Chapter 3

Persistence of a bioluminescent *Staphylococcus aureus* strain on and around degradable and non-degradable surgical meshes in a murine model

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Abstract

Biomaterials are increasingly used for the restoration of human function, but can become infected as a result of peri- or early post-operative bacterial contamination, although biomaterial-associated infections (BAIs) can also initiate at any time from haematogenous spreading of bacteria from an infection elsewhere in the body. Infecting bacteria in BAIs not only seek shelter in their own protective biofilm matrix, but also hide in surrounding tissue. This study compares staphyloccocal persistence on and around a degradable and non-degradable surgical mesh through the use of longitudinal bioluminescence imaging in a murine model, including histological evaluation of surrounding tissue after sacrifice. Surgical meshes were first contaminated with bioluminescent *Staphylococcus aureus* Xen29 and subsequently subcutaneously implanted in mice. Bioluminescent staphylococci persisted on and around non-degradable meshes during the 28-day course of the study, whereas bioluminescence returned to control levels and bacteria disappeared from surrounding tissues once a degradable mesh had fully dissolved. Thus the application of degradable biomaterials yields major advantages with respect to the prevention of BAIs, as dissolution of the implant not only is associated with elimination of the protective biofilm mode of growth of the infecting organisms, but also allows the immune system to clear the surrounding tissue from infecting organisms.
Introduction

Biomaterials are increasingly used for the restoration of function after trauma, wear or intervention surgery and as a result the number of biomaterial-associated infections (BAIs) is rising (1). BAI often results from peri- or early postoperative bacterial contamination of the implant or wound site, but BAIs can initiate at any time from haematogenous spreading of bacteria from an infection elsewhere in the body. A BAI involves bacteria adhering in their so-called biofilm mode of growth, a form of community growth through which adhering bacteria envelope themselves in a protective layer of extracellular polymeric substances (2,3). As a consequence, neither the host immune defence nor standard antibiotic treatments suffice to eradicate biofilms from a biomaterial implant surface, and often an infected implant has to be replaced.

Surgical meshes are used worldwide for the reconstruction of abdominal wall defects. Surgical meshes can be made of non-degradable materials such as polypropylene (4) or degradable materials such as porcine small intestinal submucosa (5). Recently (6), surgical meshes with different morphologies and made of different non-degradable materials have been compared with respect to their infection resistance in a murine model. Using bio-optical imaging, it was found that the bioluminescence arising from implanted surgical meshes, contaminated with a bioluminescent *Staphylococcus aureus* strain, was higher and persisted longer on multifilament polypropylene and polytetrafluoroethylene meshes than on monofilament polypropylene, polyester and Ti-coated meshes.

Although the presence of a biofilm growing on a biomaterial surface has long been considered the sole cause of BAIs, it has become increasingly apparent that the presence of a biomaterial impairs the host’s immune defence (7–10). In fact, degradable materials for surgical meshes in reconstructive abdominal wall surgery have been developed because of the reported long-term risk of infection and patient morbidity when non-degradable materials are used (5,11). A rapid restoration of the efficacy of the immune system may be anticipated once the
biomaterial has been fully degraded, despite the fact that bacteria may remain
dormantly present, even in macrophages (12), while maintaining its primary
function as a scaffold for fibrosis formation to correct abdominal wall defects. It is
hypothesized that dissolution of the biomaterial therewith will also yield
clearance of possible bacteria from surrounding tissue.

The aim of this study is to compare staphylococcal persistence on and
around a degradable and non-degradable surgical mesh through the use of
longitudinal bioluminescence imaging in a murine model, including histological
evaluation of surrounding tissue after sacrifice. To this end, we subcutaneously
implanted a degradable, porcine small intestinal submucosal and a non-
degradable, doubly filamented polypropylene surgical mesh, contaminated with
bioluminescent S. aureus Xen29 in mice. Staphylococcal persistence was
monitored for a 28-day experimental period, after which the animals were
sacrificed.

