Synergy of ultrasound microbubbles and vancomycin against *Staphylococcus epidermidis* biofilm

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**Objectives:** Device-associated biofilm infections primarily caused by *Staphylococcus epidermidis* are difficult to treat effectively with conventional antibiotics. The aim of this study was to investigate the anti-biofilm effect of ultrasound-mediated microbubbles combined with vancomycin and to explore underlying mechanisms.

**Methods:** Twenty-four hour *S. epidermidis* biofilms were established in OptiCell™ chambers to facilitate ultrasound exposure. Microbubbles were prepared and diluted to concentrations of 1% and 4% (v/v). Ultrasound was applied for 5 min at 300 kHz and 0.5 W/cm², with a 50% duty cycle. Vancomycin at the peak serum concentration of 32 mg/L was used on preformed biofilms for 24 h. Antibiotic susceptibility tests were conducted on biofilms to confirm the synergy between ultrasound and vancomycin. Biofilms exposed to ultrasound-mediated microbubbles combined with vancomycin were subjected to plate counting and microscopic examinations. A vancomycin penetration test was also performed.

**Results:** Ultrasound and ultrasound-mediated microbubbles both enhanced biofilm susceptibility to vancomycin. Ultrasound-mediated microbubbles without vancomycin could exert a bactericidal effect on biofilms. A bubble dose-dependent bioeffect was also observed. In the presence of vancomycin, biofilms exposed to ultrasound-mediated microbubbles exhibited significantly more micropores and more reduction in biofilm thickness than other treatment groups (*P* < 0.05). The transportation of vancomycin through *S. epidermidis* biofilms was significantly enhanced by ultrasound, and microbubbles could further increase biofilm permeability to vancomycin.

**Conclusions:** Ultrasound-mediated microbubbles may provide an efficient and non-invasive alternative to treat device-related biofilm infections. Future research is needed to optimize ultrasound parameters and microbubble concentrations so that this technology can be both effectively and safely applied in clinical practice.

**Keywords:** low-frequency ultrasound, scanning electron microscopy, antibiotic transport

**Introduction**

Nowadays implantable medical devices are indispensably used in clinical practice. However, device-associated infections are severe complications that can be fatal to patients and present a major financial burden. It has been increasingly recognized that bacterial biofilm development on the device surface is a significant virulent factor in these infections.¹ Among the commonly identified pathogens are coagulase-negative staphylococci (CoNS), primarily *Staphylococcus epidermidis*.² ³ As a skin commensal, *S. epidermidis* can migrate from the skin along the surface of the device into the body, forming a highly organized bacterial community known as biofilm within 24 h.⁴ So far, therapeutic alternatives targeting device-associated biofilm infections are limited to antibiotic therapies, since removal or frequent replacements of the device can sometimes be very difficult, if not impossible, to accomplish.⁵

Because of phenotypic alterations of bacteria sequestered in biofilms, slowed penetration of antibiotics through the biofilm and other unexplained mechanisms, the effect of antibiotics on biofilms is unsatisfactory and a 500- to 1000-fold increase in...
minimal bactericidal levels of many conventional antibiotics has been repeatedly reported.\textsuperscript{6,7} Therefore, other measures to reduce biofilm infections have been extensively explored. Adjunctive chemicals like farnesol and N-acetylcysteine to enhance the activity of antibiotics were demonstrated to be beneficial.\textsuperscript{8,9} However, microbes in biofilms may easily generate resistance to these treatments. On the other hand, alternatives that work via physical mechanisms are promising because microbes may not be able to evade physical interventions with genetic alterations.\textsuperscript{10} During the last decade, a series of studies have shown that low-frequency ultrasound (LFU) combined with antibiotics can significantly enhance the bactericidal activity of antibiotics, probably through the mechanism known as ultrasonic sonoporation.\textsuperscript{5,11–15} However, the reported ultrasound (US) exposure time in one intervention was considerably long, ranging from 2 to 48 h.\textsuperscript{5,11} Therefore, clinical applications of this technology may be hindered.

