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Usnic Acid, a Natural Antimicrobial Agent Able To Inhibit Bacterial Biofilm Formation on Polymer Surfaces

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In modern medicine, artificial devices are used for repair or replacement of damaged parts of the body, delivery of drugs, and monitoring the status of critically ill patients. However, artificial surfaces are often susceptible to colonization by bacteria and fungi. Once microorganisms have adhered to the surface, they can form biofilms, resulting in highly resistant local or systemic infections. At this time, the evidence suggests that (+)-usnic acid, a secondary lichen metabolite, possesses antimicrobial activity against a number of planktonic gram-positive bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*. Since lichens are surface-attached communities that produce antibiotics, including usnic acid, to protect themselves from colonization by other bacteria, we hypothesized that the mode of action of usnic acid may be utilized in the control of medical biofilms. We loaded (+)-usnic acid into modified polyurethane and quantitatively assessed the capacity of (+)-usnic acid to control biofilm formation by either *S. aureus* or *Pseudomonas aeruginosa* under laminar flow conditions by using image analysis. (+)-Usnic acid-loaded polymers did not inhibit the initial attachment of *S. aureus* cells, but killing the attached cells resulted in the inhibition of biofilm. Interestingly, although *P. aeruginosa* biofilms did form on the surface of (+)-usnic acid-loaded polymer, the morphology of the biofilm was altered, possibly indicating that (+)-usnic acid interfered with signaling pathways.

Microorganisms can colonize a wide variety of medical devices, putting patients at risk for local and systemic infectious complications, including local-site infections, catheter-related bloodstream infections, and endocarditis. The Centers for Disease Control and Prevention has estimated that approximately 80,000 central venous catheter-associated bloodstream infections occur in intensive care units each year in the United States (27). Recent studies have shown that a wide range of persistent catheter-related infections may be related to the ability of infectious bacteria and fungi to form biofilms (3, 36). Treatment of device-related infections with conventional antimicrobial agents frequently fails because microorganisms growing in biofilms are much more resistant to antimicrobial agents than planktonic cells are (34). Hypothetical mechanisms for biofilm resistance include the restricted penetration of antibiotics caused by the extracellular polymeric substance matrix (22, 37) and the slow growth of cells in biofilms (8).

In the last decade, several strategies to control biofilm growth on medical devices have been suggested, including the use of topical antimicrobial ointments, minimizing the length of time of catheterization, using catheters provided with a surgically implanted cuff (9), and coating the catheter lumen with antimicrobial agents (1, 6, 23, 24, 25, 29, 30, 32, 39). Existing antimicrobial-loaded catheters suffer from a number of limitations, including the rapid release of the adsorbed antibiotic in the first hours after implantation and, as a result, a relatively short persistence of antibacterial action (7).

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The risk of emerging multidrug-resistant pathogens is continuously growing due to the extensive use of antibiotics both in prophylaxis and long-term therapy. Consequently, catheters coated with antibiotics not used in systematic therapies of bacterial or fungal infections and the use of synergistic antibiotic combinations possessing a broad-spectrum inhibitory activity are desirable (4). One promising candidate is (+)-usnic acid (see Fig. 1). (+)-Usnic acid is 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione, a secondary lichen metabolite that possesses antimicrobial activity against a number of planktonic gram-positive bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium* (19, 33). Many secondary lichen metabolites, including (+)-usnic acid, offer protection to lichen communities against other microorganisms. The antimicrobial agent (+)-usnic acid has activity against gram-positive bacteria and mycobacteria but not against planktonic gram-negative bacteria and fungi (lichens are formed through symbiosis between fungi and algae and/or cyanobacteria). The mechanism of action expressed by (+)-usnic acid is still unknown. However, experimental evidence showed that its antiviral action is due to its ability to inhibit RNA transcription (2). Due to its low solubility in water, the use of (+)-usnic acid has been limited to oral care, topic ointments, and cosmetic formulations. In addition, (+)-usnic acid has been shown to be active against clinical isolates of *E. faecalis* and *E. faecium* and clinical isolates of methicillin- or muporicin-resistant *S. aureus*. However, there is no published data concerning its activity against microbial biofilms at this time. Since the role of (+)-usnic acid is presumably to prevent the invasion of lichens from pathogens and since lichens are surface-associated communities with commonality with other biofilms, we thought that this antibiotic may be

