Strategies to decrease biofilm formation on voice prostheses
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Effects of Quaternary Ammonium Silane Coatings on Mixed Fungal and Bacterial, Tracheoesophageal Shunt Prosthetic Biofilms

INTRODUCTION

Biofilm formation is the leading cause for the failure of biomedical prostheses, 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 tube capped on one end with a one-way valve and are placed between the oesophagus and the trachea. The valve of the prosthesis constitutes its oesophageal side and the one-way mechanism allows air to pass from the tracheal side, but fluids passing the oesophagus are blocked from entering the trachea. Micro-organisms readily form a biofilm on the oesophageal side of a prosthesis, which leads to dysfunctioning of the valve and induces leakage of fluids in the trachea or increased air-flow resistance during speech. Consequently, the useful lifetime of a voice prosthesis varies between 3-6 months.

Tracheoesophageal shunt prosthetic biofilms contain a mixture of yeast and bacteria, including Candida species, Staphylococcus and Streptococcus species, as well as Rothia dentocariosa. Especially Candida albicans, C. tropicalis and R. dentocariosa are known to reduce the lifetime of tracheoesophageal shunt prostheses in vivo. Both antimicrobials and antifungal agents have been administered to patients in order to eradicate these biofilms, but the biofilm offers effective protection against antimicrobials, to which planktonic organisms are usually susceptible. Therefore, preventive measures seem a better way to deal with these biofilms. Gottenbos et al. described the antibacterial properties of a positively charged, organosilane quaternary ammonium compound (3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (QAS), coating on silicone rubber against a variety of different bacterial strains in vitro, and moreover demonstrated effectiveness of QAS-coatings against a S. aureus biofilm in vivo. Similarly Biocidal ZF, a commercially available disinfectant, containing quaternary ammonium compounds as the only specified active ingredient, is used for coating incubators and sterile cabinets to protect cell cultures from microbial contamination. Neither the QAS-coating nor Biocidal ZF has ever been investigated for their efficacy against mixed fungal and bacterial biofilms.

The aim of this study was to evaluate the inhibitory effect of QAS- and Biocidal ZF-coatings against the development of a mixed fungal and bacterial biofilm on silicone rubber tracheoesophageal shunt prostheses in vitro, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses.
MATERIALS AND METHODS

Tracheoesophageal shunt prostheses. "Ultra Low Resistance" silicone rubber Groningen button tracheoesophageal shunt prostheses were supplied by Médin 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 210° in the hat of the oesophageal 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 ZF, tracheoesophageal shunt prostheses were sprayed twice with Biocidal ZF (WAK-Chemie Medical GmbH, Steinbach, Germany), fully covering the valve with the Biocidal, followed by exposure to ambient air and drying for 20 h, under aseptic conditions. For QAS-coating, prostheses were oxidized in a glow-discharge reactor (a DC modified Edwards sputter coater S150B) through an argon plasma treatment, done under 5 mbar argon pressure, at a power of 7 W for 5 min, followed by exposure to ambient air. Subsequently, each oxidized voice prosthesis was immediately immersed in 0.5 % QAS (Dow Coming Corporation, Carrollton, Kentucky, USA) in Millipore water. Coated tracheoesophageal shunt prostheses were allowed to react and dry at 80°C for 20 h, under aseptic conditions. Sheets of silicone rubber (SR) (25 x 76 mm) were similarly treated for surface characterization.

