CHAPTER 3

Eradicating Infecting Bacteria while Maintaining Tissue Integration on Photothermal Nanoparticle-coated Titanium Surfaces

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Abstract
Photothermal nanoparticles locally release heat, when NIR-irradiated. Clinical application initially involved tumor treatment, but currently extends towards bacterial infection-control. Application towards much smaller, micrometer-sized bacterial infections however, bears the risk of collateral damage by heat dissipating into tissue surrounding an infection site. This can become a complication when photothermal nanoparticle coatings are clinically applied on biomaterial surfaces requiring tissue integration, such as for instance titanium-made, bone-anchored dental implants. Dental implants can fail due to infection in the pocket formed between the implant screw and surrounding soft tissue (“peri-implantitis”). We address the hitherto neglected potential complication of collateral tissue damage by evaluating photothermal, polydopamine-nanoparticle (PDA-NP)-coatings on titanium surfaces in different co-culture models. NIR-irradiation of PDA-NP-coated (200 μg/cm²) titanium surfaces with adhering *Staphylococcus aureus* killed staphylococci within an irradiation-time window of around 3 min. Alternatively, when covered with human-gingival-fibroblasts, this irradiation-time window maintained surface coverage by fibroblasts. Contaminating staphylococci on PDA-NP coated titanium surfaces, as can be per-operatively introduced, reduced surface coverage by fibroblasts and this could be prevented by NIR-irradiation for 5 min or longer prior to allowing fibroblasts to adhere and grow. Negative impacts of early post-operative staphylococcal challenges to an existing fibroblast-layer covering a coated surface were maximally prevented by 3 min NIR-irradiation. Longer irradiation times caused collateral fibroblast damage. Late post-operative staphylococcal challenges to a protective keratinocyte-layer covering a fibroblast-layer, required 10 min NIR-irradiation for adverting a staphylococcal challenge. This is longer than foreseen from mono-culture studies, because of additional heat uptake by the keratinocyte-layer. Summarizing, photothermal treatment of biomaterial-associated-infection requires precise timing of NIR-irradiation to prevent collateral damage to tissue surrounding the infection site.
3.1. Introduction

Photothermal therapy (PTT) is more and more considered as an alternative bacterial infection-control strategy, in an era that alludes the end of antibiotic treatment of infection [1,2]. In a pessimistic scenario, infection by antimicrobial-resistant bacteria threatens to become the number one cause of death in the year 2050 [3]. Photothermal nanoparticles locally release heat, when photo-activated at suitable near-infrared (NIR) wavelengths [4]. The use of PTT in medicine originated as an anti-tumor strategy [5-7] and is currently finding its way towards bacterial infection-control. As a clear advantage, PTT may be expected to work indiscriminately towards different bacterial strains, regardless of Gram-character or antibiotic-resistance. Indeed, photothermal copper sulfide nanoclusters effectively killed planktonic levofloxacin-resistant Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Bacillus amyloliquefaciens [8], while photothermal N-vinylpolycaprolactam-gold nanorods killed planktonic E. coli, Acinetobacter baumannii and Enterococcus faecalis [9]. However, photothermal killing of planktonic bacteria depends heavily on the ratio of photothermal nanoparticle and bacterial concentration, along with the suspension volume in which heat generated is dissipated.

Clinically, bacterial infections are seldom caused by planktonic bacteria, but mainly by bacteria in a biofilm-mode of growth, in which bacteria adhere and adapt themselves to a substratum surface by matrix production [10]. “Surface” in this definition can either mean the surface of other bacteria, tissue cells, teeth or implanted biomaterials (joint prostheses, ocular or dental implant and many others). Effective PTT of bacterial infections requires targeting of photothermal nanoparticles to the infection site and precise NIR-irradiation. Modification of photothermal nanoparticles, such as by zwitterionic, pH-responsive molecules to target photothermal nanoparticles to a bacterial infection site is not trivial however, requiring sophisticated chemistry [11]. Moreover, bacterial infection-sites are orders of magnitude smaller in size than tumors [12], which makes precise NIR-irradiation more difficult, while heat dissipation into surrounding tissue may cause collateral tissue damage, which is less critical in larger-sized tumor control.