Microbubbles (MBs) are 1–8 μm particles, consisting of a gas core and a stabilizing shell built up by protein, lipid or biocompatible polymers.\textsuperscript{16} Being used as US contrast agents since the late 1960s, MBs have recently gained increasing attention as a promising carrier for gene and drug delivery.\textsuperscript{17,18} When exposed to US, MBs can serve as nuclei for ultrasonic cavitation and lower the energy threshold for US-induced sonoporation, exhibiting a significant bioeffect within a short time.\textsuperscript{16,17} In earlier experiments, the bioeffects of MBs were mainly targeted to use the cardiovascular system; however, non-cardiac applications of MBs (e.g. intramuscular administration, intra-articular and intraocular injection) have been extensively explored during the last decade.\textsuperscript{19–21} The efficacy of US-mediated MBs demonstrates that this ultrasonic technology can be used for any tissue or organ accessible to US,\textsuperscript{22} suggesting that US-mediated MBs may have broad applicability to acoustically accessible medical devices such as subcutaneous suture anchors, breast implants, joint prostheses and intraocular implants. So far, studies concerning the effect of US-mediated MBs on device-associated biofilms are scarce and the knowledge of underlying mechanisms has not been fully established. The aim of this study was to investigate the antibiofilm effect of MBs combining vancomycin upon short-time US application, with possible mechanisms explored as well.

Materials and methods

**Strain, media and biofilm development**

The bacterial strain used in this study was 	extit{S. epidermidis} ATCC 35984 (RP62A), a notorious clinical isolate reported to produce a robust biofilm after 24 h of incubation.\textsuperscript{5} Bacteria were streaked out on tryptic soy agar (TSA) (Oxoid, Cambridge, UK) from frozen stocks and subsequently inoculated into 5 mL of tryptic soy broth (TSB) (Oxoid) and allowed to grow overnight at 37°C with agitation (180 rpm). Cells were harvested and resuspended in TSB to a turbidity equivalent to that of a 0.5 McFarland standard. To facilitate US exposure, 	extit{S. epidermidis} biofilm was developed in an OptiCell\textsuperscript{TM} chamber (Nunc, Rochester, NY, USA) simulating the static microtitre plate model described previously.\textsuperscript{23} OptiCell\textsuperscript{TM} chambers are formed by two transparent polystyrene membranes of 50 cm\textsuperscript{2} (75 μm in thickness and 2 mm apart) attached to a frame the size of a standard microtitre plate. Ten milliliters of bacteria suspension were injected into the OptiCell\textsuperscript{TM} chamber through a side port using a syringe with an OptiCell\textsuperscript{TM} blunt tip attached. Then, the OptiCell\textsuperscript{TM} was incubated at 37°C for 24 h without disturbance. During that time, bacteria adhered to the bottom membrane and proliferated. Biofilm formation was confirmed by scanning electron microscopy (SEM) as described later.

**MB preparation**

The preparation of MBs has been described previously.\textsuperscript{18,24} We purchased 1,2-distearoyl-sn-glycerol-phosphatidylcholine (DSPC) and 1,2-dipalmitoyl-sn-glycerol-3-phosphatid ethanolamine (DPPE) from Sigma-Aldrich (St Louis, MO, USA). Briefly, 5 mg of DSPC and 2 mg of DPPE were added to 0.5 mL of 10% glycerol in 1.5 mL vials. The vials were incubated at 45°C for 30 min to fully dissolve the chemicals. Then, the headspace of a vial was filled with perfluoropropane gas and the vial was vigorously shaken for 60 s using a dental amalgamator (YJT, Shanghai Medical Apparatuses and Instruments, China). The solution was sterilized by 60Co irradiation and diluted with 0.5 mL PBS, resulting in an MB density of 1.2×10\textsuperscript{9}/mL. The diameters of prepared MBs were 4–6 μm.

