RESEARCH ARTICLE

Bacterial biofilm formation and treatment in soft tissue fillers

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The authors have developed a mouse model to evaluate tissue filler gels for their potential for sustaining bacterial biofilm infections. The model also has potential for assessment of treatment strategies.

Keywords
biofilm; prophylactic treatment; soft tissue fillers; hyaluronic acid gel; calcium hydroxyl apatite microspheres; polyacrylamide hydrogel.

Abstract
Injection of soft tissue fillers plays an important role in facial reconstruction and esthetic treatments such as cosmetic surgery for lip augmentation and wrinkle smoothening. Adverse events are an increasing problem, and recently, it has been suggested that bacteria are the cause of a vast fraction of these. We developed a novel mouse model and evaluated hyaluronic acid gel, calcium hydroxyl apatite microspheres, and polyacrylamide hydrogel for their potential for sustaining bacterial infections and their possible treatments. We were able to culture Pseudomonas aeruginosa, Staphylococcus epidermidis, and Propionibacterium acnes in all three gels. When contaminated gels were left for 7 days in a mouse model, we found sustainment of bacterial infection with the permanent gel, less with the semi-permanent gel, and no growth within the temporary gel. Evaluation of treatment strategies showed that once the bacteria had settled (into biofilms) within the gels, even successive treatments with high concentrations of relevant antibiotics were not effective. Our data substantiate bacteria as a cause of adverse reactions reported when using tissue fillers, and the sustainability of these infections appears to depend on longevity of the gel. Most importantly, the infections are resistant to antibiotics once established but can be prevented using prophylactic antibiotics.

Introduction
Injectable dermal gel fillers can rebuild volume loss and eliminate structural defects in the skin (Narins & Bowman, 2005). These fillers are injected into the dermal or subdermal tissue to give the skin a more voluminous or smooth expression (Klein & Elson, 2000; de Maio, 2004). The gels can be classified according to their biodegradability into temporary, semi-permanent, or permanent fillers, the latter referring to the lack of degradation of the gel inside the body over time.

Biologic materials such as collagen and hyaluronic acid (HA) gels are easily degraded hydrogels known as temporary fillers. They usually endure for < 12 months, and regular applications are necessary to sustain the filling effect (Rohrich et al., 2003).

Fillers consisting of an easily degradable carrier gel and slowly degradable microparticles are classified as semi-permanent fillers. The filling effect stems partially from the degrading poly-L-lactic acid, calcium hydroxyapatite (CaHA), or dextran microparticles and partially from the growing tissue response, which these microparticles elicit (Morhenn et al., 2002). The tissue response will give permanent filler effect, but as the microparticles are slowly degraded over 1–7 years in the tissue (Barber & Dockery, 2006; Tzikas, 2008), the overall filling effect is reduced accordingly.

Permanent fillers such as liquid injectable silicone oil, polyacrylamide hydrogel (PAAG), and polymethylmethacrylate (PMMA), a combination of nondegradable microspheres suspended in a degradable collagen gel, are nondegradable. The silicone oil and the polyacrylamide hydrogel contribute to the filling effect by their volume, whereas the
Table 1 Gel fillers used in the study

<table>
<thead>
<tr>
<th>Gel Filler</th>
<th>Description</th>
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<tr>
<td>Restylane and Perlane™</td>
<td>Lidocaine (Q-Med AB, Sweden)</td>
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<tr>
<td>Radiesse™</td>
<td>Volume Advantage 1.5 cc (Merz Aesthetics, San Mateo, CA)</td>
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<tr>
<td>Aquamid®</td>
<td>Reconstruction (Contura International, Søborg, Denmark)</td>
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nondegradable PMMA microspheres contribute permanently to the filling effect along with the foreign body tissue response, they elicit (Morhenn et al., 2002; Christensen et al., 2006; Christensen, 2007, 2009).

Adverse events (AE) to the different filler types have been reported, but their origin has been debated (Alijotas-Reig et al., 2010; Sperling et al., 2010; Alijotas-Reig & Garcia-Gimenez, 2011; Bachmann et al., 2011). Some have presumed the events to be due to a hypersensitivity inflammatory response to the fillers, whereas others have demonstrated that bacterial infection is the cause of AEs, at least to polyacrylamide gels (Christensen et al., 2005, 2013; Bjarnsholt et al., 2009).

Aggregating bacteria (including so-called biofilms) have recently received considerable attention. In persistent infections, these play an important role, which can be explained by their increased tolerance toward cells of the immune system and antibiotics (Alhede et al., 2011).