The need to target photothermal nanoparticles to an infection-site can be circumvented, when photothermal nanoparticles are applied as a coating on biomaterials implants [4,13-15]. Bacterial challenges form the main cause of failure of biomaterials implants, because biomaterial-associated infections are particularly hard to treat with antimicrobials, including antibiotics [16]. The use of NIR-irradiation of photothermal nanoparticle coatings to kill infecting bacteria on an implant surface bears the risk of collateral damage to tissue cells integrating the implant. Tissue integration is known to provide the best protection against post-operative infection of biomaterials implants, as arising e.g. from invasive surgery or trauma [17]. Hitherto, preserving tissue integration of a biomaterials implant has been grossly neglected in the development of photothermal nanoparticles as a novel infection-control strategy, possibly by the lack of suitable in vitro models. Suitable models for evaluating photothermal nanoparticles as a novel infection-control strategy need not only involve mono-culture studies with bacteria or cells, but also bi-culture studies with simultaneous involvement of bacteria and cells, and preferably possess three-dimensional (3D) features to account for the dissipation of the heat generated into tissue surrounding an infection-site.
Recently, we published a 3D-tissue infection-model mimicking the soft-tissue seal around a dental implant, arguably representing the most frequently applied biomaterials implant [18]. A dental implant consists of a titanium screw and a supra-gingival part. The implant screw penetrates the gingiva to become anchored in the jaw bone. Composite tooth structures are subsequently attached to the supra-gingival part of the screw. Dental implants are prone to infection (“peri-implantitis”), that occurs in the pocket formed between the implant screw and surrounding soft tissue. Formation of a soft-tissue seal consisting of fibroblasts covered with keratinocytes closely adhering to implant surface, protects the osseo-integrated implant screw against bacterial challenges [19]. The peri-implantitis model was set-up by growing keratinocytes on a membrane filter in a transwell system, while fibroblasts were adhering to a titanium surface underneath the membrane. Keratinocytes could directly contact the fibroblast underneath the membrane, as an essential feature of 3D-tissue models [20]. In the model, bacterial challenges could either be applied as a contamination on the biomaterial surface as in per-operative infections [21]. Or by adhering to the keratinocyte seal above the fibroblasts as in post-operative infections during different stages of healing [22].

In this chapter we describe the preparation of a NIR-activatable, polydopamine nanoparticle-coating on titanium, with the aim of deriving photothermal conditions for the prevention and treatment of biomaterial-associated infections that maintain tissue integration. To this end, photothermal nanoparticle-coatings will be evaluated in various mono- and bi-culture models, including the above described 3D-tissue infection-model of peri-implantitis. Tissue integration will be challenged with \textit{S. aureus} in a per- and post-operative infection-mode, evaluating both bacterial killing and collateral damage to fibroblasts integrating the surface. \textit{S. aureus} was chosen as a pathogen, as it is emerging as a causative pathogen in peri-implantitis [23]. Polydopamine (PDA) photothermal nanoparticles (NPs) were selected for coating titanium surfaces because of their good biocompatibility [24], biodegradability [25] and strong NIR-absorption [26]. Results will point to optimal NIR-irradiation times for stimulating and maintaining tissue integration, while eradicating infectious bacteria. Although carried out in an oral peri-implantitis model, results bear equal relevance to other biomaterials implants applied in the human body that require tissue integration, such as e.g. percutaneous orthopedic screws, bone-anchored joint prostheses or hearing-aids.

3.2. Materials and methods
3.2.1. Preparation of a photothermal, polydopamine nanoparticle-coating on titanium surfaces and its characterization
Photothermal, PDA-NPs were synthesized as described before [28]. Briefly, 7 mL of NH₄OH (28-30%) was mixed with 40 mL absolute ethanol and 90 mL demineralized water under mild stirring at 30 °C for 30 min. Then, 10 mL dopamine (50 mg/mL) solution was added to the solution and stirred for 24 h at 30 °C to allow formation of PDA-NPs. The PDA-NPs were harvested by centrifugation (10,000 g, 10 min, 20 °C) and washed 3 times with 96% ethanol, suspended in deionized water and stored at 4 °C for further use. Morphology of PDA-NPs was examined using a Hitachi G-120 transmission electron microscope operated at 120 kV. To this end, 20 μL of a PDA-NP suspension (20 μg /mL) in water was dropped onto a carbon-covered copper grid and dried at 60 °C for 30 min prior to insertion in the microscope. The diameter of the PDA-NPs was
measured using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Titanium samples (4 x 4 x 1 mm) were provided by Salomon’s Metalen (Groningen, The Netherlands) and washed with 9 mL NH₄OH and 9 mL H₂O₂ in 30 mL water and heated to 65°C for 20 min. Next, titanium samples were washed with demineralized water and dried with nitrogen gas. Finally, 4.6 μL PDA-NP suspension (7 mg/mL) in water was deposited on the titanium surfaces (0.16 cm²) to obtain different surface concentrations up to 800 μg/cm² PDA-NPs and samples were dried in the oven at 60 ºC.