Materials and methods

Bacterial strain, surgical meshes and biofilm formation

Bioluminescent S. aureus Xen29 originates from S. aureus ATCC12600 and
was made bioluminescent by inserting a modified Photorhabdus luminescens lux
operon into the bacterial genome (13,14). The strain was commercially obtained
from Caliper Life Sciences (Hopkinton, MA, USA). Staphylococci were cultured
from cryopreservative beads (Protect Technical Surface Consultants Ltd, UK) onto
blood agar plates at 37°C in ambient air. Prior to each experiment, one highly
bioluminescent colony was selected using an IVIS Imaging System (IVIS® Lumina II,
Imaging System, Caliper Life Sciences) to inoculate 5 ml of tryptone soy broth
(TSB, Oxoid, Basingstoke, UK) at 37°C for 24 h in ambient air. A 100 µl volume of
this culture was used to inoculate 50 ml of growth medium from which biofilms
were grown on the meshes by submerging sterilized mesh samples into TSB with a
stainless steel hook. The inoculated TSB was refreshed with sterile TSB every 24 h
during 72 h of incubation at 37°C under continuous shaking at 60 rpm. After this period, the meshes were collected and dipped once in sterile 0.9% NaCl solution, then gently dried by minimal touching with a soft tissue, avoiding distortion of the biofilm, to remove planktonic staphylococci as well as redundant TSB for *in vitro* evaluation or insertion into mice.

Two types of commercially available surgical meshes were used: Prolene® (Ethicon Inc., Sommerville, NJ, USA), consisting of non-degradable, doubly filamented polypropylene, with a surface area of $2.46 \text{ cm}^2 \text{ cm}^{-2}$ (15) and a thickness of 0.5 mm; and Surgisis Biodesign™ (Cook Biotech Inc., Bloomington, IN, USA), a degradable fleece made from porcine small intestinal submucosa with a dense fibrous structure (16) and consequently an unknown, though large, surface area per cm$^2$. Samples (8 mm diameter) were prepared out of each mesh type from a single mesh sheet using a biopsy punch, sterilized in 70% ethanol and air-dried before use.

**In vitro evaluation of biofilm**

Phase-contrast (Olympus BH, Zoeterwoude, The Netherlands) and fluorescence microscopy (Leica DM4000, Heidelberg, Germany) were used to quantify the number of live and dead bacteria on the meshes per unit mesh area. To this end, biofilms formed on the meshes were first dispersed in 0.9% NaCl saline solution, sonicated in an Eppendorf tube containing 0.5 ml of 0.9% NaCl three times for 10 s with 30 s intervals on ice-chilled water using a probing tip (Vibracell TM, Sonics & Materials Inc., Newton, CT, USA). Next, a 15 µl droplet of the bacterial suspension was placed on a glass slide and stained for 15 min in the dark at room temperature with 15 µl SYTO9 and propidium-iodide in a 1:1 ratio (Molecular Probes, Leiden, The Netherlands) (17), yielding green and red fluorescence for live and dead bacteria, respectively. Subsequently, fluorescence microscopy was applied to determine the percentage viable bacteria in the dispersions, while the total number of dispersed, dead and live, bacteria was
determined in a Bürker–Türk counting chamber using phase contrast microscopy and expressed per cm² mesh.

In addition, biofilm formation on the meshes was quantified from the bioluminescent signal arising from biofilms on the meshes, i.e. without removal and dispersal of the biofilms. Bioluminescence was imaged with a CCD camera (IVIS® Lumina II Imaging System, Caliper Life Sciences, Hopkinton, MA, USA) directly after incubation for 72 h. Bioluminescence images were obtained using a 12.5 cm field of view, binning of 4, 1/f stop, 60 s exposure time and open filters, and corrected automatically for background luminescence. The size and location of the regions of interest (ROIs) were set to coincide with the location and size of the mesh. Total photon counts over the ROIs were converted to photon flux from the ROI (p s⁻¹) by using Living Image software (IVIS® 100 Imaging System, Caliper Life Sciences) and dividing by the area of the mesh.