To investigate the role of bacteria – and possible aggregates of these – for the onset of adverse events, we tested three popular fillers, one from each class, in vitro and in vivo. We developed a novel mouse model to test whether low numbers of bacteria could initiate an infection, whether the gels could sustain a bacterial infection, and whether such an infection could be treated with relevant antibiotics.

Materials and methods

Bacterial strains

The bacterial strains used in these studies were tagged with green fluorescence protein (GFP). Pseudomonas aeruginosa (PAO1; Bjarnsholt et al., 2005), a clinical isolate of S. epidermidis 1457 from an infected central venous catheter (Mack et al., 1992) and a clinical isolate of P. acnes (isolate number 13972, Rigshospitalet, Denmark), was used.

Tissue fillers

In vitro growth and microscopy of bacteria in the tissue filler gels

A stack of silicone sheets attached to an object glass was used as cultivation chambers for bacteria and gels (Table 1). For all experiments, 100 µL of tissue filler gel, 250 µL of the bacterial suspension (400 CFU mL⁻¹), and 100 µL of medium were mixed in the chamber and incubated overnight at 37 °C.

Staphylococcus epidermidis and P. acnes were stained with syto9 (Molecular Probes, OR) and observed with the use of a confocal laser scanner microscope (CLSM; Zeiss.Z2 microscope with the connection of LSM 710 CLSM with ZEISS ZEN v.6.0 software with a 63 x/1,4 oil emersion objective). Pseudomonas aeruginosa was not stained as it carries a GFP-tag.

In vitro quantitative bacteriology and antibiotic treatment

To investigate the bacterial growth (and the possible development of biofilms) within the soft tissue fillers and their sensitivity to antibiotics, the following three experiments were performed in vitro:

1. Antibiotic treatment during propagation of bacteria.
2. Antibiotic treatment of the presumed mature biofilms in the gel.
3. No antibiotic treatment (control group).

The following final concentrations were used: 25 µg mL⁻¹ colistin, 100 µg mL⁻¹ tobramycin or the combination of the two for P. aeruginosa, and 5 µg mL⁻¹ rifampicin or 10 µg mL⁻¹ vancomycin or the combination of the two for S. epidermidis.

Samples in groups I and II were added antibiotics and incubated overnight at 37 °C. For the gels meant for late treatment (presumed mature biofilms) (II), new media were added every 24 h. After 72 h, antibiotics or saline (37.5 µL) were added and the samples were incubated overnight at 37 °C. Following incubation, the samples were added 1 mL saline, degassed, and sonicated for 5 min in an ultrasonic bath (BRANSON 1510). Serial dilutions of the samples were plated on LB plates for colony-forming units (CFU) estimation.

In vivo quantitative bacteriology

The national animal ethics committee, Denmark, approved and authorized all experiments (The animal experiments inspectorate, www.dyreforsoegstilsynet.dk permission number 2010/561-2017).

Female BALB/c mice 11–14 weeks of age were used (Taconic M&B A/S, Denmark). The mice were kept on standard mouse feed and water ad libitum in individually ventilated cage (IVC) systems (Biocenter, Copenhagen, Denmark) for at least a week before initiation of the experiments.
The mice were anesthetized by subcutaneous (s.c.) injection (0.15 mL) of Hypnorm®/Midazolam (VetaPharma, UK; Hameln Pharmaceuticals GmbH, Germany). [One part Hypnorm® (fentanyl 0.315 mg mL⁻¹), one part Midazolam (5 mg mL⁻¹), and three parts sterile water (Fresenius Kabi, Norway)]. Under anesthesia, the mice were disinfected with 70% ethanol at the injection site on their backs.

The desired gel and bacterial inoculum were mixed in the ratio of 100 μL gel to 250 μL bacterial suspension (400 CFU mL⁻¹) and then filled into a syringe. Postsurgical the mice were handled as previously described (Van Gennip et al., 2009) Four mice were euthanized immediately after injection to estimate the content of bacteria in the gel at the point of injection.

Mice were euthanized with intraperitoneal (i.p.) injection with 10 mL kg⁻¹ body weight of pentobarbital (200 mg mL⁻¹) with lidocaine hydrochloride (20 mg mL⁻¹; DAK, Denmark). For the aseptic removal of the gel, the fur was cleaned with 70% ethanol. The gel was removed and placed into a sterile 15 mL tube with 2 mL of saline and stored on ice until homogenization with a Silent Crusher M (Heidelberg, Germany). Homogenization of the samples was carried out for 30–60 s at 10,000 g. The homogenization was followed by 5 min of degassing and 5 min of sonication in an ultrasonic bath (Branson 2510) for disruption of possible bacterial aggregates. Serial dilutions of samples were plated for and incubated overnight at 37 °C.

