Effects of Quaternary Ammonium Silane Coatings on Mixed Fungal and Bacterial Biofilms on Tracheoesophageal Shunt Prostheses

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Biofilm formation is the leading cause for the failure of biomedical prostheses (9, 13), including tracheoesophageal shunt prostheses, used for speech rehabilitation in patients after total laryngectomy because of a malignant laryngeal tumor. Tracheoesophageal shunt prostheses are made of a silicone rubber (SR) tube capped on one end with a one-way valve and are placed between the esophagus and the trachea. The valve of the prosthesis constitutes its esophageal side, and the one-way mechanism allows air to pass from the tracheal side, but fluids passing the esophagus are blocked from entering the trachea. Microorganisms readily form a biofilm on the esophageal side of a prosthesis, which leads to dysfunction of the valve and induces leakage of fluids in the trachea or in the esophageal side of a prosthesis, which leads to dysfunction of the valve and induces leakage of fluids in the trachea or in the esophageal side of the prosthesis.

Two quaternary ammonium silanes (QAS) were used to coat silicone rubber tracheoesophageal shunt prostheses, yielding a positively charged surface. One QAS coating [(trimethoxysilyl)-propyltrimethoxysilane (QM)] was applied through chemical bonding, while the other coating, Biocidal ZF, was sprayed onto the silicone rubber surface. The sprayed coating lost its stability within an hour, while the chemically bonded coating appeared stable. Upon incubation in an artificial throat model, allowing simultaneous adhesion and growth of yeast and bacteria, all coated prostheses showed significant reductions in the numbers of viable yeast (to 12% to 16%) and bacteria (to 27% to 36%) compared with those for silicone rubber controls, as confirmed using confocal laser scanning microscopy after live/dead staining of the biofilms. In situ hybridization with fluorescently labeled oligonucleotide probes showed that yeasts expressed hyphae on the untreated and Biocidal ZF-coated prostheses but not on the QAS-coated prostheses. Whether this result is the positive QAS coating or is due to the reduced number of bacteria is currently unknown. In summary, this is the first report on the inhibitory effects of positively charged coatings on the viability of yeasts and bacteria in mixed biofilms. Although the study initially aimed at reducing voice prosthetic biofilms, its relevance extends to all biomedical and environmental surfaces where mixed biofilms develop and present a problem.

MATERIALS AND METHODS

Tracheoesophageal shunt prostheses. “Ultra Low Resistance” silicone rubber Groningen button tracheoesophageal shunt prostheses were supplied by Médi Instruments and Supplies (Groningen, The Netherlands). The “Ultra Low Resistance” Groningen button tracheoesophageal shunt prosthesis consists of a shaft with two flanges with a semicircular slit of 20° in the hat of the esophageal flange, functioning as a one-way valve. The prosthesis is made of implant-grade silicone rubber.

Silanization and surface characterization. The tracheoesophageal shunt prostheses were cleaned in a 2% RBS 35 detergent solution (Omniclean, Breda, The Netherlands) under simultaneous sonication and thoroughly rinsed in demineralized water, disinfected in 70% ethanol, washed with sterile Millipore-Q water, and dried overnight at 80°C under aseptic conditions. For coating with Biocidal
pH 7.0) at 10 different pressures ranging from 5.10³ to 20.10³ Pa, and each Teflon gasket. Two rectangular platinum electrodes (5.0 by 25.0 mm) were top and bottom plate were constituted by uncoated, QAS-coated, or Biocidal-the streaming potentials by employing a parallel plate flow chamber of which the rubber were prepared at 37°C for 24 h by using 12.1 ml minimum essential (Toxicon Europe NV, Leuven, Belgium). Briefly, extracts of QAS-coated silicone applications of these coatings would not be impeded because of cytotoxicity, a homemade contour monitor using the sessile drop technique.

Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). The optical density of the samples was measured at 562 nm. For the analysis of the extracts, the samples were diluted in PBS, and the absorbance was measured at 562 nm. The protein concentration was calculated using a calibration curve generated with bovine serum albumin (BSA) standards.

For surface characterization, QAS- and Biocidal-coated SR was washed for 30 min in phosphate-buffered saline (PBS), followed by rinsing with demineralized water. The chemical compositions of uncoated, QAS-coated, and Biocidal-coated SR surfaces were determined by X-ray photoelectron spectroscopy (XPS) using an S-Probe spectrometer (Surface Science Instruments, Mountain View, CA) at a spot size of 250 by 1,000 μm, and X-rays were produced using an aluminum anode. A scan of the overall spectrum in the binding energy range of 1 to 1,200 eV at low resolution (pass energy, 150 eV) was recorded, followed by scans over a 20-eV binding energy range at high resolution (pass energy, 50 eV) for C1s (carbon), O1s (oxygen), N1s (nitrogen), Si2p (silicon), and Cl2p (chloride). The area under the peak, after linear background subtraction, was used to calculate the peak intensities after correction with sensitivity factors provided by the manufacturer. The elemental surface compositions were expressed as atoms percent, by setting %C + %O + %N + %Si + %Cl to 100%.

Zeta potentials of the surfaces were derived from the protein dependence of the streaming potentials by employing a parallel plate flow chamber of which the top and bottom plate were constituted by uncoated, QAS-coated, or Biocidal-coated SR sheets fixed on Perspex plates (25 by 76 mm), separated by a 0.2-mm Teflon gasket. Two rectangular platinum electrodes (5.0 by 25.0 mm) were located at both ends of a parallel plate flow chamber (17). Streaming potentials were measured over 1 h in PBS (10 mM potassium phosphate and 150 mM NaCl, pH 7.0) at 10 different pressures ranging from 5.10³ to 20.10³ Pa, and each pressure was applied for 10 s in both directions.

Advancing type water contact angles were measured at room temperature with a homemade contour monitor using the sessile drop technique.

Determination of autoantibodies. In order to ensure that potential future applications of these coatings would not be impeded because of cytotoxicity, silicone rubber sheets with a QAS coating were sent to a reference laboratory (Toxicon Europe NV, Leuven, Belgium). Briefly, extracts of QAS-coated silicone rubber were prepared at 37°C for 24 h by using 12.1 ml minimum essential medium supplemented with serum (MEM complete) for 72.5 cm². Extracts of positive (natural rubber) and negative (bare silicone rubber) controls were also prepared to verify the proper functioning of the test system. The extracts were then tested for autoantibodies using L929 mouse fibroblast cell culture (USP 28-NF 23). QAS-coated silicone rubber may be considered noncytotoxic if none of the cultures exposed show more than mild reactivity. The toxicity measurements were performed at an authorized test institute (Toxicon, Europe NV, Leuven, Belgium) following tests for in vitro cytotoxicity (EN/ISO 10993-5).

Biofilm formation. A modified Robbins device made of stainless steel was used as an artificial throat (Fig. 1) to grow biofilms (14). Each artificial throat was equipped with three Groningen Ultra Low Resistance tracheoepiglottal shunt prostheses: an uncoated, a QAS-coated, and a Biocidal-coated prosthesion. During the experiment, the artificial throat was maintained at a temperature between 36°C and 37°C, as in a laryngectomized patient.