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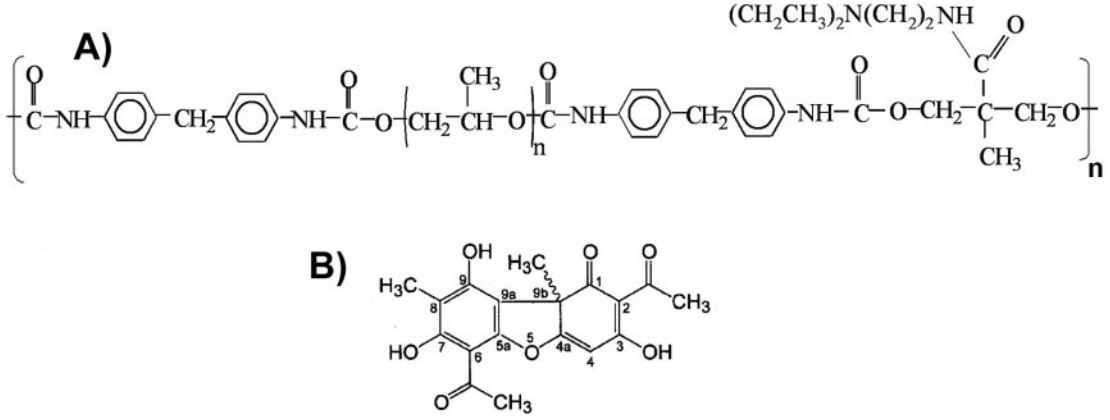


FIG. 1. (A) Repeating *n* units of the polymer (PEUADED) used in the experiments; (B) structural formula of usnic acid.

useful in the control of biofilms formed from gram-positive human pathogens.

To address this issue, we loaded polymers with (+)-usnic acid and compared the effect on biofilm accumulation with control surfaces. As (+)-usnic acid exhibits acidic properties (31), the surface of a polyether urethane acid was specifically modified to introduce basic functional groups (amino groups) able to establish electrostatic interactions with the acidic groups displayed by (+)-usnic acid. The polymers were then incorporated in a flow cell (35), designed for growing biofilm under a wide range of hydrodynamic conditions, and subsequently analyzed using confocal microscopy. The capacity of the (+)-usnic acid to control biofilm formation was assessed using *S. aureus* and the gram-negative pathogen *Pseudomonas aeruginosa*. The influence on initial adhesion was assessed after 30-min or 24-h exposure periods. Effects on longer-term growth were assessed after 3 days. Biofilm structural morphology was statistically analyzed by univariate analysis of spatial parameters from confocal images, which were quantified using COMSTAT software (14).

MATERIALS AND METHODS

Polymer. The polymer used in this study was derived from a polyether urethane acid resulting from the condensation of methylene-bis-phenyl-isocyanate, polypropylene oxide, and dihydroxymethyl-propionic acid (24). The carboxyl groups present in the side chain of this polyurethane were then amidated with 2-diethylaminoethylamine (Fluka), as previously described (6).

The resulting polymer [polyether urethane acid 2-diethylaminoethylamine (PEUADED)] (Fig. 1A) was recovered by precipitation in water, dried under vacuum at 30°C for 3 days, and then subjected to chemical analysis (acidic-alkaline titration and ^{1}H-nuclear magnetic resonance) to determine its degree of amidation.

Preparation of polyurethane disks. PEUADED polymer disks (untreated polymer), 0.5 cm in diameter and approximately 150 μm thick, were obtained by casting 100 μl of a 5% (wt/vol) tetrahydrofuran (Fluka) polymer solution on Teflon plates, followed by solvent evaporation under a vacuum at 30°C. The disks were then sterilized by UV irradiation in a laminar flow hood for 24 h. The efficacy of this procedure was then determined by examining the polymer disk with a light microscope to verify the absence of bacterial growth on disks after incubation at 37°C for 24 h in culture medium (Muller-Hinton broth; Oxoid). No modification of the polymer surface or functional groups was detected when polymers were examined by scanning electron microscopy and X-ray photoelectron spectroscopy.

