Chapter 5

The biotolerability and *in vivo* efficacy of an antifouling pNIPMAM-based nanogel coating in preventing surgical mesh associated infections

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ABSTRACT

Biomaterials pose as a substantial risk for bacterial infections. Infections are serious adverse events when associated with implanted biomaterials such as surgical meshes, where bacteria readily adhere to the surface and can form biofilms which lead to chronic infections, threatening long-term implant performance. Antifouling coatings may reduce bacterial adhesion and therewith, potentially prevent implant infections. Previously, poly-N-isopropylmethacrylamide (pNIPMAM) nanogel coatings were shown to exhibit excellent antifouling properties against Staphylococcus aureus in vitro. Here, we assessed the infection prevention capacity of pNIPMAM nanogel coatings on polyvinylidene fluoride (PVDF) meshes in a murine model with bioluminescent S. aureus Newman as well as the host response towards the nanogel coating for 7 and 13 days. Histological analyses revealed that pNIPMAM coating elicited a low-level inflammatory response comparable to that induced by pristine PVDF at day 13. The S. aureus Newman in vivo bioluminescence and ex vivo determined colony forming units were similar on both coated and uncoated filaments suggesting equivalent metabolic state independent of the nanogel coating. However, apoptosis of immune cells and biofilm formation by S. aureus Newman was limited on coated PVDF filaments for up to 7 days, suggesting that bacteria may be more vulnerable to antibiotic treatment.
1 Introduction

Bacterial infections pose a challenge to the performance and long-term efficacy of implanted biomaterials. Among implants, surgical meshes have become the gold standard for abdominal hernia repair due to advantages such as quick recovery, reduced recurrence rate and reduced postoperative pain. Aside hernia repair, surgical meshes may also be applied as a scaffold for tissue regeneration of the oesophagus and breast reconstruction. However, bacterial attachment and subsequent biofilm formation can limit host integration, promote implant loosening, and aggravate the host inflammatory response. Infection can also lead to other comorbidities such as impaired wound healing and functional loss of the abdominal wall. The presence of an implant predisposes for bacterial infection and up to 10% of surgical meshes become contaminated with bacteria. This high infection rate is related to the implant surface providing a suitable interface for bacterial adhesion, stabilized by Van der Waals, and hydrophobic interactions. Additionally, plasma proteins immediately adsorb to the implant surface after implantation and foster specific interactions with bacterial surface adhesins. Once adhesion is completed, bacteria multiply and become enclosed in a matrix of extracellular polymeric substances (EPS) to form biofilms. Bacteria prefer living in biofilms above living in the planktonic state because the biofilm mode offers protection against a multitude of external stresses such as host-derived and conventional antimicrobial compounds. Biofilms also constitute a source for bacterial dissemination, playing an important role in the persistence of infections.

The introduction of an implant into tissue induces a foreign body reaction from the host immune defence system. The foreign body reaction consists of a series of processes, namely protein adsorption, acute and chronic inflammation, foreign body giant cell formation, and fibrotic encapsulation. The in vivo immune response to synthetic biomaterials devoid of exotoxins and toxic leachables is a mild inflammatory response, ensuing in the formation of a thin fibrous capsule which isolates the implant during the lifetime of the implant. Biotolerability is a term that is often coined as the ability of an implant in a host to elicit a low degree of chronic inflammation. As such, non-absorbable medical implants, including surgical meshes can be referred to as biotolerable.

Upon infection, the efficacy of the innate immune response around the implant to clear invading microbes is limited and bacteria compete with host cells for residence on the implant surface. During an implant associated infection, bacteria such as Staphylococcus aureus are capable of promoting a profibrotic response. Therefore, chronic infections may develop due to increased fibrotic encapsulation, reduced implant performance and increased biofilm protection from an already compromised innate immune response. For these reasons, modification of the surface of implants to prevent early bacterial adhesion has great potential in decreasing the incidence of implant-associated infections, potentially enabling the implant to trigger and guide a non-fibrotic wound healing, reconstruction and regeneration of surrounding tissue leading to biocompatibility.
rather than biotolerability.

Antifouling coatings based on hydrophilic polymers provide a hydration layer that repels microorganisms by acting as a physical barrier\textsuperscript{28}. Nanogels are aqueous, crosslinked colloidal particles with high potential as antifouling agents for implants due to their ability to form hydration layers\textsuperscript{29,30}. Recently, our group developed a poly-N-isopropylmethacrylamide (pNIPMAM) nanogel coating that reduced adhesion of \emph{S. aureus} by 99\% in a parallel plate flow chamber assay\textsuperscript{30}. Nanogels are envisioned to better perform than for example polyethyleneglycol, which can undergo oxidative degradation and modulation of protein structure \textit{in vivo}\textsuperscript{28}. Specifically, the pNIPMAM nanogel coating is stable for at least 13 days \textit{in vivo}\textsuperscript{51}. The purpose of this study is to assess the \textit{in vivo} efficacy of the pNIPMAM nanogel coating in preventing \emph{S. aureus} Newman infections and biofilm formation when applied on PVDF surgical meshes using a murine subcutaneous implantation model. \emph{S. aureus} is a common etiologic factor in implant-associated infections because it expresses several surface molecules which enables attachment to surfaces and consequent biofilm formation\textsuperscript{31}. In addition to this, the tissue response towards the nanogel coating will be assessed by analyzing the early (day 7) and late (day 13) acute phase of the foreign body reaction towards coated PVDF meshes with respect to pristine PVDF meshes under both infected and sterile conditions.

