Strategies to decrease biofilm formation on voice prostheses
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Biofilm Formation on Voice Prostheses with Decreased Surface Roughness

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INTRODUCTION

In the medical field, metals, polymers, ceramics and composites can be used to make prostheses. Two important issues in the choice of a biomaterial are its mechanical properties and biocompatibility. For voice prostheses silicone rubber has turned out to be the biomaterial of choice. These prostheses are one way valves, connecting the trachea and the esophagus in patients after a total laryngectomy because of a malignant laryngeal tumor.

Silicone rubber is a polymer widely used as a biomaterial in many biomedical implants and devices. In 1955 the first silicone rubber product for medical application, a hydrocephalus shunt, was made. \(^1\) A wide range of medical devices followed through time: catheters, breast implants, contact lenses, cochlear implants etc. \(^2\) The widespread use of silicone rubber is due to its inactivity to the immune system of the human body, good possibility for sterilization, anti-adhesive and stable properties during long term residence in human body and minimum negative tissue response. \(^6\) Another positive characteristic is its ease of processing into different shapes by molding. This became even easier when liquid silicone rubber was introduced in 1979. Liquid silicone rubber has the low viscous appearance of the raw material and can be molded into any shape before curing. Curing takes a couple of days at room temperature (20ºC), but at temperatures above 140ºC the reaction is completed within a few seconds. \(^7\) The process of turning liquid silicone rubber into an end product can be described in three steps: filling, packing/holding and cooling. During the first stage, the hot polymer melt rapidly fills a cold mold reproducing a cavity of the desired product shape. During the packing/holding stage, the pressure is raised and extra material is forced into the mold to compensate for shrinkage due to temperature decreases and the development of crystallinity during solidification. The cooling stage starts at the solidification of a thin section at the cavity entrance. When the solid layer on the mold surface reaches a thickness sufficient to assure the required rigidity, the product is ejected from the mold. \(^8\)

Silicone rubber has a hydrophobicity in the so-called bio-abhesive range, which constitutes another reason for its widespread use as a biomaterial, because infection is common in the clinical application of biomaterial implants, and almost inevitably leads to removal of the implant. \(^5\)-\(^11\) This is also the case for voice prostheses despite the fact that they are made out of bio-abhesive silicone rubber. The positioning of the valve in the unsterile environment of the esophagus causes rapid biofilm formation and malfunctioning of the valve. By consequence, voice prostheses usually fail within 3-6 months. \(^12\)-\(^14\) The biofilm on voice prostheses consists of a mixed biofilm of bacteria and yeast. The variety in oropharyngeal microflora between different patients results in different mixtures of bacteria
and yeast in biofilms on voice prostheses, and probably relates to the large difference in in vivo lifetime between patients. Elving et al. identified a group of microorganisms which are significantly more present in biofilms of prostheses that failed within 4 months compared to prostheses failing after 9 months from the time of insertion.\textsuperscript{15} This group of microorganisms (\textit{Candida tropicalis}, \textit{Candida albicans}, \textit{Rothia dentocariosa}) supplemented with \textit{Staphylococcus aureus}, \textit{Staphylococcus epidermidis} and \textit{Streptococcus salivarius} are comprised in our \textit{in vitro} model to grow biofilm on the voice prostheses.\textsuperscript{16}

An important aspect of a biomaterial surface influencing biofilm formation is its roughness. The surface roughness of an end product is influenced by the material used and the surface of the mold, which is influenced by the polishing of the mold.

The aim of this study is to produce a voice prosthesis, based on the regular Groningen prosthesis, with decreased surface roughness using liquid silicone and smooth molding, that shows reduced biofilm formation in comparison with regular “Ultra Low Resistance” silicone rubber Groningen voice prostheses.

### MATERIALS AND METHODS

**Voice prostheses.** Commercially available “Ultra Low Resistance” silicone rubber Groningen voice prostheses were supplied by Médin Instruments and Supplies (Groningen, The Netherlands). The “Ultra Low Resistance” Groningen voice prosthesis consists of a shaft with two flanges with a semicircular slit of 210\(^\circ\) in the hat of the esophageal flange, functioning as a one-way valve. The prosthesis is made of implant grade silicone rubber MED-4750 NuSil Technology (Carpinteria, United States of America). It is a two-part, high tear strength silicone elastomer, that consists of dimethyl and methylvinyl siloxane copolymers and reinforcing silica. To process prostheses using MED-4750, Part B is softened first on a cooled two-roll mill, and then Part A is softened. An equal portion by weight of softened Part B is added, these components are cross blend until thoroughly mixed. The temperature of the blended material is kept as low as possible to give maximum table life. The curing process takes 10 min at 116\(^\circ\)C, and adjusting the temperature may vary the rate of cure.\textsuperscript{17}