For surface characterization, QAS- and Biocidal-coated SR was washed for 30 min in PBS followed by rinsing with demineralized water. The chemical composition of SR, QAS- and Biocidal-coated SR surfaces were determined by X-ray photoelectron spectroscopy (XPS) using an S-Probe spectrometer (Surface Science Instruments, Mountain View CA, USA) at a spot size of 250 x 1000 μm and X-rays were produced using an aluminum anode. A scan of the overall spectrum in the binding energy range of 1-1200 eV at low resolution (pass energy 150 V) was recorded, followed by scans over a 20 eV binding energy range at high resolution (pass energy 50 eV) for C1s, O1s, N1s, Si2p, and Cl2p. 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 in atomic % setting %C + %O + %N + %Si + %Cl to 100%.
Zeta potentials of the surfaces were derived from the pressure dependence of the streaming potentials employing a parallel plate flow chamber of which the top and bottom plate were constituted by SR, QAS- or Biocidal-coated SR sheets fixed on Perspex plates (25 x 76 mm), separated by an 0.2 mm Teflon gasket. Two rectangular platinum electrodes (5.0 x 25.0 mm) were located at both ends of a parallel plate flow chamber.\textsuperscript{12} Streaming potentials were measured during 1 h in PBS (10 mM potassium phosphate and 150 mM NaCl, pH 7.0), at ten different pressures ranging from 37.5 to 150 Torr and each pressure was applied for 10 s in both directions.

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

**Determination of in vitro cytotoxicity (ISO 10993-5).** 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 send to a reference laboratory (Toxicon Europe NV, Leuven, Belgium). In short, 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 cm\textsuperscript{2}. 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 cytotoxicity using L929 mouse fibroblasts cell culture (USP 28-NF 23). QAS coated silicone rubber may be considered non-cytotoxic if none of the cultures exposed show greater than mild reactivity. The toxicity measurements were performed at a notified test institute following tests for in vitro cytotoxicity EN/ISO 10993-5 (Toxicon Europe NV, Leuven, Belgium).

**Biofilm formation.** A modified Robbins device made of stainless steel was used as an artificial throat (see Fig.1) to grow biofilms.\textsuperscript{13} Each artificial throat was equipped with three Groningen Ultra Low Resistance tracheoesophageal shunt prostheses an uncoated, a QAS-coated and a Biocidal-coated prosthesis. During the experiment, the artificial throat was maintained at a temperature between 36°C and 37°C, as in a laryngectomized patient.

To grow tracheoesophageal 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 tracheoesophageal shunt prostheses. This combination comprised *Candida tropicalis* GB 9/9, *Candida albicans* GBJ 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, Great Britain) 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 DL-methionine, 20 mg DL-tryptophane, 1 g KH₂PO₄, 500 mg MgSO₄·7H₂O, 500 mg NaCl, 500 mg CaCl₂·2H₂O, 100 mg yeast extract, 500 μg H₃BO₃, 400 μg ZnSO₄·7H₂O, 120 μg Fe(III)Cl₃, 200 μg Na₂MoO₄·2H₂O, 100 μg KI, 40 μg CuSO₄·5H₂O). After inoculation, a biofilm was allowed to grow on the tracheoesophageal shunt prostheses during three days, by filling the devices with growth medium. From day four till day seven, the artificial throats were perfused three times a day with 250 ml PBS. After each perfusion the prostheses were blown through, with compressed air at three different pressures (10, 15 and 20 cm H₂O), to mimic shunt oesophageal speech and to mobilize the valve 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 during 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, similar to the situation with a stoma.¹³