\section*{2 Materials and Methods}

\subsection*{2.1 pNIPMAM nanogels}

The monomer N-isopropylmethacrylamide (97\%, NIPMAM), the cross-linker \(N,N'\)-methylene bis(acrylamide) (99\%, BIS), the initiator ammonium persulfate (98\% APS), and polyethyleneimine (PEI, branched, Mw 25,000 g/mol) were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands. The fluorescent dye methacryloxyethyl thio carbamoyl rhodamine B (MRB) used for in vivo stability assessment (not evaluated here) was purchased from Polysciences, Inc., Hirschberg, Germany. NIPMAM was recrystallized from hexane; all other chemicals were used as received without any further purification. Ultrapure water (18.2 M\textOmega, arium 611 DI water purification system; Sartorius AG, Göttingen, Germany) was used in all experiments.

pNIPMAM nanogels were prepared by a precipitation polymerization method using \(N,N'\)-methylenebis(acrylamide) as a cross-linker as previously described\textsuperscript{30}. Briefly, 626 mg of NIPMAM, 12 mg of BIS and 10 mg of MRB was dissolved in 45 mL of water using a three-necked 100 mL flask equipped with a flat anchor-shaped mechanical stirrer and a reflux condenser. The reaction mixture was degassed with \(N_2\) for 1 h. The solution was heated till 70°C and the reaction was started by injecting the degassed initiator solution APS (11 mg) in 5 mL water into the reaction mixture. The reaction was continued for another 4 h at 70°C and 300 rpm under \(N_2\) atmosphere. After 4 h, the reaction mixture was cooled to room temperature and stirred overnight. The microgel dispersion was purified by ultracentrifugation.
and washed with water (3 times at 179,200 g). The final product (pNIPMAM nanogel) was freeze-dried after purification for further use.

The nanogel particles had a zeta potential of $-16 \pm 0.4$ mV$^{30}$, as determined with a Zetasizer Nano-ZS. As shown in Fig. S1, the volume phase transition temperature (VPTT) of the pNIPMAM nanogel is around 44 °C and in good agreement with previously described values$^{32}$.

2.2 Coating preparation on the biomaterial

PVDF meshes (Dynamesh® Endolap, Feg Textiltechnik GmbH, Aachen, Germany) of 1 cm$^2$ were coated with a pNIPMAM nanogel as previously reported$^{33,34}$. In brief, the PVDF meshes were washed with ethanol and water, after which the meshes were placed in sterile cabinet to dry. Next, the meshes were plasma oxidized for 10 min at room temperature with Plasma Active Flecto 10 USB at 100 mTorr and 0.2 mbar. Afterwards, the meshes were immersed in a solution of polyethylenimine (PEI, 1.5 mg/mL, 0.15 wt %) for 20 min while the pH of the solution was adjusted to pH 7 with 0.1 M HCl. After rinsing with water 3 times, the PEI coated meshes were dried at room temperature. A pNIPMAM nanogel suspension (5 mg/mL, 0.15 wt %) in water was sprayed on the both sides of PEI-coated meshes until the entire surface was wetted to create the nanogel coating. The pNIPMAM-coated meshes were first dried at room temperature in ambient air and then overnight at 50°C. To remove unbound nanogels, the coated meshes were immersed in water for at least 5 h and the water was replaced three times during this washing step.

2.3 Bacterial strains and growth conditions

Bacterial strains were cultivated as described in Chapter 4. Briefly, bioluminescent S. aureus Newman WT (S. aureus Newman lux) were cultured on Tryptone Soy Agar (TSA) (Oxoid Ltd., Basingstoke, UK) containing 200 μg/mL kanamycin (24 h, 37°C). A preculture was made by inoculating a bioluminescent colony in 10 mL of tryptone soy broth (TSB, Oxoid Ltd.) with 200 μg/mL kanamycin (24 h at 37°C, 150 RPM). A main culture was made by inoculating 2 mL of the preculture in 40 mL TSB with 200 μg/mL kanamycin and grown for 16 h at 37°C, 150 RPM. The main culture was harvested (5 min, 10°C, 5000 g) and washed three times with phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 7.4). Then the bacterial suspension was sonicated in an ice water bath (3 times, 10 s at 30 Watts). Bacteria were resuspended in PBS to a concentration of 5x10$^9$ bacteria/mL.