**US exposure setup**

After 24 h of incubation, the medium inside the OptiCell\textsuperscript{TM} chamber was replaced with 10 mL of TSB with or without MBs. The OptiCell\textsuperscript{TM} chamber was gently flipped so that MBs were homogeneously distributed throughout the medium. Then, the OptiCell\textsuperscript{TM} was placed horizontally, as shown in Figure 1, to allow MBs to rise to the surface of the biofilm. To minimize acoustic reflection, the tank was filled with degassed water and the OptiCell\textsuperscript{TM} was submerged below the water surface. The membranes of the OptiCell\textsuperscript{TM} chamber were acoustically transparent and caused negligible change in the characteristics of the US.\textsuperscript{25,26} The gene transfer machine (UGT 1025 type) produced by the Institute of Ultrasound Imaging, Chongqing Medical University, Chongqing, China, was used throughout the experiments. Biofilms and MBs were exposed to 300-kHz pulsed waves at an acoustic intensity of 0.5 W/cm\textsuperscript{2}, with a 50% duty cycle. After 5 min of US exposure, the destruction of MBs was confirmed under a light microscope. Then, the OptiCell\textsuperscript{TM} chambers were wiped dry on the outside surfaces and incubated at 37°C for another 24 h prior to assessments.

**Antibiotic susceptibility test**

Vancomycin (Vancocin, Eli Lilly, Japan) was reconstituted in distilled water and filter sterilized. The MIC for planktonic bacteria was determined by standard broth microdilution.\textsuperscript{27} For bacteria grown in biofilms, the biofilm inhibitory concentration (BIC) was evaluated,\textsuperscript{28} with minor modifications. A series of 2-fold diluted vancomycin concentrations starting from 1024 μg/mL were tested on preformed biofilms, with or without US exposure. After 24 h antibiotic challenge, two squares of 1 cm\textsuperscript{2} were cut from the OptiCell\textsuperscript{TM} membrane in the US-exposed region (the area of the US transducer is ~3.8 cm\textsuperscript{2}). Biofilms were rinsed three times with PBS and subsequently stripped from the square surface and dispersed in TSB by sonication at 20 kHz for 5 s (Tomy UD-201, Tokyo, Japan) at room temperature. Our preliminary results indicated that this process did not affect cell culturability. Bacteria were harvested and enumerated by serial dilutions and plating techniques using TSA. BIC was defined as the lowest concentration of antibiotics that resulted in a 1 log\textsubscript{10} difference (90% reduction) in growth compared with that not treated with antibiotics.\textsuperscript{28} All MIC and BIC experiments were repeated on three different occasions.

**Protocol of biofilm treatment**

Vancomycin at the peak serum concentration reached in clinical practice (32 mg/L) was used.\textsuperscript{29,30} Preformed biofilms were treated under eight different conditions: not treated (control), MBs only, US only, US+MBs, vancomycin only, MBs+vancomycin, US+vancomycin and US+MBs+vancomycin.
vancomycin. Biofilms in the control group, MB group, vancomycin group and MB+vancomycin group received sham US exposure with the US apparatus turned off. The treatment of each group was conducted in triplicate. After treatment, three squares of 1 cm² were cut from the OptiCell™ membrane in the US-exposed region using a template. Biofilms were rinsed with PBS and subjected to plate counting, confocal laser scanning microscopy (CLSM) and SEM, respectively. Bacterial counts were expressed as the number of cfu relative to the sample area (cfu/cm²).

**CLSM and biofilm analysis**

Biofilms were stained with SYTO 9 and propidium iodide (PI) according to the instruction of the LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, USA). Bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. After staining for 30 min at room temperature in the dark, biofilms were rinsed with PBS to remove the unattached dyes and observed with a Nikon A1R laser confocal microscope (Nikon, Tokyo, Japan). Signals were recorded using the green (excitation 488 nm, emission 515/30 nm) and red (excitation 568 nm, emission 600/50 nm) channels. ISA software developed by Beyenal et al. was used to estimate biofilm thickness and areal porosity (AP) (defined as the ratio of void area to total area).