**In vivo evaluation of bacterial persistence**

*In vivo* evaluation of staphylococcal persistence on and around meshes was done in a murine model, as described by Engelsman et al. (6), and all experiments were approved by the Animals Experiments Committee at the University of Groningen (#4619K). Eightweek-old female BALB/c OlaHsd mice (Harlan Netherlands BV, Horst, The Netherlands), with an average weight of 20 g, were randomly divided into groups, corresponding with the use of Prolene® and Surgisis Biodesign™. Buprenorfine (0.03 mg kg⁻¹) was administered subcutaneously as an analgesic 30 min prior to implantation. Anesthesia was induced with 3.5% Isuflorane/O2 (Zeneca, Zoetermeer, The Netherlands) and maintained at 1.5% during mesh implantation. The back and the right flanks were shaved and disinfected with 70% ethanol before a 2 cm deep subcutaneous pocket was created through a 1 cm incision. Two experimental groups of nine mice each received a Surgisis Biodesign™ or Prolene® surgical mesh with a 72 h biofilm. Two other groups of three mice each received sterile meshes of both
types as non-infected controls. After implantation, the skin was closed with a single 7-0 Surgipro (US Surgical, Norwalk, CT, USA) suture. All groups were kept in separate, individually ventilated cages. Note that in a discarded series of experiments, bedding material used for housing turned out to contain a phosphorescent component, causing a high background signal. Information on luminescence of bedding material is not provided by manufacturers and based on our experience we suggest that future studies should indeed establish the absence of luminescent components in the bedding material prior to commencing experiments.

Bioluminescence was imaged with a CCD camera (IVIS® Spectrum Imaging System, Caliper Life Sciences) 1 day following implantation of the meshes and subsequently on selected days for 28 days. Mice were placed in the imaging chamber of the IVIS under anesthesia as described above, with their right flank exposed to the camera. After acquiring a greyscale photograph, a 5 min bioluminescent image was obtained using a 21 cm field of view, binning of 4, 1/f stop and open filters, and corrected automatically for background noise.

ROIs were manually chosen to be 1.14 cm$^2$, slightly larger than the area of the mesh in order to ensure the capture of the full amount of light produced, therewith accounting for scattering. Total bioluminescent photon counts over the ROIs were converted to the photon flux from the ROI (p s$^{-1}$) using Living Image software (Caliper Life Sciences). Bioluminescence fluxes for the experimental groups are reported after subtraction of the bioluminescence flux in the control groups, i.e. in the absence of staphylococcal contamination.

**Microbiological and histological ex vivo evaluations after sacrifice**

After sacrificing the animals at day 28, tissue samples were taken. First, an incision was made at the ventral side of a mouse, after which the subcutaneous layer with adjacent skin was prepared free to make the mesh visible and a standardized biopsy specimen of 12 mm diameter, comprising the mesh with
surrounding tissue, was taken from the implantation site. The biopsy specimens were cut into halves. One half was used for a quantitative microbiological culturing of the mesh and surrounding tissue. To this end, the cut mesh-half was carefully separated from the tissue and kept in 300 µl of reduced transport fluid (RTF; 0.9 g l\(^{-1}\) NaCl, 0.9 g l\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.45 g l\(^{-1}\) KH\(_2\)PO\(_4\), 0.19 g l\(^{-1}\) MgSO\(_4\), 0.45 g l\(^{-1}\) K\(_2\)HPO\(_4\), 0.37 g l\(^{-1}\) Na\(_2\)EDTA, 0.2 g l\(^{-1}\) L-cysteine HCl (pH 6.8)), while tissue was stored in 10 ml of 0.9% NaCl solution. The other mesh-half was used for histological analysis.

For microbiological culturing, the mesh-halves were sonicated for 30 s in RTF, after which the supernatants were serially ten-fold diluted and plated on blood agar plates. Agar plates were incubated for 24 h and the number of colony forming units (CFUs) was counted. Tissue samples were sonicated by a tip sonicator. Resulting suspensions were subsequently divided into three 100 µl aliquots and separately plated on blood agar plates. CFUs were enumerated after 24 h of incubation.

For histological analysis, the other mesh-half of the biopsy specimens were fixed in 10% phosphate buffered formaldehyde, embedded in plastic (methylmethacrylate/butylmethacrylate; Merck Schuchart, Hohenbrunn, Germany), and cut into 3 µm sections. These sections were stained with hematoxylin–eosin and examined by light microscopy.