The presence of a PDA-NP coating on titanium samples was demonstrated using scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). For SEM, titanium surfaces prior to and after PDA-NP coating were examined in Zeiss Supra 55 microscope (Carl Zeiss, Germany) at an accelerating voltage of 10 kV. Prior to microscopy, surfaces were spray-coated with a 10 nm thick gold-layer.

For XPS, a Surface Science Instrument (Mountain View, CA, USA), equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator was employed. The angle of photoelectron collection was 55 degrees with the sample surface and the electron flood gun was set at 14 eV. A survey scan over a binding energy range of 1100 eV was made with a 1000 x 250 μm spot and a pass energy of 150 eV. Binding energies were determined by setting the binding energy of the C1s peak (carbon bound to carbon) at 284.8 eV.

3.2.2. Photothermal effects of PDA-NP coated titanium surfaces

To determine photothermal effects of PDA-NP coated titanium surfaces, different volumes of water, phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 7.0) and DMEM-HG medium ranging from 10–600 μL were added on PDA-NP coated (200 μg/cm²) titanium samples in a 24 well-plate. Each sample was NIR-irradiated for 10 min at 808 nm (Thorlabs, Newton, NJ, USA) at a power density of 1 W/cm². During irradiation, temperature was recorded using an infrared imaging camera (Fluke TiX580 Infrared Camera, Eindhoven, The Netherlands), imaging the entire sample surface.

3.2.3. Integration of PDA-NP coated titanium surfaces by HGFs in mono-culture

Human gingival fibroblasts (HGF) were obtained from the American Type Culture Collection (HGF-1, ATCC-CRL-2014, Manassas, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM-HG) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Breda, The Netherlands) at 37 °C in 5% CO₂. Cells from passages 15–25 were used. HGFs (100 μL, 1 x 10⁵ HGF/mL) were seeded on sterile PDA-NP coated titanium samples with different surface concentrations of PDA-NPs in a 96 well-plate and incubated at 37 °C in 5% CO₂. After 72 h of growth, the HGF cells were stained with phalloidin-TRITC and DAPI and analyzed using a fluorescent microscope (Leica DM4000, Leica Microsystems Ltd., Wetzlar, Germany). Surface coverage and number of cells per unit area were subsequently derived from the images using image J software.

3.2.4. HGF integration and staphylococcal killing upon NIR-irradiation of PDA-NP coated titanium surfaces in respective mono-cultures

In order to observe photothermal effects on HGF integration of PDA-NP coated titanium surfaces after NIR-irradiation, HGFs were grown in mono-culture on PDA-NP coated (200 μg/cm²)
titanium surfaces for 48 h, essentially as described above. After 48 h, the samples were transferred to a 24 well-plate and immersed in different volumes of DMEM-HG medium (between 10 and 600 μL). These volumes encompass the salivary volume embracing a tooth surface (21 - 100 μL) [28] and are slightly larger than the volume of embracing crevicular fluid, depending on the periodontal status of a patient (0.12 - 0.93 μL) [29]. Subsequently, samples were irradiated at 808 nm (1 W/cm²) for 1, 3, 5 and 10 min. After irradiation, 24 h were allowed for cell growth, after which surface coverage by HGFs was determined, as described above (section 2.3).

For photothermal bacterial killing, *S. aureus* ATCC 12600 was inoculated onto blood agar plates and incubated at 37 °C. After 16 h, one colony was transferred in 10 mL of Tryptone Soya Broth (TSB, OXOID, Basingstoke, UK) and incubated for 24 h at 37 °C. Subsequently, 10 mL bacterial culture was added to 200 mL growth medium and incubated for 16 h at 37 °C. Then, staphylococci were harvested by centrifugation at 6300 g for 5 min at 10 °C and washed twice with sterile PBS and suspended in PBS. Finally, the staphylococcal suspension was sonicated (3 x 10 s at 30 W) in order to break bacterial aggregates in an ice/water bath. The bacterial concentration in suspension was enumerated in a Bürker–Türk counting chamber and the suspension further diluted in PBS to a concentration of 5 x 10⁴ bacteria per mL.