**Evaluation of biofilms.** On day eight of an experiment, tracheoesophageal shunt prostheses were removed from the artificial throats. Biofilm formation on the valve side of the prosthesis was assessed by determining the number of colony forming yeast and bacteria (CFUs). To this end, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating the serial dilution 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 prosthesis was inserted as a control and the number of bacterial and yeast colony forming units on the oesophageal surface of each prosthesis was determined separately and expressed as a percentage with respect to that 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 prostheses with Confocal Laser Scanning Microscopy (CLSM). Voice prostheses of one artificial throat were visualized after Fluorescence in Situ Hybridization (FISH) with rRNA-targeted oligonucleotide probes. FISH was performed using a modification of previously described protocols. After removal from the artificial throat the tracheoesophageal shunt prostheses 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 an ethanol/PBS (1:1) solution. After those preparation steps the valves of the prostheses were cut into small cross-sections and attached on glass slides. The fixed samples were hybridized (in a closed moist chamber by 50°C) in a volume of 200 µl pre-warmed (50°C) hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, and 0.01% SDS) mixed with 10 µl 100 ng/µl rhodamine labeled EUB-338 probe (5'-GCTGCCTCCCGTAGGAGT-3') for detecting bacteria and 10 µl 100 ng/µl fluorescein-isothyocyanate (FITC) labeled EUK-516 probe (5'-ACCAGACTTGCCCTCC-3') for visualization of the yeasts (Eurogentec, Seraing, Belgium). After 17-19 h the slides were washed in a pre-warmed (50°C) washing buffer for 15 min to remove unbound probes, rinsed with sterile Millipore water, carefully dried with tissues, mounted in Vectashield® medium for fluorescence (Vector Laboratories, Inc. Burlingame CA 94010) and covered with a coverslip. The confocal images were obtained using a 20x oil immersion objective of a CLSM model LEICA TCS SP2 (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Samples from the second artificial throat intended for imaging were subjected to live/dead baclight® (Molecular Probes, Leiden, the Netherlands) staining kits for yeasts and bacteria. Directly after removing the prostheses from the artificial throat, prostheses were stained for 15 min in the dark at room temperature with both the live/dead viability stain containing SYTO 9 dye (3.34 mM) and propidium-iodide (20 mM) and the live/dead Yeast viability kit containing FUN-1 cell stain (10 µM) and Calcofluor White M2R staining (25 µM), but in our experience the two-color fluorescent probe, FUN-1, sufficed for determining yeast viability. Series of about 20 images were made of each biofilm on the prostheses using a 20x water immersion objective and stacked into overlay projections.
Statistical analysis. All experiments in the artificial throats were done quadruple and the quantitative data were statistically compared with respect to the control. A Wilcoxon signed rank test was used for the statistical analysis, accepting $P < 0.05$ as statistically significant.

RESULTS

The surface characteristics of the silicone rubber prior to and after QAS- and Biocidal-coating are summarized in Table 1. The presence of a QAS-coating increases %N and %Cl relative to the uncoated SR, whereas the presence of the Biocidal-coating is not evident from the XPS data, likely because its layer thickness is too thin for detection by XPS. Water contact angles are similar on QAS-coated (100 degrees) and uncoated SR (108 degrees), but the Biocidal-coating creates a more hydrophilic SR surface (40 degrees). Most importantly, the zeta potential of SR, authentically negative, becomes positive after QAS- and Biocidal-coating (16 and 29 mV, respectively). The Biocidal-coating however, becomes negatively charged within one hour whereas the QAS coating stays positively charged.

Table 1. Chemical surface composition, equilibrium water contact angles (degrees) and zeta potentials (mV) in PBS of untreated silicone rubber, Quaternary Ammonium Silanized silicone rubber (QAS-coated SR) and Biocidal-coated SR.

<table>
<thead>
<tr>
<th>Surface property</th>
<th>Untreated</th>
<th>QAS-coated SR</th>
<th>Biocidal-coated SR</th>
</tr>
</thead>
<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</td>
<td>-15 mV</td>
<td>+16 mV</td>
<td>+29 mV</td>
</tr>
</tbody>
</table>
The cytotoxicity of the QAS coated silicone rubber was tested and mild biological reactivity (Grade 2) was observed in the L929 mammalian cells at 48 h, post exposure. The observed cellular response obtained from the positive control extract (Grade 3) and the negative control extract (Grade 0) confirmed the suitability of the test system. The QAS coated silicone rubber can therefore be considered non-cytotoxic.

The percentages of viable yeast and bacteria harvested from QAS- and Biocidal-coated SR are shown in Table 2, as well as the total number of micro-organisms cultures per cm² prosthesis surface. Significantly (p< 0.05) less viable bacteria and yeast are harvested from the QAS coating than from authentic silicone rubber prostheses, while the Biocidal-coating shows a reduction as well, that is however not significantly. These numbers are confirmed qualitatively in Figure 2 showing CLSM images of the prostheses surfaces after live/dead staining. Note some hyphae on the Biocidal-coating are observed, which are absent on the QAS coated prosthesis.

