Impact of surface roughness on long-term candida biofilm colonization of prosthetic silicone: A pilot study.
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Abstract
Introduction
Voice prostheses made of silicone are gold standard in voice rehabilitation of laryngectomized patients. However, the in situ device lifetimes are limited due to candida biofilm infestation. Recent in vitro studies report surface roughness as possible promoting factor for initial phases of biofilm formation, but do not evaluate its impact on later colonization processes.

The goal of this study was to investigate the impact of surface roughness of prosthetic silicone on growth kinetics and colonization patterns of a candida biofilm over an observation period of 19 days.

Material and methods
Six platelets of medical grade silicone were roughened to half and incubated in a 2-species in vitro biofilm model of Candida albicans and Streptococcus salivarius over 19 days. Biofilm growth kinetics and local surface distribution of macroscopic biofilm deposits on the platelets were assessed 3 times a week using an image analysis tool (“biofilm mapping”).

Results
On all platelets a stable in vitro biofilm formation could be achieved without any difference in quantity and localization between the roughened and the smooth half. Interestingly, the biofilm mapping technique identified preferred biofilm formation on the upper edges of all test platelets, regardless of the surface finish.

Conclusion
Surface roughness of silicone might enhance initial microbial adhesion and early phases of biofilm formation. Local factors seem to have more impact on long-term biofilm colonization patterns than surface characteristics.

Introduction
In laryngectomized patients voice prostheses have become the gold standard for voice rehabilitation. They are implanted in surgically punctured tracheoesophageal fistulas. The valve mechanism and the oesophageal flange are exposed to a constant afflux of microbes from the upper airways, as well as to saliva and incorporated nutrition. Due to multispecies biofilm formation, especially in the niches around the valve flap, the valve mechanism becomes dysfunctional and oesophageal contents leak through the prosthesis into the lower airways1 (Figure 1). Laryngectomized patients with leaking prostheses are forced to rapidly visit an ENT specialist to avoid risk of severe pneumonia by aspiration. Mixed bacterial and fungal biofilms have gained growing clinical attention because they challenge standard drug therapy schemes due to increased microbial resistance and have been identified to cause severe device associated infections2,3,4,5. Average device lifetimes of standard voice prostheses are reported as 3-6 months6. Interestingly, the frequency of prosthesis replacements does not seem to be associated to lifestyle habits, it varies from few days to years in situ, even in the same patients6. Expensive prostheses with advanced valve designs such as magnetic closure should help to prolong the device lifetime, but remain restricted to patient cases of repeated heavy biofilm infestation7.

In a previous study, differences between biofilm formation on medical grade silicone and polyurethane have been observed on explanted voice prostheses8. The macroscopic and microscopic morphology, the colonization patterns of biofilm deposits and the stability of the extracellular polysaccharide matrix (EPS) were speculated to be associated with the different polymer materials or the different surface roughness. As in vivo studies allow only limited objective assessment of biofilm growth kinetics, an in vitro model to study candida-based biofilms on polymer test surfaces was needed. Therefore a new functional extension of the software Biofilm Cartographer was used to continuously quantify biofilm surface coverage and to assess the local distribution pattern of biofilm deposits in this study (“biofilm mapping”).

The goal of the presented in vitro study was to investigate the long-term impact of surface roughness of silicone on growth kinetics and distribution patterns of a 2-species biofilm.

Materials and Methods
The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Preparation of test surfaces
Six polymer platelets of 8 mm diameter and 3 mm thickness made of medical grade silicone were used as test surfaces to evaluate biofilm colonization. Each platelet was cut in shape, pierced in the centre and mounted on a surgical steel tip as illustrated in figure 2. The vertical positioning of the platelets during incubation avoids accumulation of

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planktonic cells on the surfaces by gravity. The particular shape of a clipped circle prevents tilting and rolling of the platelet during incubation on the orbital shaker. This ensures that the top edge remains in cranial position. The 2 corners of the shape were defined as landmarks for exact overlaying of the images during biofilm mapping. The left half of each platelet was abraded manually with sandpaper (Silicon Carbide Paper FEPA #320, Struers GmbH, Willich, Germany). The difference in surface roughness was confirmed by scanning micrography and is illustrated in figure 3. The wettability was tested before and after surface roughening of one separate platelet using the sessile drop method. Measurement was repeated 3 times and a mean of contact angles of both sides of the platelet was calculated. It showed a mean contact angle of 89.7° for the smooth and 91.3° for the rough surface. All platelets were steam sterilized at 121°C (20 min) and coated with foetal bovine serum (FBS, PAA Laboratories GmbH, Cölbe, Germany) over night at 37°C before primary microbial inoculation.