In addition to the commercially available prostheses, a smoother “Ultra Low Resistance” variant was made by a simple injection molding technique utilizing a single cavity hardened steel mold. To produce a smooth mold surface, an extreme level of polishing was applied to the relevant mold surfaces. The highly smooth finish was achieved by polishing using pure diamond particles of varying sizes from 45 \(\mu\text{m}\) down to 1 \(\mu\text{m}\) to create a
final surface roughness of between 0.05-0.2 Ra (µm). Prostheses prepared in this mold were made of silicone rubber MED-4850 (NuSil Technology Carpinteria, United States of America) which is a two part translucent silicone system, with a viscous liquid physical state. To produce the prostheses, the materials were mixed in a 1:1 ratio in a vacuum mixer before being loaded into a 10 ml syringe. The mold surfaces were sprayed with MACSIL silicone release agent (Polymed limited, Cardiff, United Kingdom) and the three parts of the mold were tightly bolted together. The MED-4850 was then injected through the injection port and the mold was placed in a dry oven at 100°C for 2 h to ensure full curing. After curing, the prostheses were left in dry air for cooling.

**Surface characterization.** Elemental surface compositions of the silicone rubber surfaces of the commercially available and newly made voice prostheses were determined by X-ray photoelectron spectroscopy (XPS). The XPS used was an S-Probe spectrometer (Surface Science Instruments, Mountain View CA, USA) with a spot size of 250 x 1000 µm and monochromatic X-rays were produced using an aluminum anode. A scan of the overall spectrum in the binding energy range of 1-1100 eV at low resolution (pass energy 150 V) was recorded for concentration measurements.

The roughness of the silicone rubber surfaces of the commercially available and newly made prostheses were measured using Atomic Force Microscopy (AFM). The AFM used was a Nanoscope III Dimension™ 3100 Digital Instruments, (Santa Barbara, CA, USA) operated in the contact mode using a Si3N4 cantilever tip with a spring constant of 0.06 Nm⁻¹. The valves of the voice prostheses with their concave sides up were put below the cantilever of the AFM to obtain height images in three dimensions at six places per sample, from which its mean roughness ($R_a$) was calculated. $R_a$ indicates the average distance of the roughness profile to the centre plane of the profile.

Advancing type water contact angles were measured at room temperature using the sessile drop technique. Droplets were placed with a syringe and advancing angles were obtained by placing the needle in the water droplet (1-1.5 µl) and carefully moving the sample until the advancing angle appeared to be maximal. The contact angles were calculated from droplet profiles determined with a home-made contour monitor. On the different valves of the voice prostheses five droplets were placed over the surface.

**Biofilm formation** A modified Robbins device made of stainless steel was used as an artificial throat (Fig. 6.1) to grow biofilms. Each artificial throat was equipped with original Groningen Ultra Low Resistance voice prostheses and a smooth variant in order to evaluate
both in the same artificial throat. During the experiment, the artificial throat was maintained at a temperature between 36°C and 37°C, as in a laryngectomized patient.

![Figure 6.1. Schematic presentation of the modified Robbins device, used as an artificial throat, containing three Groningen voice prostheses.](image)

To grow voice 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 voice prostheses. This combination comprised *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9 and *R. 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 H₂BO₃, 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 voice 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 phosphate buffered saline (PBS). 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. The experiments were carried out in quadruple for quantitative biofilm evaluation.
Biofilm evaluation. On day eight of the experiment, voice prostheses were removed from the artificial throats to assess the number of colony forming yeast and bacteria (CFUs) on the valve side of the prostheses. To this end, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating, the serial dilutions 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, original and newly made silicone rubber prostheses were inserted. The number of bacterial and yeast colony forming units on the esophageal surfaces of the newly made prostheses was determined and expressed as a percentage of the mean number of bacterial and yeast colony forming units of the original prostheses.

Statistical analysis. The experiments in the artificial throats were done in quadruple. The quantitative data were statistically compared with respect to the mean of the original voice prostheses, using a Wilcoxon signed rank test and accepting $P < 0.1$ as statistically significant.

RESULTS

The physico-chemical surface characteristics of the original and newly made smooth Ultra Low Groningen voice prostheses are summarized in Table 6.1. The chemical surface characteristics and water contact angles are comparable. The main difference between both prostheses is in their surface roughness. The mean surface roughness of the newly made

<table>
<thead>
<tr>
<th>Surface property</th>
<th>%C</th>
<th>%O</th>
<th>%Si</th>
<th>Water contact angle (degrees)</th>
<th>Surface roughness Ra (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>48</td>
<td>26</td>
<td>26</td>
<td>108</td>
<td>46</td>
</tr>
<tr>
<td>Smooth</td>
<td>48</td>
<td>27</td>
<td>25</td>
<td>112</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 6.1. Elemental surface composition, water contact angles (degrees) and roughness (nm) of original and newly made smooth silicone rubber prostheses.
prosthesis amounts 8 nm, which is significantly smoother than the original prosthesis with a roughness of 46 nm.