2.4 Animals, surgical procedure and bioluminescence imaging

Permission for all animal experiments was obtained from the competent authority (Centrale Commissie Dierproeven, CCD in the Netherlands) (IvD protocol 197305-01-001), and experiments were performed according to their guidelines. Surgical implantation and infection of PVDF meshes was executed as described in Chapter 4. Briefly, PVDF meshes, with and without a pNIPMAM nanogel coating, were subcutaneously implanted in 6 week old female Balb/c OlaHsd mice weighing around 20 g. A 1 cm$^2$ coated or uncoated PVDF mesh was inserted in a
subcutaneous pocket created in the dorsal cervical area of animals. For the sham group, no mesh was inserted. The wounds were closed with Histoacryl followed by an injection of 20 µL of sterile PBS or *S. aureus* Newman lux (5x10⁹ bacteria/mL) in the pocket. Temgesic (0.05 mg/kg) was subcutaneously administered for pain relief. Six groups of mice were created: (1) a group with coated mesh and *S. aureus* Newman lux (n = 7 for day 7, n = 3 for day 13), (2) a group with coated mesh and PBS (n = 4 for day 7, n = 3 for day 13), (3) a group with uncoated mesh and *S. aureus* Newman lux (n = 7 for day 7, n = 3 for day 13, data is taken from Chapter 4), (4) group with uncoated mesh and PBS (n = 4 for day 7, n = 3 for day 13, data is taken from Chapter 4), (5) group with sham surgery and *S. aureus* Newman lux (n = 3 for both day 7 and 13, data is taken from Chapter 4) and (6) group with sham surgery and PBS (n = 3 for both day 7 and 13, data is taken from Chapter 4).

At day 7 and day 13, the meshes and the surrounding tissues were collected from the subcutaneous pockets under anaesthesia. Each sample was dissected into two parts, one half was utilized for colony forming units (CFU) determination and the other half was fixed overnight in 4% paraformaldehyde (PFA) at 4°C, dehydrated using a series of ethanol in ascending concentrations (70%, 80%, 90%, 100%), washed with xylene and embedded in paraffin for histological analysis. Mice were terminated using cervical dislocation, after sample collection. Bioluminescence was measured with an *In Vivo* Imaging System (IVIS Lumina II Imaging System, PerkinElmer, Waltham Massachusetts, USA) at 0, 1, 3, 7, 10 and 13 days post implantation and injection with bacteria or PBS, except for the sham group with a PBS injection, where bioluminescence was measured for up to day 7, as described in Chapter 4.

### 2.5 Determination of bacterial infection by CFU counting

Tissue biopsies with or without mesh implants from all groups were stored in sterile eppendorf tubes on ice containing glass beads (1 mm) and 1 mL of Reduced Transport Fluid for maximum 2 h. Samples were subsequently homogenized by a bead-beater (3000 bpm, 1 min, Sartorius, Goettingen, Germany), followed by 30 s of vortexing, 5 min of sonification in a sonification bath, and another 30 s of vortexing. Then samples were serially diluted 1:10 and three 10 µL droplets of the first seven dilutions (10⁰-10⁷) were plated on TSA. After 16 h of incubation at 37 °C all plates were imaged in the IVIS to count the number of bioluminescent colonies.

### 2.6 Histological analysis of the tissue response

All staining procedures were performed on 5 µm thick slices that were cut from the paraffin embedded samples. The slices were deparaffinized in xylene (EMSURE, Darmstadt, Germany) for 10 min. Followed by ethanol rehydration steps in descending concentrations of ethanol (100%, 96%, 70%). After washing in demineralized water, hematoxylin and eosin staining was executed as described in Chapter 4. Briefly the slices were stained with Mayer’s hematoxylin (Sigma, ST. LOUIS, USA) for 5 min and washed several times with demineralized water and tap water to develop the blue colour of the stain. Afterwards, eosin (Eosin Y) was performed for 40 s, after which the samples were dehydrated and incubated in
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xylene. Finally, the slices were covered with Permount mounting medium (Fisher Scientific, New Jersey, USA) and a glass cover slip and allowed to dry overnight in a fume hood at room temperature. Images were obtained with a bright field light microscope.

2.7 Detection of S. aureus protein A with immunohistochemistry

All immunohistochemical staining procedures were performed on 5 µm thick slices that were deparaffinized and rehydrated as described above. The slices were subjected to heat induced epitope retrieval to unmask antibody binding sites in citrate buffer (10 mM citric acid, 0.05% Tween20, pH 6) in a water bath (95°C, 50 min). After this, the samples were washed twice with PBS supplemented with 0.05% Tween20. Blocking of endogenous binding sites was performed by incubating the slices in PBS supplemented with 5% goat serum (Bio-Rad, Hercules, California, USA). Subsequently, slices were incubated with primary antibody: Rabbit anti-