Preparation of microbial suspensions
The test organisms Candida albicans (C. albicans) and Streptococcus salivarius (S. salivarius) were isolated from an explanted Provox®2 voice prosthesis of a patient with frequent valve dysfunction due to biofilm infestation. Harvesting was performed by vortexing the explanted prosthesis in 5 ml of phosphate buffered solution (PBS). From this suspension, C. albicans was isolated using ChromAgar (Mast Diagnostic, United Kingdom) and identified using the API20C-AUX system (bioMerieux-Vitek, Marcy l’Étoile, France). S. salivarius was identified using the API Strep 20 (bioMerieux-Vitek, Marcy l’Étoile, France). Pre-testing of the strains in a well plate assay with RPMI 1640 Medium showed increased biofilm formation in 48 hours observation time for the 2 combined strains. Microbial suspensions were prepared as followed: C. albicans was...
incubated on Sabouraud agar (Becton Dickinson, Franklin Lakes, USA) for 48 hours at 37°C. A loopful of C. albicans colonies was inoculated in 20 ml of yeast peptone glucose (YPG) medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone 2% [wt/vol] glucose, Oxoid LTD, Basingstoke, Hampshire, England) and preincubated overnight in an orbital shaker at 100 rpm and 37°C.

C. albicans cells were then separated from the preincubation medium by centrifugation (3500 rpm, 5 min, 8°C) and S. salivarius was harvested from the agar. Both species were suspended in sterile phosphate buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride [pH 7.4], Morphisto, Frankfurt am Main, Germany). Cellular density of the prepared suspension was 1×10⁶ colony forming units per milliliter (cfu/ml). S. salivarius was suspended in 0.9% PBS to 10⁸ cfu/ml. The platelets placed in well plates, inoculated with 1.5 ml of the mixed microbial suspension and incubated for 4 hours at 37°C. The microbial suspension was then replaced by tryptone soya broth (TSB, Tryptone Soya Broth, Oxoid, Hampshire, England) and incubated overnight. This protocol was continued daily for the next 5 days in order to support streptococcal growth. After 5 days the medium was switched to RPMI 1640 (Sigma-Aldrich, Chemical Co, St. Louis, Montana, USA) and buffered with morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich, Chemical Co, St. Louis, Montana, USA) and refreshed daily until the end of the study.

In vitro generation of 2-species biofilm
The applied in vitro model to generate candida biofilm on polymer surfaces is based on incubation cycles including microbial reseeding with planktonic cell forms and was described previously by Leonhard et al.⁹. In this study, the reseeding was performed daily on weekdays. The study was conducted over an observation period of 19 days during which the platelets were photographed 3 times a week (Monday - Wednesday - Friday) top down under standard lighting conditions with a digital microscopy camera (dnt® DigiMicro, Dietzenbach, Germany). Image analysis was performed using the Biofilm Cartographer Software (Version 2.63), which enables the examiner to calculate the percental surface biofilm cover and document the local distribution of biofilm deposits on the platelets. The

![Figure 3: Scanning electron micrography of a silicone test platelet (A). Left half shows increased surface roughness after abrasion with sandpaper (B), right half of the platelet shows smooth surface structure (C).](image)

![Figure 4: Image processing of the macroscopic biofilm covers: biofilm deposits are marked on the images every 2 days. Images are merged into a color-coded image to visualize distribution patterns and frequency of biofilm coverage.](image)
Table 1: Assessment of biofilm surface cover as percentage (%) of total platelet surface

<table>
<thead>
<tr>
<th>Day of assessment</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>19</th>
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<tr>
<td>Biofilm surface cover (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface 1</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Surface 2</td>
<td>0</td>
<td>11</td>
<td>5</td>
<td>9</td>
<td>17</td>
<td>10</td>
<td>16</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Surface 3</td>
<td>0</td>
<td>18</td>
<td>12</td>
<td>15</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>Surface 4</td>
<td>0</td>
<td>18</td>
<td>6</td>
<td>23</td>
<td>15</td>
<td>11</td>
<td>31</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Surface 5</td>
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<td>19</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>5</td>
<td>11</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Surface 6</td>
<td>0</td>
<td>11</td>
<td>7</td>
<td>18</td>
<td>19</td>
<td>9</td>
<td>24</td>
<td>30</td>
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</table>

Results

On the platelets, the 2-species biofilms evolved over the observation period of 19 days. During this time, the size of biofilm covered areas varied between 3 % to 30 % of the total platelet surface (mean 13.75 %, ±6.75, Table 1). Phases of biofilm detachment were observed on days 5, 12 and 19, but a slow overall increase in macroscopic biofilm colonization was detected (Figure 5). In order to compare biofilm formation on the rough and the smooth halves of the platelets, the mean percental biofilm surface coverage over the observation period was calculated separately for each platelet half (Figure 6). No increase of biofilm growth or special affinity for the rough surfaces could be confirmed (p=0.595). Three platelets showed more biofilm formation on the rough and 3 on the smooth half. However, biofilm mapping revealed that the top edges of all platelets, which were not submerged constantly in agar, showed increased microbial colonization, whereas the bottom edges remained macroscopically free.