Loading of the antimicrobial agent in the polymer. The loading of (+)-usnic acid (Aldrich) (Fig. 1B) in PEUADED was performed by preparing an acetone solution containing either (+)-usnic acid (2% [wt/vol]) or PEUADED (5% [wt/vol]). (+)-Usnic acid-loaded PEUADED disks (treated polymer) were obtained by casting of the solution described above on Teflon plates, followed by solvent evaporation under a vacuum at 30°C. A high-affinity antibiotic-polymer interaction was established by combining polyurethane provided with basic tertiary amino groups in the side chain and an antimicrobial agent, (+)-usnic acid, displaying acidic groups (31). ^{1}H-nuclear magnetic resonance analysis and acidic-alkaline titration of the amidation reaction efficacy revealed a content of 75% of amino groups present in the polymer side chain (data not shown).

(+)-Usnic acid release from the polymer. The kinetics of the release of antibiotic from polymer disks in water was determined by measuring the concentration of (+)-usnic acid in the water the disks were immersed in by spectrometry, looking at the absorbance at 270 nm (38) every 24 h for 6 days (the planned experimental period). Because of the limited solubility of usnic acid in water, standard solutions were prepared in a solution of 95% water and 5% acetone. No leaching of (+)-usnic acid was detected over this period (data not shown).

Strains and nutrients. The *S. aureus* strain used in these experiments was Seattle 1945 transformed with a green fluorescent protein (GFP)-producing plasmid to produce 1945^{GFPuvr}. The plasmid was constructed by inserting the promoter for the global regulator *sarA* into the upstream region of a promoterless GFP adapted for maximum expression in *S. aureus*, GFP*uvr*, as previously described (20). The *sarA*-GFP*uvr* sequence is contained in the pSK236 plasmid, which carries a chloramphenicol resistance cassette and a gram-positive bacterial origin of replication (16). The *P. aeruginosa* strain was pMF230, characterized by the presence of a constitutive (GFP)-producing plasmid. The plasmid was constructed to carry a carbenicillin resistance cassette. The media used for *P. aeruginosa* and *S. aureus* biofilm growth were Luria-Bertani (LB) broth and tryptic soy broth (both diluted 1/50), respectively.

Determination of the MIC of (+)-usnic acid. The MICs of (+)-usnic acid for *P. aeruginosa* and *S. aureus* were determined by the microdilution method (26). Because of the limited solubility of (+)-usnic acid in water, acetone was used as the solvent mediator for the antimicrobial agent, after ruling out any intrinsic activity of acetone by plating viability. A 0.2% (wt/vol) solution of (+)-usnic acid was prepared and then diluted to the desired concentrations with LB broth for *P. aeruginosa* and with tryptic soy broth for *S. aureus*. An inoculum of 5×10^5 CFU/ml was used for both species. The MICs of (+)-usnic acid were 32 μg/ml for *S. aureus* 1945^{GFPuvr} and 256 μg/ml for *P. aeruginosa* strain pMF230.

Biofilm flow cell system. To observe the morphology of biofilms, coupons of the treated or untreated polymer (control) were incorporated into a flow cell compatible with time-lapse and scanning confocal laser microscopy (SCLM). The flow cell consisted of a channel machined into a polycarbonate body sealed with a glass coverslip observation window with lumen dimensions of 25.4 mm by 12 mm by 0.127 mm (length by width by height). The flow cell was connected to nutrient and waste carboys by silicone rubber tubing, and nutrients were delivered by peristaltic pump (Masterflex; Cole Parmer, Niles, Ill.). The reactor system and nutrients were sterilized in an autoclave.

The flow cell was inoculated with $\sim 3 \times 10^9$ CFU of either *S. aureus* or *P. aeruginosa* at the logarithmic stage of growth and allowed to attach to the polymer surface for 30 min without shear. Subsequently, the nutrient influent flow rate was applied at 1.0 ml/min. Biofilm growth experiments were conducted at room temperature (21 °C ± 1°C). Flow was laminar with a Reynolds number