**Prophylactic treatment of P. aeruginosa and S. epidermidis in vivo**

To observe the effect of prophylactic treatment, one group of mice (n = 8) were treated with 100 μg g⁻¹ tobramycin or 20 μg g⁻¹ rifampicin (Rifadin infusion® – rifampicin 600 mg, Sanofi-adventis, UK) two hours prior to injection of PAAG, which had been inoculated with P. aeruginosa or S. epidermidis. A control group (n = 8) received isotonic saline instead of tobramycin or rifampicin (Region Hovedstadens Apotek, Denmark).

**Late treatment of established P. aeruginosa/S. epidermidis infections in vivo**

Another group of mice (n = 8) were treated with 100 μg g⁻¹ tobramycin (P. aeruginosa) or 20 μg g⁻¹ rifampicin (S. epidermidis), which was given at day 7–14 (7 days) postsurgery. The control group (n = 8) received only isotonic saline.

**Treatment with Triamcinolone Acetoneid steroid**

Another group of mice (n = 8) were injected 7 days postsurgery with 20 μL 40 mg mL⁻¹ Triamcinolone Acetoneid® (Region Hovedstadens Apotek) for 6 days, while the control group (n = 8) was given isotonic saline. After 6 days of treatment, the mice were euthanized and the gel implants were evaluated for CFUs.

**Statistical analysis**

Statistical significance was evaluated by Mann–Whitney test for nonparametric data and one-way analysis of variance (ANOVA) test with post-test for linear trend for parametric data. P-values > 0.05 were considered statistically significant. If no total clearance was seen in the treatment group, a Wilcoxon signed-rank test (nontreated median vs. 0) was carried out. The tests were carried out using with PRISM (GraphPad Software).

**Results**

To test whether the different types of filler could support bacterial growth in vitro, we inoculated the fillers with the opportunistic pathogen and biofilm model organism, P. aeruginosa. As seen in Fig. 1A, a dense bacterial population and numerous aggregates were seen inside all the three gels after 24 h. The amount and size of aggregates were even more pronounced after 48 h, suggesting that these tissue gels make up a perfect habitat to sustain biofilm growth.

In addition to culturing P. aeruginosa inside all of the fillers, we also tested two clinically relevant bacterial strains S. epidermidis and P. acnes. As we had not seen any differences in growth of P. aeruginosa between the three fillers, we chose to test growth of the two strains only in the permanent PAAG. As with P. aeruginosa, high numbers of bacterial aggregates indicated that also S. epidermidis and P. acnes were capable of forming biofilm-like structures within the gel (Fig. 1b).

As all three fillers were capable of supporting bacterial growth, and we were able to visualize aggregates of P. aeruginosa, S. epidermidis, and P. acnes, we decided to study whether the bacteria within the gels had adapted an antibiotic tolerant biofilm phenotype. After the three gel fillers had been inoculated with a suspension of P. aeruginosa, a high concentration of tobramycin (100 μg mL⁻¹) was added either simultaneously or 72 h after the inoculation, which would allow a dense and possible antibiotic tolerant biofilm to form. As seen in Fig. 2a, tobramycin was able to clear the bacteria within 24 h when administered together with the bacteria (P < 0.0005). In contrast, after 72 h, the antibiotic did not eradicate the bacteria completely, although their number was significantly reduced compared with the nontreated control (P < 0.01; Fig. 2b).

Knowing that biofilms of P. aeruginosa are more susceptible to combinations of antibiotics (Famp et al., 2008; Alhede et al., 2011), we tested a combination of two clinically relevant types of antibiotics, which are known to kill both growing and nongrowing bacteria (tobramycin 100 μg mL⁻¹ and colistin 25 μg mL⁻¹, respectively). As seen in Fig. 2c, high concentrations of either colistin or tobramycin were able to reduce the number of bacteria significantly but not to clear them, the two antibiotics in combination reduced bacterial count further but not significantly.

As it was possible to prevent growth of P. aeruginosa within the gel by administering prophylactic antibiotics, we tested the more clinically relevant bacterium, S. epidermidis using 5 μg mL⁻¹ rifampicin and 10 μg mL⁻¹ vancomycin and a combination of the two antibiotics. Similar to the results obtained for P. aeruginosa, a 24 h treatment with the antibiotics administered prophylactic cleared the growth of S. epidermidis and treatment with rifampicin 72 h postinoc-
ulation also completely eradicated the *S. epidermidis* biofilm-like clusters in the gel. This was in contrast to the vancomycin treatment, which did not have any effect on the bacteria at all, likely due to its poor penetration (see Fig. 3).