**Examination of bacterial morphology by SEM**

Biofilm specimens were fixed for at least 2 h at 4°C with 2% glutaraldehyde and rinsed twice in PBS. Dehydration was performed in progressively increasing concentrations of ethanol to 100%. After being lyophilized, each sample was coated with gold using a gold sputter and observed with an S-3000 Hitachi SEM (Hitachi High-Technologies, Tokyo, Japan).

**Antibiotic penetration**

The model to evaluate the diffusion of vancomycin through S. epidermidis biofilm was similar to that described by Dunne et al., with modifications. Our model involved placing a Millicell hanging cell culture insert (Millipore, Billerica, MA, USA), which contains a 12 μm-thick polystyrene membrane with a pore size of 0.4 μm and a pore density of 1×10⁶/cm², into a culture plate well. As instructed, 4.2 mL of TSB and 2 mL of bacterial inoculum (turbidity equivalent to that of a 0.5 McFarland standard) were added to the basolateral side of the well and the inside of the insert above the membrane, respectively, so that the liquid levels inside and outside the insert were equal. After incubation at 37°C for 24 h, a dense biofilm covered the inner surface of the membrane. Then, the insert was washed with PBS and transferred to another well. Two millilitres of vancomycin at 200 mg/L were loaded onto the inside of the insert and 4.2 mL of TSB were added to the basolateral side of the well, and the model was left in an incubator at 37°C for the next 12 h. Vancomycin had to diffuse through the biofilm and subsequently the membrane to reach the plate well. At predetermined time intervals, samples of 10 μL were aseptically removed from the well to load onto 6 mm discs, and the concentration of vancomycin was determined by a disc diffusion method. To test the effect of US and US+MBs on antibiotic diffusion, the US transducer was placed just below the well via a coupling gel and US was transmitted through the bottom of the plate and subsequently the membrane to reach the biofilm. Prior to US+MB treatment, 2 mL of MB solution was added to the well so that the membrane with preformed biofilm was directly exposed to MBs. After 5 min of US exposure (the US parameters were similar as mentioned earlier), the membrane was gently washed to remove the attached MBs, and vancomycin and TSB were loaded as mentioned above. Control experiments were conducted with sterile insert membranes exposed to vancomycin alone, US+vancomycin and US+MBs+vancomycin. Experiments were performed in triplicate.

A standard relationship between the antibiotic concentration and zone of inhibition (ZOI) was obtained by loading known concentrations of vancomycin onto discs and performing the bioassay. The ZOIs measured were converted to concentrations of vancomycin by using the standard curve.

**Data analysis**

Data from this study were expressed as means±SD. Independent unpaired data were analysed using Student’s t-test. One-way analysis of variance (ANOVA) tests were used for multigroup comparisons. Error bars shown in the figures are standard deviations of duplicate samples from experiments conducted in triplicate. Statistical analyses were
performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). \( P<0.05 \) was considered to be statistically significant.

**Results**

**Antibiotic susceptibilities of biofilms**

The MIC for planktonic *S. epidermidis* was 2 mg/L. Antibiotic susceptibilities of biofilm-grown bacteria treated under different conditions are presented in Table 1. When growing in a biofilm mode, bacteria showed a significant resistance to vancomycin as compared with their planktonic counterparts. Different from the previous study,\(^8\) we used cfu instead of the optical density to assess the growth of bacteria because *S. epidermidis* is able to form microcolonies within 2 h when suitable surfaces are provided,\(^9\) which leads to inaccuracies of microtitre plate readings. The results showed that the BIC for *S. epidermidis* biofilm was reduced by 75% in the presence of US, and a further reduction in BIC was achieved after US+MB treatment. MB dose-dependent synergy was also observed.