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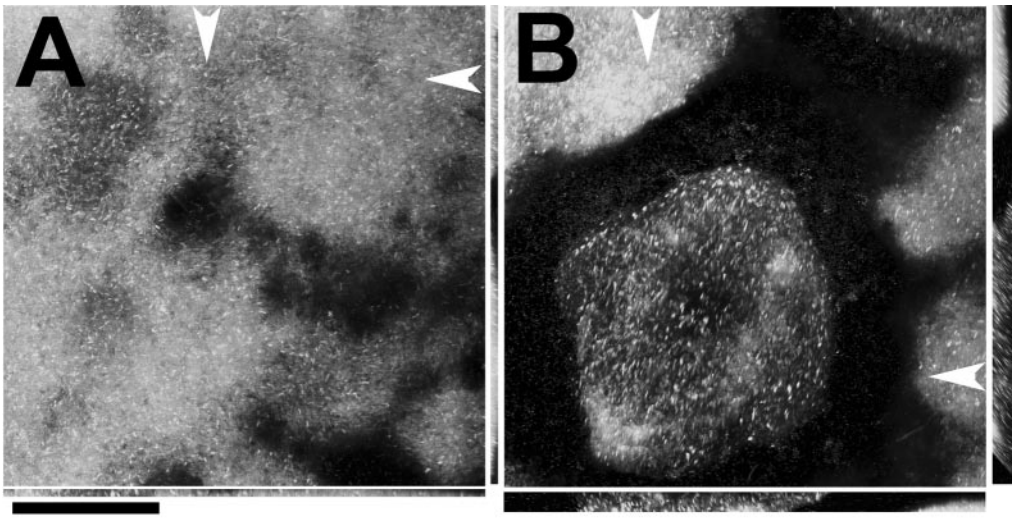


FIG. 2. *P. aeruginosa* biofilm expressing GFP growing on the surfaces of control polyurethane disks (A) and (+)-usnic acid loaded-polyurethane disks (B), 3 days postinoculation in *x-y* plane view (square panel) and in horizontal and vertical cross sections (positions are indicated by the arrowheads) through the biofilm. Bar, 10 μm.

of 3, a maximum flow velocity of 0.01 m/s, and a corresponding wall shear stress of 0.26 Pa.

Microscopy. The developing biofilm was visualized by SCLM using a Leica TCS-NT confocal microscope. Metamorph software (Universal Imaging Corporation) was used for image processing, and Scion Image software (free download at the website www.scioncorp.com) was used for image analysis. Biofilm surface area coverage was measured with digital images taken with a 20× objective. The average biofilm surface area coverage was obtained from a minimum of five measurements taken at randomly positioned locations over the polymer surface. Means ± 1 standard deviation (SD) were reported for three independent experiments. In addition, to evaluate differences between biofilms growing on the treated and control polymers, COMSTAT software (14) was used to quantify biofilm thickness and roughness coefficient from three-dimensional confocal stacks.

Biofilm cell concentration. At the end of each experiment, 0.2-cm^2 polymer segments were collected, sonicated for 5 min, and put in a vortex mixer for 10 s in test tubes with 10 ml of Ringer's solution to remove biofilm cells. This procedure was repeated three times for each sample. A serial dilution was prepared, and six 10-μl aliquots were plated on LB agar plates with carbenicillin (300 μg/ml) for *P. aeruginosa* cells and on tryptic soy agar plates with chloramphenicol (20 μg/ml) for *S. aureus* cells. Plates were then incubated at 37°C, and bacterial CFU were counted after 18 h. The possible (+)-usnic acid release in the Ringer's solution as a consequence of sonication was excluded by checking the absorbance of the solution at 270 nm and comparing it to a standard curve as described above.

Influence of (+)-usnic acid on initial bacterial adhesion. The flow cell was inoculated with ~3×10^9 CFU of *S. aureus* in the logarithmic stage of growth and allowed to attach to the polymer surface either for 30 min or 24 h without shear and in the presence of nutrients. Subsequently, flow was applied for an hour to rinse out cells not adhered to the polymer surface. Bacteria were then stained using a Live/Dead BacLight viability kit (Molecular Probes). This kit differentially stains live cells versus dead cells by using two nucleic acid stains, SYTO 9 and propidium iodide. The SYTO 9 green fluorescent nucleic acid stain labels all cells whether living or dead, while the red fluorescent nucleic acid stain, propidium iodide, enters only cells with damaged membranes. The staining procedure consisted of injecting 3 ml of a staining solution containing both SYTO 9 and propidium iodide in a 1:1 molar ratio into the flow cell. The flow cell was incubated at room temperature for 20 min in the dark. After incubation, residual stain was rinsed off using Ringer's solution. Confocal images were taken using a 488-nm-wavelength excitation laser and a 525- to 550-nm-wavelength band filter for the SYTO 9 stain and a long-pass 645-nm-wavelength filter for the propidium iodide stain.