*S. aureus* antibody (1:50, Bio-Rad) for 20 h at 4°C to target S. aureus protein A. As a negative control, primary antibody was replaced with equal amount of antibody diluent (PBS supplemented with 1% goat serum). After washing with PBS supplemented with 0.05% Tween20, the slices were treated with 0.3% H$_2$O$_2$ for 10 min in darkness to quench endogenous peroxidase activity. After washing with PBS supplemented with 0.05% Tween20, bound antibodies were detected with polyclonal, biotinylated Goat Anti-Rabbit immunoglobulin (1:2000, Dako, Santa Clara, CA, USA) secondary antibodies for 45 min at room temperature. After washing with PBS supplemented with 0.05% Tween20, slices were treated with Avidin-Biotin Horseradish Peroxidase complex (ABC Peroxidase staining kit, Thermo scientific, Illinois, USA) according to the manufacturer’s instructions. Slices were then stained with 3,3’-diaminobenzidine tetrahydrochloride (DAB, Thermo Scientific) and counterstained with Mayer’s hematoxylin as described in Chapter 4. The samples were mounted in Permount mounting medium, covered with a glass cover slip and allowed to dry overnight at room temperature in a fume hood. Brightfield images were acquired with a light microscope. Four mesh filaments in each sample were utilized to determine the mean grey value of the brown channel after colour deconvolution with ImageJ unless fewer mesh filaments were found.

2.8 Masson’s trichrome staining for collagen determination

5 µm slices of paraffin-embedded tissues from mice with uncoated or pNIPMAM-coated PVDF meshes with a bacterial inoculum or PBS, were deparaffinized and rehydrated as described above. The samples were stained for 10 min in Weigert’s hematoxylin solution to stain nuclei brown-black. The hematoxylin stain was developed in running tap water for 10 min. Next, the slices were washed in demineralized water. The samples were then incubated in Biebrich scarelet-acid fuchsin (Sigma-Aldrich, Darmstadt, Germany) for 12 min. This step was followed by incubating the slices in a 1:1 mixture of phosphomolybdic (Sigma-Aldrich) and phosphotungstic acid (Sigma-Aldrich) for 12 min which stains collagen, connective tissue and cytoplasm red. This step was followed by incubating the slices in decolorizing agents, a 1:1 mixture of phosphomolybdic (Sigma-Aldrich) and phosphotungstic acid (Sigma-Aldrich), for 12 min. Collagen bundles were stained
blue after incubating in aniline blue (Sigma-Aldrich) for 10 min. After washing with demineralized water, the slices were dehydrated in increasing concentrations of ethanol (70%, 96%, 100%) and finally a 6 min incubation in xylene, both at room temperature. The slices were covered with Permount mounting medium and covered with a glass cover slip and allowed to dry overnight at room temperature in a fume hood. Images were obtained with a brightfield light microscope. For quantitative analysis of the blue collagen stain, a blue filter of a home-made software was used to isolate the blue stain of collagen in acquired brightfield images. Afterwards, the mean gray value was determined with ImageJ.

2.9 Statistical analysis

Statistical evaluation was performed with Graphpad Prism 9 (Graphpad, San Diego, United States). Statistical analysis was done using the Mann-Whitney test where P < 0.05 was considered significant (P < 0.0125 after Bonferroni correction).

3 Results

3.1 Bacterial infections

Bioluminescence measurements by IVIS were utilized to follow the course of infection during the study period (Fig. 1A). The total bioluminescence was constant (approximately 10⁵ p/s) at all measured time points for the control groups, which are mice that received a coated mesh, an uncoated mesh or underwent a sham surgery and received a PBS injection (Fig. 1A). The total bioluminescence associated with an S. aureus Newman lux infection in mice that underwent a sham surgery, received a coated or uncoated mesh remained stable from day 0 till day 7. From day 7, there was a gradual decline in bioluminescence up to day 12, where it reached the level of the coated or uncoated mesh without bacteria and the sham without bacteria. At day 13, a slight increase in bioluminescence occurred. There was no significant difference between the total bioluminescence from the S. aureus Newman lux infection on coated PVDF meshes and the total bioluminescence from mice with uncoated PVDF meshes (Fig. 1A). The bioluminescence measured for the sham group of mice with an S. aureus infection was 10-fold lower than that detected in the group of mice with a coated or uncoated mesh with a bacterial infection. Fig. 1B shows ex vivo CFU from tissues with or without a mesh from all groups of mice at day 7 and day 13. Similar amount of CFUs (10⁸) were determined for mice with a coated or uncoated mesh and an infection at both 7 and 13 days. Note that tissue from mice inoculated with sterile PBS contained also some bacteria, this contamination was probably caused during the explantation process. Such small contaminations could not have an effect on the analysis of the infected groups since the number of CFU’s in these groups were 10⁴-10⁵ times higher. A small number of colonies on the agar plates obtained from biopsies from the infected mice have been tested for nuclease production and no WT bacteria were found in the group infected with the ΔnucI mutant strains or vice versa (data not shown), which indicates that no cross contaminations took place during implantation and inoculation. Nuclease production was assessed by a nuclease activatable fluorescence probe⁴³.
Figure 1. Total bioluminescence and CFU counts from mice with or without a coated or uncoated PVDF mesh implant after inoculation with *S. aureus* Newman lux bacteria (1x10^8 bacteria/mL) or sterile PBS. (A) Total bioluminescence as a function of time. (B) Colony forming units (CFU) obtained from biopsies taken from the mice (n = 3) after termination at 7 or 13 days post mesh implantation. Note that the data from the uncoated mesh + WT, Sham + WT, Sham + WT and Sham + PBS are taken from Chapter 4, Figure 1 and 2.