**Recovery of bacteria after treatments**

Bacteria recovery in the non-treatment group and the MB-alone-treated group was \( 8.03 \pm 0.04 \log_{10} \text{cfu/cm}^2 \) and \( 8.04 \pm 0.04 \log_{10} \text{cfu/cm}^2 \), respectively (\( P>0.05 \)), indicating that MBs not exposed to US did not have a bactericidal effect on *S. epidermidis* biofilm. The number of bacteria in the US-alone-treated group (\( 8.07 \pm 0.02 \log_{10} \text{cfu/cm}^2 \)) was slightly more than in the controls, and the increase was not significant (\( P>0.05 \)). On the other hand, US+MBs exhibited an antibacterial activity dependent on the dose of MBs. MBs at a 1% concentration did not affect biofilm viability, whereas a 4% concentration of MBs reduced the bacterial counts to \( 83.06\% \pm 1.68\% \) of that in the controls. In Figure 2, vancomycin at the highest achievable serum concentration only reduced the number of biofilm bacteria by half. US application for 5 min significantly enhanced the bactericidal action of vancomycin by reducing the bacterial count to \( 38.98\% \pm 3.95\% \) of that in the controls. The antibacterial effect was more pronounced in the US+MBs+vancomycin group than in US+vancomycin group, and bacteria recovery from biofilms exposed to 4% MBs was significantly less than that from biofilms exposed to 1% MBs (\( 6.10\% \pm 1.31\% \) versus \( 22.29\% \pm 2.99\% \), \( P<0.05 \)).

**Assessment of biofilms by CLSM**

A dense and compact biofilm was observed in the non-treatment and MB-alone groups (Figure 3). Application of US and US+MBs loosened the biofilm structure by creating many micro pores. Changes in biofilm viability as indicated by fluorescent stains were consistent with those in Figure 2. As presented in Figure 4, variations in biofilm thickness were parallel to that in AP. Biofilms exposed to US+MBs showed a greater reduction in thickness, more dead bacteria and more micro pores than those in other groups, whether vancomycin was present or not (\( P<0.05 \)).

**Bacterial morphology**

Different experimental factors were tested upon biofilms. The size and shape of the bacteria exposed to vancomycin and MBs alone were similar to those in the non-treatment group, so only the micrograph of the control biofilm is presented (Figure 5). US intervention did not change the bacterial morphology, whereas US combined with 4% MBs resulted in bacterial damage, and cell debris was interspersed among intact bacterial clusters in the visual field. Bacterial residues in biofilms treated with US+1% MBs were faintly seen. Observed under a higher magnification, the cell debris was a mass of amorphous material mingled with bacteria with disrupted membranes.

**Antibiotic penetration**

We chose a much higher concentration of vancomycin in this part of the experiment due to the detecting limits of the disc diffusion method. Figure 6 delineates the temporal trend of vancomycin transportation through sterile membranes and biofilm-grown membranes. Under a certain treatment condition, the amount of vancomycin in the discs increased rapidly before 8 h, while the amounts in samples at 8, 10 and 12 h did not differ from each other (\( P>0.05 \)), indicating that vancomycin accumulation would reach a plateau at around 8 h. At each time interval, the vancomycin permeability of non-sonicated sterile membranes was similar to that of sonicated ones in the presence or absence of MBs (\( P>0.05 \)). Without biofilms on the insert membrane, the plate well contained \( 61.49\% \pm 3.98\% \) mg/L vancomycin after 12 h, which equals \( 64.57\% \pm 4.19\% \) transportation of the loaded vancomycin. When biofilm was present, the vancomycin contained in the plate well was not detectable at 2 h (ZOI = 6 mm), and the 12 h transportation was \( 41.47\% \pm 1.6\% \) of the load, indicating that the presence of biofilm retarded the penetration of vancomycin (\( P<0.05 \)). US significantly increased biofilm permeability to vancomycin (\( P<0.05 \)), and US+MBs facilitated more vancomycin to penetrate through the biofilms than US did at most time intervals. However, an increase in MB concentration did not correlate with an enhancement in vancomycin transport.