Statistics. Linear regression analysis and analysis of variance comparisons were performed using MiniTab. Differences were considered significant for P values of <0.05. Data were reported as means ± 1 SD.

RESULTS

Effect of (+)-usnic acid on biofilm formation by *P. aeruginosa*. SCLM image analysis demonstrated that *P. aeruginosa* formed biofilms on the surfaces of both the untreated polymer and the usnic acid-treated polymer 3 days postinoculation (Fig. 2). The biofilm surface area coverage increased significantly after 72 h of growth ($P < 0.01$) (Fig. 3), with the surface coverage reaching 54.6% ± 2.7% on the surface of the control polymer and 28.8% ± 3.3% for the biofilm growing on the surface of the treated polymer.

In contrast, there was no statistically significant difference ($P > 0.1$) between the biofilm cell concentrations, which were 8.9 ± 0.8 $\log_{10}$ CFU/cm^2 for the untreated PEUADED and 8.1 ± 0.8 $\log_{10}$ CFU/cm^2 for the (+)-usnic acid-treated polymer, respectively (Fig. 4). However, (+)-usnic acid did have a

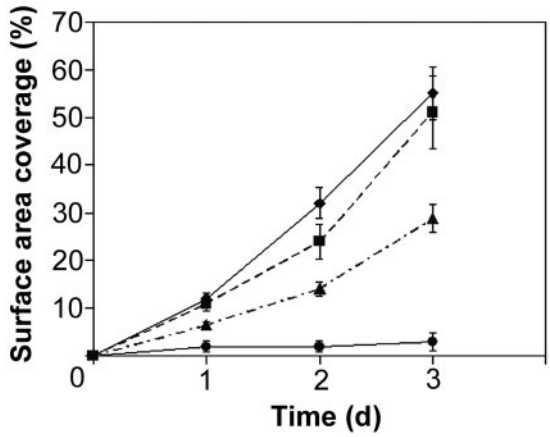


FIG. 3. Surface area covered by biofilm over the 3-day (d) growth period. *P. aeruginosa* growing on control (◆) and (+)-usnic acid-treated polyurethane disks (▲) and *S. aureus* growing on control (■) or treated (●) polyurethane disks.

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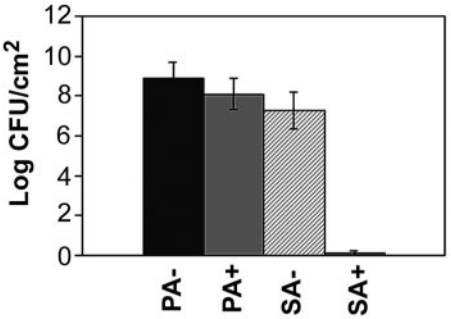


FIG. 4. Biofilm surface cell concentration of *P. aeruginosa* (PA) and *S. aureus* (SA) growing on control (−) and usnic acid-treated (+) polyurethane disks.

marked influence on biofilm morphology, including biofilm thickness, density, and pattern of growth. On the control polymer, *P. aeruginosa* formed a flat biofilm which contained some open patches of exposed substratum (Fig. 2). The biofilm thickness was 14.53 μm with a SD of 26.2% of the mean (Fig. 5). However, on the treated polymer, the biofilm formed a structure consisting of mound- and mushroom-shaped microcolonies separated by interstitial void areas (Fig. 2). The biofilm was significantly thicker ($P < 0.05$) than on the untreated control with a mean thickness of 26.61 μm and a SD of 53.4% of the mean (Fig. 5). The increased SD relative to the mean biofilm thickness reflected the increased roughness and heterogeneity of the biofilm growing on the treated polymer. Analysis with COMSTAT software confirmed this difference and showed that the roughness coefficient (dimensionless) of the biofilm grown on the treated polymer (0.73 ± 0.30) was significantly less ($P < 0.01$) than that of control (1.52 ± 0.30).

