine study on pneumonic biofilms (Chapter 3, [4])

Four- to five-week-old healthy female BALB/c nude mice were purchased from HFK Bioscience Co., Ltd. (Beijing, China) and subjected to experiments in accordance with the Guidelines for Care and Use of Laboratory Animals of Nankai University. Experiments were approved by the Animal Ethics Committee of Nankai University (Tianjin, China). A staphylococcal pneumonia was induced by intranasal infection with an *S. aureus* $ATCC12600^{GFP}$ suspension (10^9 bacterial per mL, 50 μL).

To monitor the biofilm eradication *in vivo*, mice were injected intravenously with PBS or PEG/PAE-DNase micelles (at a DNase equivalent concentration 40 μg mL^{-1}, 50 μL to reach a dose of 0.1 mg DNase per kg body weight). The

injection scheme was repeated daily till the end of the experimental period (**Figure S1c**). Detailed description of the animal experiments, as well as extensive results on biofilm eradication and tissue analysis have been published previously.[4] Body temperatures of the mice were recorded, but for the reason given above never published. Therefore, we here present unpublished data obtained during the above described study of the body temperature of the mice during treatment with PBS and PEG/PAE-DNase micelles.

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2 Outlook

Even though using dispersants is a promising new strategy to combat infectious biofilms, there are many aspects to be addressed when moving from bench to bed. Too little is known in biofilm dispersal about the physiological properties of bacteria dispersed out of "Pandora's box". For future research, the following questions need to be answered before entering further clinical translation:

- How many dispersed bacteria can the host immune system handle?
- How does this number depend on the strength and concentration of a dispersant applied, how does it depend on the type of infection?
- Can the immune system be boosted to handle dispersed bacteria or does dispersal imperatively have to be accompanied by antibiotic administration?
- Finally, if it comes to septic side-effects, what is the predictive value of rodent models, responding differently to sepsis than humans?[1]

As we gain a better understanding of infectious biofilms and their dispersal, more rational polymeric micelles can be designed to act as dispersants or dispersant-carriers for specific types of infection, making biofilm dispersal effective in the control of biofilm-related infections, preferably without additional use of antibiotics (which may be a far dream).

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