To grow tracheoepiglottal shunt prosthetic biofilms as found in laryngectomized patients, artificial throats were inoculated for 5 h with a combination of bacteria and yeasts previously isolated from explanted Groningen tracheoepiglottal shunt prostheses. This combination comprised Candida tropicalis GB 9/1, Candida albicans GB 13/4A, Staphylococcus aureus GB 2/1, Staphylococcus epidermidis GB 9/6, Streptococcus salivarius GB 24/9, and Rothia dentocariosa GBJ 52/2B and was cultured in a mixture of 30% brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) and 70% defined yeast medium (per liter, 7.5 g glucose, 3.5 g (NH₄)₂SO₄, 1.5 g L-asparagine, 10 mg l-histidine, 20 mg L-methionine, 20 mg L-tryptophan, 1 g KH₂PO₄, 500 mg MgSO₄·7H₂O, 50 mg NaCl, 500 mg CaCl₂·2H₂O, 100 mg extract yield, 500 μg H₂BO₃, 400 μg ZnSO₄·7H₂O, 120 μg Fe(III)Cl₃, 200 μg Na₂MoO₄·2H₂O, 100 μg KI, and 40 μg CuSO₄·5H₂O). After inoculation, a biofilm was allowed to grow on the tracheoepiglottal shunt prostheses for 3 days, by filling the devices with growth medium. From day 4 to day 7, the artificial throats were perfused three times a day with 250 ml PBS. After each perfusion, the biofilms were blown through with compressed air at three different pressures (10, 15, and 20 cm H₂O) to mimic shunt epiglottal speech and to mobilize the test system.

Subsequently, the prostheses were left in the moist environment of the artificial throats. At the end of each day, the devices were filled with growth medium for 30 min and left overnight in the moist environment of the drained artificial throats. The tracheal sides of the prostheses were left in ambient air, a situation similar to that with a stoma (14).

Evaluation of biofilms. On day 8 of an experiment, tracheoepiglottal shunt prostheses were removed from the artificial throats. Biofilm formation on the valve side of the prosthesions was assessed by determining the number of colony-forming yeasts and bacteria (CFU). To this end, biofilms were removed by scraping and sonication and were subsequently serially diluted. After the serial dilutions were plated on MRS (de Man, Rogosa, and Sharpe) agar plates for yeasts and blood agar plates for bacteria, plates were incubated at 37°C in an aerobic incubator for 3 days prior to enumeration. In each experimental run, an untreated silicone rubber prosthesion was inserted as a control, and the number of bacterial and yeast CFU on the epiglottal surface of each prosthesis was determined separately and expressed as a percentage of that for the control to ensure consistency of biofilm formation in each run.

Two artificial throats were used for imaging biofilm formation on the valve side of the prosthesions by confocal laser scanning microscopy (CLSM). Voice prosthesions of one artificial throat were visualized after fluorescence in situ hybridization (FISH) with (RNA-targeted oligonucleotide probes. FISH was performed using a modification of previously described protocols (1, 6, 12). After removal from the artificial throat, the tracheoepiglottal shunt prosthesions underwent the following preparation steps: conservation for 24 h in sterile PBS, fixation for 24 h in a 4%-paraformaldehyde solution at 4°C, and conservation for at least 24 h in ethanol-PBS (1:1) solution. After those preparation steps, the valves of the prosthesions were cut into small cross-sections and attached to glass slides for analysis.

### Table 1. Chemical surface composition, equilibrium water contact angles, and zeta potentials in PBS of untreated, QAS-coated, and Biocidal-coated SR

<table>
<thead>
<tr>
<th>Surface property</th>
<th>Untreated SR</th>
<th>QAS-coated SR</th>
<th>Biocidal-coated SR</th>
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<tbody>
<tr>
<td>%C</td>
<td>49</td>
<td>63</td>
<td>49</td>
</tr>
<tr>
<td>%O</td>
<td>26</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>%Si</td>
<td>25</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>%N</td>
<td>0</td>
<td>2.6</td>
<td>0</td>
</tr>
<tr>
<td>%Cl</td>
<td>0</td>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>Equilibrium water contact angle (°)</td>
<td>108</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-15</td>
<td>+16</td>
<td>+29</td>
</tr>
</tbody>
</table>
TABLE 2. Decreases in percentages of viable bacteria and yeasts isolated from the tracheoesophageal shunt prostheses coated with QAS or Biocidal with respect to untreated prostheses

<table>
<thead>
<tr>
<th>Coating</th>
<th>% of:</th>
<th>Total microorganisms (CFU/cm²)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total bacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Total yeast&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>QAS</td>
<td>36 ± 16</td>
<td>12 ± 9</td>
</tr>
<tr>
<td>Biocidal</td>
<td>27 ± 32</td>
<td>16 ± 15</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results were obtained in four independent experiments and are means ± standard deviations. For both bacteria and yeasts, the number of organisms of the untreated prostheses was set at 100%. Asterisks indicate results significantly different from those for untreated prostheses (P < 0.05 by the Wilcoxon signed-rank test).