(+)-Usnic acid inhibits biofilm formation by *S. aureus*. Three days postinoculation, *S. aureus* formed a biofilm on the surface of the control polymer. The biofilm was patchy and consisted of mushroom-like structures (Fig. 6). However, no evidence of significant biofilm growth was observed on the (+)-usnic acid-loaded polymer, and only a few isolated cells and small groups (up to 10 cells) were observed (Fig. 6). The culturable biofilm cell concentration on the untreated polymer was 7.3 ± 0.9 $\log_{10}$ CFU/cm^2 compared to 0.9 ± 0.1 $\log_{10}$ CFU/cm^2 on the (+)-usnic acid-containing polymer (Fig. 5).

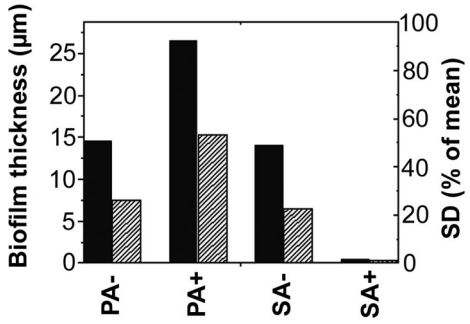


FIG. 5. Mean biofilm thickness (black bars) and standard deviation (hatched bars) expressed as a percentage of the mean for *P. aeruginosa* (PA) and *S. aureus* (SA) growing on control (−) and usnic acid-treated (+) polyurethane disks.

Since the accumulation of *S. aureus* biofilm was almost completely inhibited by the presence of (+)-usnic acid in the polymer, we challenged the (+)-usnic acid-loaded polymer a second time by a second inoculation. Results of SCLM image analysis demonstrated again that *S. aureus* biofilm growth was inhibited for the second 3-day growth period, confirming the ability of (+)-usnic acid to inhibit biofilm growth after multiple challenges.

Effect of (+)-usnic acid on initial attachment. After 30-min and 24-h exposure without shear and in the presence of nutrients, *S. aureus* adhered to the surface of the (+)-usnic acid-treated polymer (Fig. 6) but did not grow to form a mature biofilm. Viability staining showed that the relative proportion of attached live cells decreased from approximately 80% after 30 min to less than 1% after 24 h (Fig. 6).

DISCUSSION

Our experimental results suggest that (+)-usnic acid may be used in the development of antimicrobial catheters to resist biofilm formation by *S. aureus* and possibly other gram-positive organisms. The modified polymer successfully inhibited the formation of *S. aureus* biofilm for a period of up to 6 days under flow conditions with multiple challenges of high concentrations of bacteria.

In contrast, *P. aeruginosa* biofilms did form on the surfaces of both the control and (+)-usnic acid-treated polymer. Over a 3-day period of growth, there was no statistically significant difference in the biofilm cell concentration cultured from the two control and treated polymers. This result is consistent with the MICs for plankton, which showed a higher sensitivity by *S. aureus* (32 μg/ml) relative to *P. aeruginosa* (256 μg/ml). However, (+)-usnic acid did affect the morphology of the *P. aeruginosa* biofilm as quantified by thickness and roughness. On the control polymer, the biofilm was relatively thin and flat with some void areas of exposed substratum, similar to wild-type *P. aeruginosa* PAO1 biofilms grown in our laboratory (28). Interestingly, in the presence of (+)-usnic acid, the biofilm was significantly thicker and rougher and consisting of mushroom- and mound-shaped microcolonies separated by interstitial void areas.

The quorum-sensing (QS) molecule *N*-3-oxo-dodecanoyl homoserine lactone (OdDHL) has been reported to have a similar effect on the structure of *P. aeruginosa* biofilms, although the control was mushroom shaped and an OdDHL mutant was flat (5). A number of studies have shown similar contradictions as discussed elsewhere (28). Also, it has been shown that natural halogenated furanone compounds isolated from the alga *Delisea pulchra* inhibit QS and biofilm development in *P. aeruginosa* biofilms (13, 17), and synthesized furandione analogues of natural compound products have been shown to inhibit QS-dependent swarming motility in *Serratia liquefaciens* (15). In particular, the activity of these compounds in inhibiting QS was influenced by their chemical structure.