3.2 Tissue response

The host response to the nanogel coating was examined after hematoxylin and eosin staining of *ex vivo* samples taken 7 or 13 days after implantation of the coated or uncoated mesh with *S. aureus* Newman lux or sterile PBS. The mesh filaments (M) were identified as disc-shaped openings with a diameter of 144 ± 18 µm (n = 15), falling within the 85-165 µm diameter range of the PVDF filaments (Fig. 2). A high influx of immune cells, as identified by their large dark blue nuclei, were found in tissues from mice with a coated mesh implant and an *S. aureus* Newman lux infection (Fig. 2-1, Fig. 2-5) at day 7. In case of the uncoated mesh with an
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*S. aureus* Newman lux infection, the tissue surrounding the filaments stained strongly for eosin while almost no hematoxylin stained nuclei were identified, supposing the absence of immune cells in the surrounding tissues. Bacteria were identified as purple granules enclosed in protein fibres both at the mesh-tissue interface and within the surrounding tissues (Fig. 2-2, Fig. 2-6, black arrowheads). No foreign body giant cells (giant multinucleated cells) were identified in tissues surrounding coated or uncoated mesh with an *S. aureus* Newman lux infection and there were more coated filaments with blood vessels (39%) than there were uncoated filaments (12%) (Table 1). The immune cell infiltration in mice that received a coated mesh and a PBS injection was lower than that of mice with a coated mesh implant and a bacterial infection at day 7 (compare Fig. 2-3 and Fig. 2-7 with Fig. 2-1 and Fig. 2-5, blue hematoxylin stained nuclei).

The immune cells surrounding the coated filaments with PBS, mainly consisted of neutrophils, as identified by their irregular nuclei (Fig 2-3 and Fig. 2-7, yellow arrowheads). At day 7, blood vessels (Table 1) and clouds of hematoxylin stained DNA (Fig. 2-3, white arrow) were discovered near the sterile coated filaments while no foreign body giant cells were identified (Table 1). At day 7, a lower immune cell infiltration was present in the group of mice that received an uncoated mesh implant with PBS (Fig. 2-4) in comparison to tissues surrounding the coated sterile mesh (Fig. 2-3). Tissue surrounding the filament in the latter group consisted of loose connective tissue (LC, Fig. 2-4), scattered with possibly macrophages (blue arrowheads) and fibroblast-like cells (orange arrowhead) as identified by their round nuclei and elongated morphology respectively (Fig. 2-4, Fig. 2-8). Also, foreign body giant cells and blood vessels were identified in the vicinity of the mesh filaments (Table 1).

At day 13, eosin staining was still intense in tissue samples of mice that received a coated or uncoated mesh and were infected with *S. aureus* Newman lux bacteria, but the tissue surrounding the coated mesh filaments were devoid of immune cells as there was a lack of the blue hematoxylin stained nuclei (compare Fig. 2-9 and Fig. 2-13 with Fig. 2-10 and Fig. 2-14). In addition to this, bacteria were found as purple granules enclosed in eosinophilic tissue at the mesh-tissue interface as well as within the surrounding tissues of both coated and uncoated meshes with an *S. aureus* Newman lux infection (Fig. 2-9, Fig. 2-13 and Fig. 2-10, Fig. 2-14, black arrowheads). At this time point, there were still no foreign body giant cells around coated or uncoated filaments with an *S. aureus* Newman lux infection and blood vessels were only found in tissues surrounding the uncoated filaments (Table 1). For mice that received a coated or uncoated mesh with a PBS injection, inflammatory cell infiltration around the filaments was low (Fig. 2-11 and Fig. 2-12) with mainly macrophages (blue arrowheads) and the tissue consisted of loose connective tissue. Blood vessels (single layer of endothelial cells surrounding red eosin-stained erythrocytes, green arrowhead, Fig. 2-11, Fig. 2-15), as well as foreign body giant cells (red arrowhead Fig. 2-12, Fig. 2-16), were recognized near coated and uncoated filaments with a PBS injection (Table 1).

**Table 1.** Percentages of tissue samples with foreign body giant cells (FBGCs) and blood vessels around filaments with and without a coating and with and without an *S. aureus* Newman lux infection.
contamination evaluated on day 7 and 13. Note that the data from the uncoated mesh + S. aureus Newman lux and uncoated + PBS are taken from chapter 4.