**Statistical analysis**

Data are reported as means with standard deviation and analysed using SPSS software version 16.0 (SPSS, Chicago, IL). For comparison of both mesh-types, *in vitro* data from one bacterial culture were analysed pairwise using a two-sided Student t-test to assess significant differences in colonization. *In vivo* and *ex vivo* data were analysed using permutation statistics. *p*-Values <0.05 were considered statistically significant.
Results

Biofilm evaluation \textit{in vitro}

The numbers of viable bacteria in, as well as the bioluminescence arising from 3 days old \textit{in vitro} biofilms of \textit{S. aureus} Xen29 on surgical meshes, which were similar as on meshes prior to implantation in the mice, are presented in Fig. 1. Both the numbers of viable bacteria in, and the bioluminescence flux arising from, the biofilms were significantly (paired Student \textit{t}-test) higher on the degradable mesh than on the non-degradable mesh.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{biofilm_evaluation}
\caption{Log number of viable \textit{S. aureus} Xen29 on (left panel) and bioluminescence flux arising from (right panel) 3-day-old biofilms on non-degradable (Prolene) and degradable (Surgisis) surgical meshes. Bacterial numbers are represented as the log\textsubscript{10} number viable bacteria per cm\textsuperscript{2}, while bioluminescence is expressed as log\textsubscript{10} number of p s\textsuperscript{-1} cm\textsuperscript{2}. Data are presented as means ± standard deviations for five independent experiments. * indicates significant differences (\textit{p} < 0.05) in the numbers of viable bacteria, as well as in the bioluminescence flux between the bio-degradable and the non-degradable mesh.}
\end{figure}
Biofilm evaluation \textit{in vivo}

The bioluminescence flux generated from the staphylococci at the implantation site allows monitoring of bacterial persistence longitudinally in one and the same mouse (Fig. 2). Bioluminescence from mice carrying degradable meshes contaminated with bacteria was significantly (Student $t$-test, $p < 0.03$) higher at each time point during the first 20 days than from mice in the control groups, i.e. with a non-contaminated mesh of either type, the latter showing a photon flux generated by camera noise and auto-luminescence of the mice (average bioluminescence flux in control group: $1.6 \times 10^4 \pm 4.0 \times 10^3$ p s$^{-1}$). During the same period, the bioluminescence flux from mice with an implanted degradable mesh was significantly (Student $t$-test, $p < 0.03$) higher than from mice with a non-degradable mesh, which corresponds with the observation that only mice implanted with a contaminated degradable mesh showed clinical signs of infection, i.e. soft tissue swelling and pus formation. This difference in bioluminescence between both meshes exceeded the difference in bioluminescence observed \textit{in vitro} after 3 days of biofilm growth, by a factor of ten. The bioluminescence from contaminated, non-degradable meshes remained slightly, but significantly (Student $t$-test, $p < 0.03$), higher than control levels during the entire experimental period up to 28 days (average bioluminescence flux in control group: $1.7 \times 10^4 \pm 1.0 \times 10^4$ p s$^{-1}$). Bioluminescence from contaminated degradable meshes steadily decreased between days 5 and 20, to a level slightly higher than around the contaminated non-degradable meshes. A Student $t$-test on the bioluminescence decrease for each of the individual mice with degradable meshes over this time period showed this decrease to be significant ($p < 0.03$).

Interestingly, \textit{ex vivo} tissue evaluation after 28 days (see below) demonstrated that the degradable mesh had fully disappeared during the course of the investigation in seven mice, while in two mice a partly degraded mesh could still be retrieved. Accordingly, the course of bioluminescence during the
entire experimental period was separately plotted in Fig. 2 for these two groups of mice, demonstrating that when the mesh had been fully degraded, bioluminescence returned to control levels, i.e. lower even than in the group with a bacterially contaminated non-degradable mesh. In case the mesh had not been fully degraded, a steady or slightly increasing bioluminescence was observed after ~20 days. This resulted in a statistically significant correlation ($p < 0.005$) between the bioluminescence of the groups with complete or non-complete degradation during the last 2 days of the experiment, as obtained from permutation statistics.