**Infection in mice injected with infected tissue filler gels**

To investigate whether the gels also support bacterial growth *in vivo* female BALB/c mice were injected with *P. aeruginosa*-infected tissue filler gels containing c. 40 CFU per mouse. According to Fig. 4, it is clear that the *in vivo* growth results were very different from those observed *in vitro*. After 7 days, we were not able to detect any bacteria within the HA gel, and only a limited amount was found in the CaHA gel. By comparing the medians with nonparametric statistic tests, we found that only the PAAG supported further bacterial growth.

As the formulation of HA gel contains 3 mg mL⁻¹ lidocaine hydrochloride, both the CaHA gel (not shown) and the PAAG (Fig. 4) were supplemented with the same concentration of lidocaine hydrochloride prior to the bacterial inoculation. Lidocaine was not found to have any significant effect on the bacterial growth (Fig. 4).

**Effect of antibiotic treatment of infected PAAG gel in mice**

As the *P. aeruginosa* bacteria were able to grow within the PAAG *in vivo*, we further tested whether it was possible to either prevent an infection by prophylaxis or to treat an established infection successfully with antibiotics. Thirty-six female BALB/c mice were divided into two groups (I + II) and their respective control groups, which were treated with isotonic saline:

1. Prophylactic treatment with tobramycin (100 μg g⁻¹) two hours prior to injection of contaminated PAAG.
2. Initiation of tobramycin treatment (100 μg g⁻¹) starting at day 7 postinjection of contaminated PAAG and administered once a day for 7 days.

As seen in Fig. 5, the group that received prophylactic treatment with tobramycin was able to completely eliminate the bacteria within the gel, whereas the nontreated group was inadequate in clearing the bacteria (Fig. 5a).

In accordance with the *in vitro* studies, a delayed long-term treatment with tobramycin did not clear the bacteria but a statistically significant reduction was observed ($P = 0.0335$; Fig. 5b).

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**Fig. 1** (A) *In vitro* growth of *Pseudomonas aeruginosa* in tissue filler gels. Microscopic images of tissue filler gels inoculated with GFP-tagged *P. aeruginosa*. The pictures were obtained with the use of a Zeiss Z2 microscope with the connection of LSM 710 CLSM with ZEISS ZEN v.6.0 software with a 63×/1.4 oil objective and prepared in Imaris. The data bar represents 15 μm in all images. (B) Microscopic images of PAAG inoculated with *P. aeruginosa*, *P. acnes*, or *Staphylococcus epidermidis* in the silicone setup. The bacteria were grown for 24, 48, and 72 h, respectively, as biofilm on the PAAG. The gel and bacteria inoculated into chamber cut in the silicone sheets and incubated in a moisture chamber at 37 °C. The pictures were obtained with the use of a Zeiss Z2 microscope with the connection of LSM 710 CLSM with ZEISS ZEN v.6.0 software with a 63×/1.4 oil emersion objective and prepared in Imaris. Biofilm appears after (A) 24 h for *P. aeruginosa*, (B) 48 h for *P. acnes*, and (C) 72 h for *S. epidermidis*. 

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Similar experiments were performed with the skin bacterium, *S. epidermidis*. Like in the *in vitro* experiment, the antibiotic used was rifampicin (Rifadin 5 µg g⁻¹).

Also prophylactic treatment of *S. epidermidis*-infected gels was able to clear the bacteria (Fig. 5c) and the late treatment after 7 days did not clear the bacteria, although the CFU per mouse was reduced to a statistically significantly lower level (*P* = 0.0140; Fig. 5d).

**Effect of treatment with the steroid Triamcinolone Acetonide**

To investigate the effect of steroid treatment on infected gels (Christensen *et al.*, 2005; Bjarnsholt *et al.*, 2009), we initiated a 6-day-long treatment with the highest tolerable dose (40 µg g⁻¹ bodyweight) of triamcinolone acetonide 7 days postinfection with *P. aeruginosa*-infected PAAG. Following this, the mice were either euthanized or left alone for 4 days before we estimated CFU per gel.

With this concentration of triamcinolone acetonide, we did not find any effect of the treatment at all (Data not shown). However, higher doses (100 µg g⁻¹ bodyweight) of the steroid left the mice in a poor condition (ruffled fur and squinted eyes) and promoted bacterial growth in the gels (data not shown).