Discussion

Multi-species biofilms on prostheses have gained clinical attention because they have been identified to cause medical device associated infections and develop resistance to standard drug therapy schemes. Device associated biofilms might function as intra-corporeal reservoirs of pathogens, which can cause local infections and even disseminate into further body compartments causing septic conditions with high morbidity rates. Beside the risk of implant surrounding tissue inflammation, the prosthetic material itself can be infested and damaged by microbes, which can lead to loss of function. Many medical devices, such as intravenous catheters, heart valves, urinary catheters, tracheostomy tubes and voice prostheses are made of biocompatible polymers (silicone, polyurethane, polyvinylchloride) with different surface structures and material characteristics. Depending on the function, biofilm colonization and prosthetic dysfunction can impose a crucial risk on the patient. The problem of biofilm associated prosthetic dysfunction is well illustrated by voice prostheses, which have become standard in voice rehabilitation after laryngectomy. From the moment of implantation, the implant surface is covered with an aqueous salivary conditioning film composed of proteins, which facilitates further colonization by combinations of microbiota. Candida species are regularly identified fungal colonizers of prosthetic polymers and are mainly encountered as parts of multi species biofilms in vivo. These microbial communities show highly evolved colonization skills. The production of an extracellular polysaccharide matrix shields and organizes the variety of co-existing species in drug resistant biofilm deposits. Over weeks, the biofilm deposits infest the valve structures and cause leakage of oesophageal contents into the lower airways.

Optimization of polymer materials towards biofilm resistance is a strategy of utmost interest for further prosthesis development. Physical properties of polymers, such as surface roughness and hydrophobicity have been controversially discussed to play key roles in initial steps of microbial adhesion and the presence of saliva, serum, proteins, rhamnolipids, adhesins can neutralize or even invert anti-adhesive effects of artificial surfaces. However studies differ greatly in materials (soft denture liners, acrylic resins) and microbial species employed in the protocols.

![Mean biofilm coverage of platelet surfaces](image)

**Figure 5:** Growth kinetics of the 2-species biofilm over 19 days on all test platelets. The total surface coverage slowly increases over the observation period. Phases of biofilm detachment can be seen on days 5, 12 and 19.
Irregular surface roughness tends to be considered to promote microbial colonization because a larger surface is offered for adhesion. Surprisingly, most of these in vitro investigations have been conducted with observation times of only hours to days, which seem hardly comparable to documented months of in situ device life time. In vivo studies on biofilm formation are limited by the difficult access to the prostheses and an uncontrollable number of in vivo parameters. The devices can only be examined thoroughly at implantation and explantation, while the actual biofilm growth kinetics remains unexamined. These facts contribute to the lack of documentation on long-term effects of biofilm formation on medical devices.

In the presented study, the technique of “biofilm mapping” is introduced to evaluate biofilm growth kinetics and colonizing patterns on medical grade silicone of rough and smooth surface structures. Localization and two-dimensional surface spread are captured without alteration of the living biofilm over time by images, which are digitally merged into overlays and color-coded depending on the frequency of locally assessed biofilm deposits. With this method, no impact of surface structure of medical grade silicone could be found for long-term biofilm formation. Surface colonization was equally distributed on the roughened and on the smooth areas of the platelets. Although initial microbial adhesion might be promoted by rough surfaces, it does not seem to have an impact on later phases of biofilm formation. This shows, that the predictive value of short-term evaluations, such as up to 72 hours, do not necessarily resemble the later biofilm formation process and emphasizes the importance of long-term testing periods for evaluation of biofilm resistance of materials or surfaces. It is also remarkable, that the top platelet edges, which were not constantly submerged in the liquid medium, were preferably colonized with biofilm deposits on all platelets, whereas the bottom edges showed almost no signs of biofilm colonization. Two effects might explain this: physical particles in turbulent liquids accumulate in surface layers of the fluid medium and are therefore more likely to adhere to surfaces there. This physical effect is likely to apply to planktonic cells too. Further, inside the well, the top region of the platelet is characterized by turbulent changes of fluid and air contact, which might result in higher degrees of mechanical sheer forces and oxygen levels. It might be possible, that a similar effect takes place in vitro in a microscopic scale: due to the turbulences, planktonic cells accumulate at the top edge, the sheer forces select the “fittest” cells for adhesion and improve perfusion and stability of the biofilm matrix, so that an optimum habitat of nutrition and metabolism is created. The results show that local conditions possibly have to be considered to have greater impact on long-term formation of local biofilm deposits than the surface structures of materials.

Conclusion

The novel technique of biofilm mapping could document the microbial colonization and distribution pattern of a 2-species biofilm on medical grade silicone. Local factors proved to have more impact on long-term biofilm formation than surface condition and should be taken into account in investigations on material resistance to biofilms. Also, the predictive values of short-term examinations on microbial material adhesion should be questioned for prostheses, which have to endure for months.

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References


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All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.