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<th>Days after implantation</th>
<th>Total number of examined tissue samples around filaments</th>
<th>Percentage tissue samples around filaments with FBGCs</th>
<th>Percentage tissue samples around filaments with blood vessels</th>
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3.3 *S. aureus* detection with immunohistochemistry

Antibodies targeting *S. aureus* surface protein A were utilized to detect bacteria in *ex vivo* samples taken 7 days after implantation of a coated or uncoated mesh in combination with an *S. aureus* Newman lux or PBS injection. Tissues from mice with a coated implant in combination with an *S. aureus* Newman lux infection showed very low bacteria (protein A staining), as the characteristic brown stain from DAB was very low (black arrowhead, Fig. 3A-1). However, at day 13, detected bacteria (protein A) had accumulated at the mesh-tissue interface as well as in the surrounding tissues (black arrowheads, Fig. 3A-5). In the group of mice with an uncoated mesh and an *S. aureus* Newman lux infection, bacteria (protein A) accumulated at the filament-tissue interface and within the surrounding tissues at both 7- and 13-days post implantation (Fig. 3A-2 and Fig. 3A-6). Very low bacteria (protein A) were detected in samples from the group of mice that received a coated implant with PBS at both day 7 (Fig. 3A-3) and day 13 (Fig. 3A-7), as there was low brown DAB precipitate.

Also, the detected *S. aureus* (protein A) in tissues from the group of mice with an uncoated implant and PBS was low and diffuse both at day 7 (Fig. 3A-4) and day 13 (Fig. 3A-8). Negative controls with only secondary antibody did not show the characteristic brown DAB precipitate (Fig. 3A-9, through 16). Analysis of the mean grey value of the DAB stain, showed that at day 7 the detected bacteria (protein
A) in mice with an uncoated mesh and an *S. aureus* Newman lux infection was significantly higher (P = 0.0072) than that of mice with a coated mesh and an *S. aureus* Newman lux infection as well as related to mice with PBS and an uncoated mesh (P= 0.001) (Fig. 3B). At day 13, no significant difference was found between bacteria (protein A) detected in samples from mice with a coated mesh and an uncoated mesh with an *S. aureus* Newman lux infection (Fig. 3C). Statistical analysis of the mean grey value of the DAB stain performed for the group with a coated mesh and an *S. aureus* Newman lux infection at day 13 is based on data from two out of the three tissues selected for histological analysis, as the third sample lacked filaments.

**Figure 2.** Representative images of hematoxylin (dark blue), eosin (pink) staining of mice tissue biopsies at positions surrounding a single filament of a nanogel-coated or uncoated PVDF surgical mesh implant (M) in combination with an *S. aureus* Newman lux or sterile PBS injection, 7 and 13 days after implantation (n=3) at 200X and 1000X magnification. Black arrowheads show locations of bacteria, yellow arrowhead shows locations of neutrophils, orange arrowhead shows location of fibroblast-like cells, blue arrowheads show locations of macrophages, green arrowheads show locations of blood vessels and white arrowhead shows hematoxylin stained DNA clouds. Scale bar 200X = 100 µm and scale bar 1000X = 25µm. Note that the data from the uncoated mesh + *S. aureus* Newman lux and uncoated +
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PBS are taken from chapter 4.

Figure 3. (A) Representative images (3A-1 through 3A-8) immunohistochemical staining (brown) of *S. aureus* protein A in mice tissue biopsies at positions surrounding a single filament of a nanogel-coated or uncoated PVDF surgical mesh implant (M) in combination with an *S. aureus* Newman lux or sterile PBS injection, 7 and 13 days after implantation (*n* = 3). Black arrowheads show locations of bacteria. As a negative control for each antibody staining (3A-9 through 16), primary antibody was
replaced with equal volumes of antibody diluent. Images with negative controls correspond to the same mouse and the same location in the sample as used for immunohistochemical staining with the primary antibody. Scale bar = 100 µm. Semi-quantitative analysis of immunohistochemically determined *S. aureus* protein A at (B) 7 days and (C) 13 days post PVDF surgical mesh implantation and infection (n = 3). Data points represent data from tissue from 3 different mice where 4 filaments (n = 17) from each mouse were analysed, except when only 1 (n = 2) or 2 filaments (n = 3) were present. Error bars show the standard error of the mean. Statistical significance between mice with coated and uncoated mesh with a sterile PBS injection is indicated with asterisks, **P ≤ 0.001. Note that the data from the uncoated mesh + *S. aureus* Newman lux and uncoated mesh + PBS were generated utilizing mice tissue taken from chapter 4.