**Discussion**

The present study was motivated by the need for an effective and non-invasive method to treat device-related biofilm infections. The prevalence of methicillin-resistant *S. epidermidis* is reported to be on the rise, and vancomycin is used as the last resort for methicillin-resistant staphylococcal strains.\(^{15,36}\) Our experiments demonstrated that LFU could enhance the antibacterial activity of vancomycin at a clinical serum concentration in a very short period of time. Consistent with a previous study, our

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**Table 1.** BICs for biofilm-grown bacteria treated under different conditions

<table>
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<tr>
<th>Treatment conditions</th>
<th>Vancomycin (mg/L)</th>
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<tbody>
<tr>
<td>Vancomycin alone</td>
<td>512</td>
</tr>
<tr>
<td>US+vancomycin</td>
<td>128</td>
</tr>
<tr>
<td>US+1% MBs+vancomycin</td>
<td>64</td>
</tr>
<tr>
<td>US+4% MBs+vancomycin</td>
<td>16</td>
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results showed that US+MBs+vancomycin could further increase the bactericidal effect of vancomycin and form more micropores within biofilms as compared with US+vancomycin treatment. We also showed that US+MBs in the absence of vancomycin could exert an antibacterial activity, which was also dose dependent. The inconsistency observed could be explained by three factors. First, we established the laboratory model by using the OptiCell™ chamber so that the biofilm and MBs were in close contact. In the previous study, the MB solution was added into wells where biofilms were developed, resulting in a certain amount of liquid between MBs floating above and the biofilm underneath, which might attenuate the effect of US-mediated MBs. Second, the average diameter of MBs in this experiment was larger than that of Sonovue (2.5 μm) used previously. Therefore, the acoustic energy released by MB collapse might be higher herein. Third, although the number of MBs in the 4% concentration approximated that used previously, the cell-to-bubble ratio was much lower herein (~10:1 versus 500:1). In view of the above, a more significant bioeffect induced by US-mediated MBs could be achieved in our study, although the acoustic energy applied in the previous study was much higher (0.08 MHz, 1 W/cm², 10 min).

When bacteria switch from a free-living mode to a biofilm mode, the conventional MICs of antibiotics targeting planktonic bacteria often fail to effectively antagonize biofilm-associated infections. Although the RP62A strain is classified as vancomycin susceptible according to the planktonic MIC, the concentration of vancomycin needed to reduce 90% of bacterial growth in the biofilm was well beyond the highest achievable serum level. Application of US decreased the BIC for S. epidermidis biofilm, and the addition of MBs to the ultrasonic field further reduced the BIC to the range of clinical serum concentrations, indicating that US-mediated MBs hold promise in the treatment of S. epidermidis biofilm infections by acting synergistically with vancomycin. It should be noted that we only applied 5 min of US to the biofilm, which is a much shorter exposure time than previously reported and could be well tolerated in clinical practice.

Ultrasonic enhancement of antibiotic action on biofilms was termed a ‘bioacoustic effect’, where sonoporation induced by ultrasonic cavitation was speculated to play an important role. Despite an abundance of research during the last decade, a detailed mechanistic description of sonoporation and its full range of bioeffects remains largely unexplained. Upon application of US under the cavitation threshold, MBs form by dissolved gas in the liquid medium and oscillate in size without bursting (stable cavitation). The fluctuating shear stress and liquid velocity generated by bubble oscillation could create transient holes on cell membranes (sonoporation) and increase their permeability to extracellular substances while keeping cells
Figure 3. Confocal laser scanning micrographs of biofilms treated under different conditions (×400). Red, non-viable cells; green, viable cells. Bar = 20 µm. More dead bacteria and micropores were observed in biofilms exposed to US combined with MBs. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Figure 4. Comparison of biofilm characteristics under different treatments. (a) AP. (b) Average thickness. Data represent the average of three image stacks collected from randomly selected areas plus 1 SD. Triangles denote a statistically significant difference from the control (P<0.05). Circles denote a statistically significant difference from the vancomycin-alone treatment (P<0.05). Squares denote a statistically significant difference from the US+vancomycin treatment (P<0.05).
The addition of exogenous MBs to liquid medium significantly decreases the cavitation threshold and may trigger a violent process referred to as transient cavitation, during which bubbles collapse and generate high-energy microjets, resulting in an amplified effect of sonoporation as compared with stable cavitation. Sonoporation facilitated the flux of nutrients as well as antibiotics through bacterial membranes, restoring the active growth phase of bacteria while increasing the antibiotic concentration intracellularly, thus bacterial susceptibility to antibiotics was enhanced. Because the ultrasonic energy in our
experiment was below the reported value of the cavitation threshold, we posit that the different bioacoustic effect observed between the US + vancomycin group and the US + MBs + vancomycin group could be attributed to the difference between stable cavitation and transient cavitation. Additionally, during transient cavitation, cells in the vicinity of collapsing bubbles may undergo tremendous membrane perforation and subsequent death while cells distant from bubbles may survive due to ultrasonic energy attenuation across the sectional distance, which could explain our SEM results that US + MBs resulted in bacterial disruption among clusters of intact cells. However, US-mediated interactions between bubbles and bacteria are very complex, and future studies are needed to elucidate the underlying mechanisms.