Table 2. Decrease in percentage of viable bacteria and yeast isolated from the tracheoesophageal shunt prostheses coated with QAS or Biocidal with respect to untreated prostheses, as obtained in four independent experiments (± SD). Both for bacteria and yeasts, the number of organisms of the untreated prostheses was set at 100%.

<table>
<thead>
<tr>
<th>Coatings</th>
<th>Percentage of total bacteria*</th>
<th>Percentage of total yeast*</th>
<th>Total micro-organisms (CFU/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100a</td>
<td>100a</td>
<td>2.5 × 10⁶ ± 1.5 × 10⁶</td>
</tr>
<tr>
<td>QAS</td>
<td>36 ± 16</td>
<td>12 ± 9</td>
<td>0.8 × 10⁶ ± 0.3 × 10⁶</td>
</tr>
<tr>
<td>Biocidal</td>
<td>27 ± 32</td>
<td>16 ± 15</td>
<td>0.6 × 10⁶ ± 0.7 × 10⁶</td>
</tr>
</tbody>
</table>

* = the number of viable bacterial and yeast colony forming units on untreated silicone rubber prostheses amounted respectively 2.1 × 10⁶ and 3.8 × 10⁵ per cm² on the oesophageal side of the Low Resistance Groningen Button tracheoesophageal shunt prostheses; * = significantly different from untreated prostheses (Wilcoxon signed rank test, p < 0.05) from the control.

Figure 3 shows CLSM images of cross-sections of biofilms on the valve of the silicone rubber tracheoesophageal shunt prostheses prior to and after coating. As can be seen, the biofilm on the untreated prosthesis is thicker than on the coated ones. The high
magnifications for the control and Biocidal coating show ingrowth of hyphae into the silicone rubber. No hyphae of yeasts in the biofilm on the QAS-coated surface have been observed.

Figure 2. Confocal Laser Scanning Microscopy images (CLSM) of surfaces of Groningen button tracheoesophageal shunt prostheses, after live/dead staining for yeasts and bacteria. The arrows are pointing to hyphae. Bar denotes 40 μm. A) Untreated prosthesis; B) Quaternary Ammonium Silanized Silicone Rubber (QAS) coating; C) Biocidal coating.

Figure 3. Confocal Laser Scanning Microscopy images (CLSM) of cross-sections of tracheoesophageal shunt prosthetic biofilms after in situ hybridization with fluorescence-labelled oligonucleotide probes, making bacterial cells appear red and yeast appear green. The arrows in the magnifications are pointing to hyphae. Bar denotes 40 μm for the overview and 10 μm for the magnification. A) Untreated prosthesis; B) Quaternary Ammonium Silanized Silicone Rubber (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 effect 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 the prevention of microbial colonization of silicone rubber of voice prostheses but also of medical devices in general and can be helpful in prevention of resistance of micro-organisms against 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 (3-5 nm). In contrast, water contact angles and the zeta potential, both measuring on the outer surface layer with an information depth of several Å, clearly demonstrated the presence of the coating. The zeta potential of the Biocidal-coating quickly becomes negative, indicating the instability of the coating. 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 opposite to the chemical bonding established for the QAS-coating.

Gottenbos et al. 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 reduces also the number of viable bacteria and yeast 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. 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). Alternatively, little is also known about the influence of the bacterial presence on the expression of hyphae in yeast. Consequently, the absence of hyphae could either be 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 yeast and bacteria in mixed biofilms is affected by positively charged QAS-coatings on silicone rubber. Since a QAS-coating is non-toxic, clinical application could increase the useful lifetime of tracheoesophageal shunt prostheses by decreasing biofilm formation in vivo, as ingrowth of
yeasts is mainly held responsible for deterioration of the silicone rubber in vivo. The relevance of the current findings extends, however, to all biomedical and environmental applications where mixed biofilms develop and form a problem.
REFERENCES