3.4 Collagen deposition

Collagen deposited around the mesh filaments (M) in biopsies from mice in all groups was stained blue with Masson’s trichrome staining (Fig. 4A). At day 7, there was no blue stained collagen in tissue surrounding coated meshes in mice with an *S. aureus* Newman lux infection and the cytoplasm of tissue surrounding the mesh filaments was mainly stained red by Biebrich scarlet-acid fuchsin (Fig. 4A-1). Mice that received an uncoated mesh with an *S. aureus* Newman lux infection showed deposited collagen (Fig. 4A-2, pink arrowheads), bordered by tissue stained red by Biebrich scarlet-acid fuchsin. Collagen deposition in the control groups with coated (Fig. 4A-3, blue stain) or uncoated mesh (Fig.4A-4, blue stain) under sterile conditions was low at this time point. However, long collagen fibres aligned perpendicular (Fig. 4A-4) to filaments were identified more frequently around uncoated (71%) than around coated mesh filaments (19%) (Table 2). At day 13, very low blue collagen stain was detected around coated (Fig. 4A-5) or uncoated meshes (Fig.4A-6) with an *S. aureus* Newman lux infection and tissues surrounding the filaments in these groups were mostly stained red by Biebrich scarlet-acid fuchsin. Under infected conditions, long collagen bundles that aligned perpendicular to the filaments were identified in 30% and 39% of the coated and uncoated samples respectively (Table 2). Under sterile conditions higher percentages (61% and 70% of samples) were found positive in aligned collagen bundles (Fig. 4-7 and 4-8 and Table 2). Quantitative analysis of the mean grey value of blue collagen stain (after blue filter) however did not reveal significant differences between either of the groups at both 7 and 13 days after implantation (Fig. 4B).

4 Discussion

The purpose of this study was to assess the *in vivo* efficacy of the pNIPMAM nanogel coating in preventing *S. aureus* Newman infections around an implanted mesh. Three independent assessment methods were applied to study the persistence of the induced infection: bioluminescence imaging, CFU counting and histology. Since it was anticipated that biofilm formation on coated surfaces was affected by the presence of the coating due to reduced bacterial adhesion, we also wanted to investigate biofilm formation. It is difficult to clearly distinguish biofilm formation from bacterial presence in histological hematoxylin-eosin stained samples (See Fig. 2-6 and 2-14). However, since the *ex vivo* immunohistochemical staining of *S. aureus* Newman lux mainly showed biofilm formation at or in close
proximity to the surface of uncoated meshes. Therewith, we provided evidence inferring that the pNIPMAM coating prevented the formation of detectable biofilms on PVDF meshes for at least 7 days \textit{in vivo} (compare Fig. 2 and 3). At day 13, biofilms detected at the filament-tissue interface as well as within the surrounding tissues may possibly indicate detachment of the pNIPMAM nanogel coating potentially as a result of a disruption of the electrostatic interaction between the nanogel and PEI-modified PVDF mesh\textsuperscript{37}. Similar to the differences in biofilm formation between coated and uncoated materials, differences were observed in the immune response towards the bacteria around coated and uncoated PVDF meshes on both days 7 and 13 (compare Fig. 2-5 and Fig. 2-6 with Fig. 2-13 and Fig. 2-14). The infection around uncoated meshes at day 7 led to apoptosis of immune cells as was not observed for coated meshes (Fig. 2-5 and Fig. 2-6), implying that this form of immune evasion by \textit{S. aureus} bacteria as observed in Chapter 4 was reduced around the coating.

**Figure 4.** (A) Collagen deposition as determined with Masson’s trichrome stain in tissue biopsies at positions surrounding a single filament of a nanogel-coated or uncoated PVDF surgical mesh implant (M) in combination with \textit{S. aureus} Newman lux infection or sterile PBS injection, 7 and 13 days after implantation (n=3). Blue colour shows location of collagen bundles, red colour shows location of cytoplasm and muscles (white arrowhead), pink arrowheads show locations of collagen films and brown-black colour shows location of nuclei. Scale bar=100 µm (B) Semi-quantitative analysis of collagen. Datat points represent datat from tissue from 3 different mice where 6 or 4 filaments from each mouse
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were analysed. Error bars show the standard error of the mean. Statistical significance
between detected collagen at day 7 and day 13 is indicated with asterisks, ** \( P \leq 0.001 \).

**Table 2.** Long collagen bundles around mesh filaments. Note that the data from the
uncoated mesh + *S. aureus* Newman lux, and uncoated mesh + PBS were taken from
Chapter 4.

<table>
<thead>
<tr>
<th>Group of mice implanted with a PVDF mesh with or without coating</th>
<th>Sterile (PBS) or infected with <em>S. aureus</em> Newman lux (+)</th>
<th>Days after implantation</th>
<th>Total number of examined tissue samples around filaments</th>
<th>Percentage tissue samples around mesh filaments with long collagen bundles aligned perpendicular to the filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coated</td>
<td>+</td>
<td>7</td>
<td>17</td>
<td>0%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>+</td>
<td>7</td>
<td>16</td>
<td>0%</td>
</tr>
<tr>
<td>Coated</td>
<td>PBS</td>
<td>7</td>
<td>26</td>
<td>19%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>PBS</td>
<td>7</td>
<td>14</td>
<td>71%</td>
</tr>
<tr>
<td>Coated</td>
<td>+</td>
<td>13</td>
<td>10</td>
<td>30%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>+</td>
<td>13</td>
<td>14</td>
<td>39%</td>
</tr>
<tr>
<td>Coated</td>
<td>PBS</td>
<td>13</td>
<td>17</td>
<td>61%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>PBS</td>
<td>13</td>
<td>18</td>
<td>71%</td>
</tr>
</tbody>
</table>