<sup>b</sup> The numbers of viable bacterial and yeast CFU on untreated silicone rubber prostheses amounted, respectively, to 2.1 × 10<sup>6</sup> and 3.8 × 10<sup>5</sup> per cm² on the esophageal side of the Low Resistance Groningen Button tracheoesophageal shunt prostheses.

FIG. 2. CLSM images of surfaces of Groningen button tracheoesophageal shunt prostheses after live/dead staining for yeasts and bacteria. Red and green indicate dead and live cells, respectively, of both yeasts and bacteria. Bar, 40 μm. (A) Untreated prosthetic; (B) QAS coating; (C) Biocidal coating.
DISCUSSION

In this study, silicone rubber tracheoesophageal shunt prostheses were coated with QAS and Biocidal ZF coatings to evaluate their inhibitory effects against the development of a mixed fungal and bacterial biofilm on these prostheses. QAS coatings turned out to be stable coatings that were not cytotoxic in a first evaluation due to the stable bound state of the QAS molecules (note that the Biocidal ZF coating is not stable and was therefore not tested for its cytotoxicity). Thus, QAS coatings constitute a new strategy for prevention of microbial colonization of silicone rubber surfaces in voice prostheses, but also for prevention of microbial colonization of medical devices in general, and can be helpful in prevention of resistance of microorganisms to antibiotics or antimycotics.

The surface characteristics of the coated tracheoesophageal shunt prostheses showed that the Biocidal coating was not evident from the XPS data, probably because the Biocidal coating is thinner than the depth of information of XPS (3 to 5 nm). In contrast, water contact angles and the zeta potential, both measured on the outer surface layer with an information depth of several angstroms, clearly demonstrated the presence of the coating. The zeta potential of the Biocidal coating quickly becomes negative, indicating its instability. In this respect it should be noted that the commercially available antimicrobial fluid, Biocidal ZF, is normally used as a coating for incubators, which have to be cleaned and recoated every 14 days. This is in contrast to the chemical bonding established for the QAS coating.

Gottenbos et al. (8) reported that the positively charged QAS coating affects the viability of gram-negative bacteria as well as of gram-positive bacteria in single-strain bacterial biofilms. Here it is demonstrated that such a coating also reduces the number of viable bacteria and yeasts in mixed biofilms, as demonstrated by plate counting and CLSM after live/dead staining. Immobilized QAS molecules are known to interact with cell membranes of adhering bacteria, presumably causing membrane leakage and cell death (8, 11). The mechanisms of action of QAS causing death in yeast is not known, but it seems to impede the formation of hyphae (see Fig. 3). Little is known also about the influence of the bacterial presence on the expression of hyphae in yeast. Consequently, the absence of hyphae could be either a direct effect of the coating or an indirect effect caused by the absence of bacteria on QAS-coated surfaces.

This study demonstrates for the first time that the viability of both yeasts and bacteria in mixed biofilms is affected by positively charged QAS coatings on silicone rubber. Because QAS coatings are nontoxic, clinical application could increase the useful lifetime of tracheoesophageal shunt prostheses by decreasing biofilm formation in vivo, since ingrowth of yeasts is held mainly responsible for deterioration of the silicone rubber in vivo (4). The relevance of the current findings extends, however, to all biomedical and environmental applications where mixed biofilms develop and present a problem.

REFERENCES