Figure 2. Bioluminescence flux after subtraction of the bioluminescence of the respective control groups as a function of time after implantation for mice with a degradable and non-degradable surgical mesh, in presence of staphylococcal contamination on the meshes prior to implantation. Note that data for mice having received a bacterially contaminated, degradable mesh were separated into two subgroups in this analysis, depending on whether the mesh appeared to have been fully (seven mice) or partly degraded (two mice) at sacrifice on day 28. Data points for these subgroups represent the mean bioluminescence in each subgroup. Data points represent the means ± standard deviations over each group of nine mice (or subgroups).
Microbiological and histological ex vivo evaluations

After sacrifice of the animals at day 28, bacteria were recovered from the meshes and surrounding tissues and quantified by plate counting. No viable bacteria were cultured from meshes in either control group. Viable *S. aureus* Xen29 were retrieved from mice implanted with a contaminated non-degradable mesh and surrounding tissue (Fig. 3). Interestingly, in seven out of the nine animals with a bacterially contaminated, degradable mesh, the mesh had completely disappeared when the mice were sacrificed, and tissues from three of these animals were culture negative.

![Figure 3. Log CFUs of *S. aureus* Xen29 retrieved from surrounding tissue biopsies and meshes from mice implanted with a bacterially contaminated degradable and non-degradable mesh, after sacrifice at day 28. In seven out of the nine mice implanted with a contaminated degradable mesh, the mesh had been completely degraded upon sacrifice (indicated by open circle for tissue data).](image-url)
Representative results of the histological analysis of the tissue around the meshes are presented in Fig. 4. The biopsy samples were randomly assigned for histological evaluation. Similar, mild inflammation was evident in mice carrying either of the control (non-contaminated) meshes. Around the contaminated, non-degradable mesh strong infiltration of inflammatory cells, predominantly neutrophils and macrophages, was observed. The group of mice with contaminated degradable meshes was divided into mice in which the mesh had been fully degraded (seven mice) and mice in which remains of the mesh were still present (two mice). In the latter group, the remaining filaments of the mesh were surrounded by inflammatory cells, neutrophils and macrophages, and around this layer novel fibroblasts were seen with extracellular matrix.

**Figure 4.** Hematoxylin-eosin stained sections of biopsies retrieved from mice implanted with degradable and non-degradable meshes, obtained at sacrifice after 28 days of implantation. A highly dense inflammatory infiltrate composed of neutrophils and macrophages around a bacterially contaminated non-degradable mesh can be seen, as compared to a far less intense inflammatory response around a contaminated, degradable mesh. Arrows indicate the remains of the meshes in the tissue. Bars indicate 50 µm.
Discussion

The persistence of BAI is related to the ability of infecting organisms to form biofilms on the implant materials offering protection against the host immune system and antibiotic treatment (18) and to find shelter in surrounding tissues (19). The presence of a biomaterials implant frustrates phagocytic activity (20) and deranges the host immune response, enhancing susceptibility to infection (21). Thus in the current study we evaluate the hypothesis that bacterial persistence on and around an implant is different for degradable and non-degradable meshes using bioluminescence imaging in a murine model. Murine models are generally accepted for studies on the pathogenesis and prevention of BAI, such as infection of surgical meshes (9,14). Obviously the results of our murine model, like of any other animal model, should be extrapolated with care as a preclinical evaluation model of human infections, because each animal model has its own species-specific response to infection (22). The persistence of \textit{S. aureus} was above control levels throughout the course of this study for a non-degradable mesh, but was reduced to control levels once meshes manufactured of degradable porcine small intestinal submucosa had been fully degraded. This suggests that degradation of the biomaterial not only removes the biofilm as a source of infection, but also enables the immune system to clear surrounding tissue from infecting organisms.

This study uses bioluminescence imaging to monitor the spatiotemporal progression of \textit{S. aureus} persistence in combination with traditional microbiological and histological end-point analyses after sacrifice of the animals. As an advantage, bio-optical imaging enables longitudinal monitoring of bacterial persistence in single and the same animals without sacrifice, which is an enormous statistical advantage over the use of multiple groups of animals for analyses at different time points. The technique thus allows statistically significant conclusions to be drawn with relatively low numbers of animals. It also meets ethical considerations and the ensuing societal pressure to reduce the number of
animals used in scientific experiments. As a consequence of following individual animals longitudinally over time, data requiring sacrifice can only be obtained at the last time point.