Interestingly, the prevention of *S. aureus* Newman lux biofilm formation at day 7
due to the presence of the coating, did not reflect in a reduction of bacterial infection
as observed by bioluminescence imaging and CFU counting (compare Fig 3A-1, Fig. 3A-2 and Fig. 3B with Fig.1A and Fig. 1B). This seeming contradiction was
mentioned in earlier reports showing that prevention of bacterial adhesion does not
necessarily abolish biomaterial-associated infections, as surrounding tissue may
also shelter accumulated bacteria\(^{38,39}\). However, in this study, detectable biofilms
were not identified in surrounding tissues of pNIPMAM nanogel-coated PVDF on
day 7 (compare Fig. 2-5 with Fig. 2-6 and Fig. 3A-1 with Fig. 3A-2), nor at deeper
locations in the tissues surrounding the filaments (Fig. S2). Bacteria in a biofilm
mode of growth have a lower bioluminescence due to decreased metabolic activity
possibly as a result of limited nutrient availability or a reduction in the NADPH
redox pool to prevent respiration related bacterial cell damage\(^{40,41}\). Therefore, the
similar bioluminescence and CFU counts around coated and uncoated filaments
at day 7, suggest that the infection around both coated and uncoated filaments
consisted of similarly metabolically active bacteria. However, bacteria around the
pNIPMAM-coated PVDF meshes were in a more dispersed state, implying that the
infection may be more vulnerable to for example antibiotic treatment. Our
suggestion is supported by a recent study by Zhang et al.\(^{42}\) where *S. aureus* Xen29
infection of animals with uncoated and poly(sulfobetaine methacrylate)-coated
metallic pins. They observed equivalent bioluminescence and CFU counts for
uncoated and coated pins, but coated metallic pins showed increased susceptibility
to vancomycin with respect to the infection in mice as compared to uncoated metallic pins\textsuperscript{42}.

Apart from the effect of the coating on biofilm formation, our results indicate that the pNIPMAM coatings affected the foreign body reaction around the filaments as well. Adhesion of macrophages is a prerequisite for the formation of foreign body giant cells and can be influenced by material surface properties\textsuperscript{43,44}. Based on the percentage of samples under sterile conditions in which foreign body cells were identified (Table 1), the coating seemed to have concealed the filaments and prevented the triggering of a proper foreign body reaction. Possibly, as a consequence of the prevention of macrophage adhesion by the hydration layer formed by the pNIPMAM nanogel coating, as previously reported for other hydrophilic coatings such as hyaluronic acid\textsuperscript{37} and poly(sulfobetaine methacrylate)\textsuperscript{45,46}. In line with this, collagen formation observed after 7 days around filaments was randomly oriented by the presence of the coating (Table 2), indicating the possibility of reduced scarring associated with aligned collagen fibres\textsuperscript{47,48}. Under infected conditions, foreign body giant cell formation was not observed in tissues surrounding coated and uncoated meshes, probably due to lack of macrophage adhesion or consequent prolonged acute phase of the foreign body response\textsuperscript{49}, delaying macrophage recruitment. Similarly, collagen deposition was not augmented under infected conditions. These observations are in disagreement with earlier reports that fibrotic encapsulation of biomaterials is enhanced during an infection\textsuperscript{27,50}. Also, the differences in foreign body giant cell formation around filaments between coated and uncoated filaments was not reflected in the amount of blood vessels identified (Table 1).

**Conclusion**

This study provided evidence that the pNIPMAM nanogel could not reduce the overall infection as both bioluminescence and CFU were high for coated and uncoated PVDF meshes after subcutaneous implantation in mice. However, the nanogel coating limited the formation of detectable biofilms as well as the apoptosis of immune cells in tissues surrounding coated PVDF surgical meshes for at least 7 days under infected conditions. Therefore, the infection is suggested to be more susceptible to antibiotic treatment than bacteria in biofilms around uncoated PVDF surgical meshes. Under sterile conditions, the tissue response to coated and uncoated PVDF was similar, specifically at day 13, marked by equivalent immune cell infiltration, foreign body giant cell formation, collagen deposition and formed blood vessels. It can therefore be concluded that the pNIPMAM-based nanogel is as biotolerable as pristine PVDF.
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Supplementary figures

Figure S1. Hydrodynamic Diameter ($D_h$) as a function of temperature (T) of pNIPMAM nanogel.
Figure S2. Images showing accumulation of bacteria (black arrowhead) in the tissue further away from the mesh filaments (M) in mice with uncoated PVDF surgical mesh and S. aureus Newman lux infection 7 days after implantation. Scale bar = 600 µm. Note that the data from the uncoated mesh + S. aureus Newman lux, and uncoated mesh + PBS were taken from chapter 4.
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References


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52. Forson *et al.*, Micrococcal nuclease is essential for *Staphylococcus aureus* biofilm formation in a murine implant infection model (2022).