US can stimulate the transfer of antibiotics not only through bacterial membranes, but also across biofilm extracellular matrix. We demonstrated that the presence of S. epidermidis biofilm significantly retarded the penetration of vancomycin, as previously reported. Possible explanations include physical trapping of vancomycin by the biofilm matrix and chemical reactions between glycopeptides and exopolysaccharides.

Consistent with Carmen et al., our study showed that US can increase the penetration of antibiotics through biofilm. It is speculated that the hydrodynamic changes generated by ultrasonic cavitation may accelerate antibiotic transportation in pores and channels within biofilms so that trapping or reaction sites saturate more quickly, thus a larger amount of antibiotics is retained in discs within the allotted time. Because the acoustic energy released by bubble destruction is much higher than that by bubble oscillation, US + MBs could give a greater boost to vancomycin transportation than US. It is interesting that the change in MB concentration failed to influence vancomycin penetration, probably due to the fact that the number of pores on the insert membrane is fixed and only a certain amount of acoustic energy was allowed through the membrane to reach the biofilm.

There are several significant aspects of this study. First, we successfully established S. epidermidis biofilm in vitro by using the OptiCell chamber, which is routinely applied as a cell culture system. The ultrasonic setup we designed facilitated close contact between MBs and bacteria, improving the accuracy of the investigation of the bioacoustic effect. Second, we revealed results different from previous studies that US combined with MBs
can exert an antibacterial effect in a bubble dose-dependent manner. Third, unlike the conventional method, antibiotic susceptibility tests were performed on biofilms other than planktonic bacteria to show synergy between LFU and antibiotics. Last but not the least, to the best of our knowledge, this is the first demonstration of US-mediated MBs enhancing vancomycin penetration through S. epidermidis biofilm.

Research on the synergistic bactericidal action of LFU is still in its infancy, and more work is required in order to develop this technology. It is recognized that the species of bacteria and the age of the biofilm can influence the efficacy of US-mediated antibiotics. Meanwhile, US parameters including frequency, intensity, duty cycle and the exposure time are considered to be important in producing the bioacoustic effect. Sonoporation efficiency is negatively related to frequency and improves with increased values for intensity, duty cycle and duration. Additionally, the presence of MBs could help to enhance US bioeffects. In order to obtain an ideal bactericidal effect against various biofilms encountered in clinical practice, US parameters need to be optimized based on a thorough evaluation of a myriad of parameter value combinations. On the other hand, animal studies are necessary to investigate the effect of US-mediated MBs with antibiotics on biofilms in vivo, for in vitro experiments fail to account for the role of immune function. Furthermore, ultrasonic sonoporation may act differently on mammalian cells than on prokaryotic cells, probably due to the differences in cell microstructures. Concerns arise that LFU-mediated approaches may exert harm on mammalian cells, probably due to the differences in cell microstructures. In conclusion, US-mediated MBs can act synergistically with antibiotics against biofilms. With the rapid development in MB-based therapeutic approaches, the hope is that this technology may be transferred into clinical practice to treat biofilm infections.

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Transparency declarations
None to declare.

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