**Discussion**

This study has confirmed the growing evidence that some tissue fillers can support resistant bacterial aggregates (so-called biofilms) and that this is the real cause of the adverse reactions observed in the clinic (Christensen *et al.*, 2013). To our knowledge this is the first time, tissue fillers have been studied experimentally in a systematic manner to compare bacterial growth and elucidate the effect of possible treatments on induced bacterial infection. We were able to culture and establish *P. aeruginosa* biofilms inside all of the fillers. Although only the PAAG was used for *S. epidermidis*

**Fig. 2** *In vitro* prevention and treatment of *Pseudomonas aeruginosa* in tissue filler gels. (a) Effect of treatment with tobramycin (100 µg mL⁻¹) simultaneously with the inoculation of *P. aeruginosa* in the gels. CFU was enumerated after 24 h. *N* = 12. The horizontal bars represent the median values and *P* < 0.05 using Wilcoxon signed-rank test. (b) The effect of treatment with tobramycin (100 µg mL⁻¹) 72 h after the inoculation of *P. aeruginosa* in the gels. CFU was enumerated after 24 h treatment. *N* = 12. The horizontal bars represent the median and *P* < 0.05 using Mann–Whitney test. (c) *In vitro* effect of combined antibiotics of *P. aeruginosa* biofilm infected PAAGs. The effect of late treatment with colistin (25 µg mL⁻¹), tobramycin (100 µg mL⁻¹) or a combination of the two were given 72 h after the inoculation of *P. aeruginosa* in the PAAG gel. CFU was enumerated after 24 h treatment. *N* = 12 per group. The horizontal bars represent the median value.
and *P < 0.05 using Wilcoxon Signed-Rank Test.

Interestingly, a combination of colistin and tobramycin did not have the same significantly additive treatment effect as is usually seen in *P. aeruginosa* infections (Herrmann et al., 2010; Alhede et al., 2011). Although rifampicin (5 µg mL⁻¹) in contrast to vancomycin, was able to both prevent and treat gel infection (at 72 h) with *S. epidermidis* in vitro, our animal model stresses the importance of using prophylactic antibiotics when injecting a contaminated gel. Even high successive doses of tobramycin and rifampicin failed to eradicate the bacteria harbored in the gel, in spite of the antibiotics had full access to the bacteria and that their protection against these antibiotics was most likely explained by the enhanced biofilm mode of growth, which we observed in the microscope (Figs 1 and 2).

The route of bacterial infection is likely to be during injection of the filler. In our experiments, as little as 40 single bacteria were enough to cause an infection, studies have shown that bacteria are present on the human skin in microcolonies of up to 10⁵ bacteria. Thus, contamination of the needle during skin penetration could be the cause. We were able to establish a gel infection in the mice by contaminating the needle by briefly immersing it in a dilute bacterial culture (data not shown).

The FDA has now approved the local anesthetic agent lidocaine hydrochloride to be included in the gel formulation. In the present study, we evaluated the HA, Restylane Perlane® Lidocaine, and compared it to the both the PAAG, Aquamid® and the CaHa, Radiesse®, supplemented with lidocaine. As expected, our comparison studies did not show any antibacterial effect of the Lidocaine in vivo.

Adverse foreign body reactions toward tissue fillers have previously been interpreted as inflammatory reactions of noninfectious type and treated with steroids to take the swelling (Christensen et al., 2005; Bjarnsholt et al., 2009). Treating our mice carrying contaminated gels with a low dose (40 µg g⁻¹ bodyweight) of triamcinolone acetonide for 6 days did not influence bacterial load within the gel, but a high dose treatment (100 µg g⁻¹ bodyweight) left the mice in a poor condition, and bacterial growth in the gel was promoted. Thus, we cannot recommend treating infected gels with steroids – in the best case, it has no effect and in the worst case, the bacteria will grow freely in the absence of immune cells.
Conclusion

We here present solid evidence that tissue filler gels do support the growth of bacterial into clusters (as biofilms) and that these might be the cause of most adverse events seen in patients with gel fillers. Even low numbers of contaminating bacteria were sufficient for a tolerant biofilm to form, and steroids as well as late initiated antibiotic treatments (including combinations) were not effective in eradicating these. Only prophylactic treatment with relevant antibiotics seems to have effect. To prevent such infections, we therefore recommend a strict aseptic injection technique, thorough skin decontamination and proper prophylaxis.

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