The percentages of viable yeast and bacteria harvested from the original and the newly made, smooth Ultra Low Groningen voice prostheses are shown in Table 6.2. Percentages of viable bacteria and yeast were expressed with respect to the mean numbers found on the original prostheses, for which the number of organisms was set at 100%. A reduction of about 40% in both bacterial and yeast prevalence is seen for the smooth prostheses, as compared with the original Groningen button voice prosthesis, despite large differences between runs.

Table 6.2. Percentage of viable bacteria and yeast isolated from original and newly made, smooth voice prostheses. Results were obtained in four independent experiments (± SD); percentage was expressed with respect to the mean of the original prostheses for which the number of bacteria and yeast was set at 100%.

<table>
<thead>
<tr>
<th>Voice prostheses</th>
<th>Percentage of total bacteria</th>
<th>Percentage of total yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smooth Run I</td>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>Smooth Run II</td>
<td>85</td>
<td>102</td>
</tr>
<tr>
<td>Smooth Run III</td>
<td>80</td>
<td>42</td>
</tr>
<tr>
<td>Smooth Run IV</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td><strong>60 ± 31</strong>&lt;sup&gt;−&lt;/sup&gt;</td>
<td><strong>60 ± 29</strong>&lt;sup&gt;(**)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = the number of viable bacterial and yeast colony forming units on original silicone rubber prostheses amounted 2.0 x 10<sup>7</sup> and 8.8 x 10<sup>5</sup> per cm<sup>2</sup>, respectively; <sup>−</sup> = Significantly different from original prostheses (Wilcoxon signed rank test, p < 0.1) from the control; <sup>(**)</sup> = Significantly different from original prostheses (Wilcoxon signed rank test, p < 0.15) from the control.
DISCUSSION

In this study the original Groningen ultra low resistance silicone rubber voice prosthesis was modified through the use of a different mold and liquid silicone rubber filling, which resulted in a decreased surface roughness. Roughness has been shown in literature to be an important aspect in biofilm formation. In dentistry, a roughness above 200 nm is said to facilitate biofilm formation on restorative materials.\textsuperscript{19} Bruinsma et al. described an increase in deposition of \textit{Pseudomonas aeruginosa} when rigid gas permeable contact lenses had a surface roughness exceeding 14 nm.\textsuperscript{20} Verran et al. contributed the effect of a rougher surface to an increase in surface area available for microbial attachment, and the provision of protection against shear off forces in the environment.\textsuperscript{21} Moreover, Whitehead et al. noted that the shape and size of microorganisms may play an important role in relation to the shape and dimension of the surface features in order to establish strong attachment. \textit{S. aureus} (a 1 μm coccal bacterium) was more easily removed from titanium oxide surfaces with a mean surface roughness ($R_a$) of 8.7 nm compared to a 500 nm featured surface, while \textit{P. aeruginosa} (a 1 μm x 3 μm rod shaped bacterium) showed an opposite result.\textsuperscript{22} Surface roughness of voice prostheses however, has never been subject of research before.

In this study, a large decrease in roughness from 46 nm to 8 nm was established on the newly made, smooth voice prostheses compared to the original prostheses. This decrease was created by using a different silicone rubber MED-4850 instead of MED-4750 in combination with another mold. These two silicone rubbers differ significantly in their physical state: rubber-crepe for MED-4750, which is a high consistency elastomer, and viscous liquid for MED-4850, which is a liquid silicone rubber. Because of this difference in physical state, MED-4850 is easier to mix and more fully filling the mold. Moreover, an extreme level of polishing was applied to the surface of the mold, using pure diamond particles of varying sizes from 45 μm down to 1 μm to create the smooth finish of the mold with $R_a$ values in the range of 0.05-0.2 (μm). Standard polish levels for injection mold tools are in a $R_a$ range of 0.4-1 (μm).

In this study a voice prosthesis was produced, based on the regular Groningen prosthesis, with decreased surface roughness using liquid silicone rubber and smooth molding. These smooth prostheses showed reduced biofilm formation in comparison with regular “Ultra Low Resistance” silicone rubber Groningen voice prostheses \textit{in vitro}, without the need of major adjustments in the production process or coating of prostheses after production. The next step is to investigate the effect of this smooth prosthesis on \textit{in vivo} biofilm formation and lifetime in patients.
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