Previously, it has been shown that in vivo bioluminescence strongly correlates with results of ex vivo culturing of explanted, bacterially contaminated non-degradable meshes (23) and catheter segments (14), despite the fact that bioluminescence depends not only on the number of bacteria present on and around an implanted mesh, but also on the metabolic state of the individual, single organisms in a biofilm. Bacteria in a biofilm mode of growth are generally considered to form a heterogeneous community of organisms in different growth phases. Because the bioluminescent reaction needs ATP and NADPH to maintain sufficiently high levels of aldehydes, metabolically active bacteria predominantly contribute to the emission of light. Thus it may be concluded that bacteria in different growth phases as existing in a biofilm present an average metabolic activity that enables quantification of bacterial presence from the bioluminescence signal. This is confirmed by our in vitro data (see Fig. 1) showing a higher number of viable staphylococci in 3-day-old biofilms as well as a higher bioluminescence signal on the degradable mesh as compared to the non-degradable mesh.

Mice implanted with bacterially contaminated degradable meshes showed an initially higher bioluminescent flux than mice implanted with contaminated non-degradable meshes (see Fig. 2). This might be due in part to a higher bacterial burden on the degradable mesh, compared to the non-degradable mesh (compare Fig. 1) as a result of the dense fibrous structure of the degradable mesh (16) in comparison with the more open structure of the non-degradable mesh. Yet bioluminescence never returned to control levels for the non-degradable mesh, while upon full degradation of the degradable mesh, bioluminescence did return to control levels, despite the initially higher bacterial contamination. In this regard, it should be considered that the persistence of
bacteria might develop differently for different bacterial strains or species, depending on their virulence and their propensity to disturb immune responses around biomaterials. Microbiological and histological analyses after sacrifice led us to separate the group of mice with a degradable mesh into a subgroup of mice with a completely degraded mesh and mice with a partially degraded mesh. Importantly, full degradation of the mesh led to clearance of infection, as judged not only from the bioluminescence flux (see Fig. 2), but also from the cultures of the surrounding tissue (see Figs. 3 and 4). Whereas all mice with non- or partly degraded meshes were culture positive, bacteria could only be detected in 50% of the tissue samples from mice with fully degraded meshes. This suggests that in mice with non- or partly degraded meshes clearance was compromised, or that it would require a longer period to be completed. Literature indicates that in general 60 and 100% of submucosal degradable meshes degrade within 4 weeks following implantation in a dog bladder (24) and murine subcutaneous pockets (25). From Fig. 2 it can be concluded that when degradation is slow and not completed within ~20 days, bioluminescence remains steady or even slightly increases within the first week after an initial decrease. This might attest to increased bacterial metabolic activity and growth, possibly in the surrounding tissue. However, when a hernia mesh is fully degraded, bacteria disappear completely, likely due to a higher efficacy of the immune response in the absence of the biomaterial. This is indirectly evidenced by the decrease of the bioluminescent signal and the reduction of culture positive ex vivo samples, and is in line with the abundant presence of neutrophils and macrophages in the areas where the implant likely had been present (see Fig. 4). Novel fibroblasts were observed as well in this area, indicating that the foreign body reaction had progressed to the stage of tissue repair, apparently following the successful elimination of the bacteria.


**Conclusion**

The persistence of a bioluminescent *S. aureus* strain on and around surgical meshes develops differently for degradable than for non-degradable mesh materials. Despite the open structure of non-degradable meshes, allowing macrophage infiltration, *S. aureus* persisted on and around non-degradable meshes during the entire 28 day course of the study, whereas bioluminescence returned to control levels once the degradable mesh had fully been degraded. Thus this study shows, for the first time, that degradation of an implant not only is associated with the elimination of a biomaterial surface providing a niche for the protective biofilm mode of growth of the infecting organisms, but also appears to create the conditions in which the immune responses are sufficiently effective again to clear the surrounding tissue from infecting staphylococci.
References