We hypothesize that (+)-usnic acid, which belongs to the chemical class of dibenzofurandiones may also influence QS in *P. aeruginosa*. This may be important from a clinical perspective, since natural and synthetic QS inhibitors have been found to attenuate *P. aeruginosa* virulence and increase susceptibility to tobramycin (12). Additionally, (+)-usnic acid has an anti-

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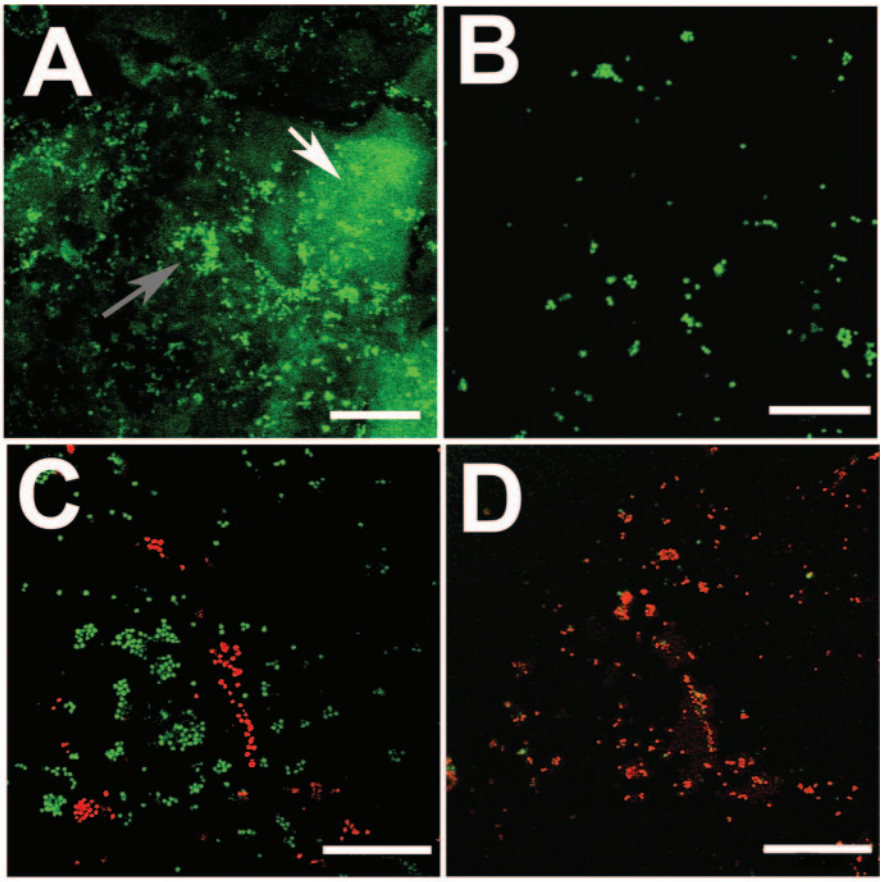


FIG. 6. Confocal images showing *S. aureus* biofilm on the surfaces of the control polyurethane (A) and (+)-usnic acid-loaded polyurethane (B) disks 3 days postinoculation. The grey arrow indicates a cluster of cocci, and the white arrow indicates the biofilm slime matrix. Bar, 55 μm. *S. aureus* adhered to the usnic acid-loaded polyurethane disk after 30 min (C) and 24 h (D) stained with the Live/Dead BacLight viability kit. Bar, 125 μm. The predominance of red cells after 24 h indicated a progressive loss of viability.

inflammatory activity comparable to the known anti-inflammatory agent ibuprofen (40). Consequently, it is likely that the presence of (+)-usnic acid in medical devices will not cause an inflammatory response. There is little data on its toxicity in humans. The only reported adverse effects are local irritation and allergic contact dermatitis. In vitro studies were performed by Lodetti et al. (21) on primary and continuously cultured cells to evaluate the cytotoxic action of usnic acid alone or as a component of oral-care formulations. The compound did not show any sign of cytotoxicity, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red, and modified neutral red cytotoxicity tests. Evidence of toxic effects of usnic acid were also absent both in pharmacokinetics studies (18) and after oral administration (10, 11).

Our results open the possibility of using (+)-usnic acid-modified materials for the construction of biofilm-resistant catheters, in particular intravascular catheters, given its specific activity against gram-positive bacterial strains.

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