Staphylococci were adhered to a PDA-NP coated (200 μg/cm²) titanium surface by adding 1 mL of a bacterial suspension into 24 well-plates containing a PDA-NP coated titanium sample. After bacterial sedimentation for 1 h, samples were washed with PBS and transferred into a new well and NIR-irradiated, as described above for HGFs in mono-culture. After irradiation, the samples were placed on a hydrated 3M™ Petrifilm™ Rapid Aerobic Count (RAC) Plate (3M Microbiology, St. Paul, Minnesota, USA) for culturing of viable staphylococci. The Petrifilm™ plating system was incubated for 48 h at 37 °C after which colonies formed were enumerated. Staphylococcal killing was expressed with respect to the number of CFUs observed on samples in absence of NIR-irradiation.

### 3.2.5. Tissue integration of PDA-NP coated titanium surfaces upon staphylococcal challenges and NIR-irradiation in bi-cultures and in a 3D-tissue infection-model

*Staphylococcal* challenges were applied to PDA-NP coated titanium surfaces to mimic different stages of healing. To mimic per-operative contamination prior to tissue integration, 1 mL of a staphylococcal suspension (5 x 10⁴/mL) in PBS was added on a PDA-NP coated (200 μg/cm²) titanium surface in a 24 well-plate and bacteria allowed to sediment for 1 h. After 1 h, the samples were transferred to a new well, washed three times with PBS yielding approximately 1 x 10⁵ bacteria/cm² on the implant surface and again transferred to a new well. Different volumes of DMEM-HG medium (10, 50 and 100 μL) were added on the samples and NIR-irradiated (808 nm) at a power density of 1 W/cm² for different times. Subsequently, the samples were transferred to a 96 well-plate and 100 μL HGF suspension of (1 x 10⁵/mL) was added and wells incubated at 37 °C in 5% CO₂. After 24 h of growth, HGFs were stained and analyzed as described before.

In a bi-culture mimicking an early post-operative bacterial contamination before the development of a human oral keratinocytes (HOK) sealing, but with fibroblasts in direct contact with an implant surface, a 48 h grown HGF layer on the samples was challenged by adding 1 mL *S. aureus* suspension in PBS (5 x 10⁴/mL) and allowing sedimentation for 1 h. After 1 h, the samples were transferred to a new well, washed three times with PBS and transferred again to a
new well. Then, different volumes of DMEM-HG medium (10, 50 and 100 μL) were added on the samples, and the samples were NIR-irradiated at a power density of 1 W/cm² for different times. After irradiation, 1 mL DMEM-HG medium was added and the samples with bacteria and cells were incubated at 37 °C in 5% CO₂. After 24 h of growth, the HGFs were stained and analyzed, as described before.

In a late, post-operative infection-model, mimicking a staphylococcal challenge to a fully developed soft tissue seal around an implant, 3D-tissue infection-model was applied. HOK were purchased from ScienCell company (Carlsbad, CA, USA) and grown in Oral Keratinocyte Medium (OKM, ScienCell) supplemented with Oral Keratinocyte Growth Supplement at 37 °C in 5% CO₂. Cells from passages 3–5 were used in this chapter. 100 μL HOKs suspended in OKM medium (5 x 10⁵/mL) were seeded on the transwell membrane (PET transparent, Greiner Bio-One, Frickenhausen, Germany) and incubated for 48 h at 37 °C in 5% CO₂. After 48 h, the cell culture medium was removed and 100 μL S. aureus suspension in PBS (5 x 10⁴/mL) was added in the transwell and staphylococci allowed to sediment for 1 h. After 1 h, the membranes with adhering HOKs were washed three times with PBS and transferred to a new well containing a PDA-NP coated titanium sample covered with a layer of HGFs, grown as described above. Different volumes (10, 50 and 100 μL) of mixed cell culture medium (DMEM-HG and OKM, in a 1:1 ratio [30]) were added in the transwell and the well was NIR-irradiated (808 nm) at a power density of 1 W/cm² for different times. After irradiation, 600 μL of mixed cell culture medium was added in the well containing the HGFs covered samples and 100 μL of mixed cell culture medium was added in the transwell containing HOKs and bacteria. After 24 h incubation at 37 °C in 5% CO₂, HGFs were stained and analyzed as described before.

3.2.6. Statistical analyses
All data were plotted in Graphpad Prism and Origin. One-way ANOVA with a Bonferroni post-hoc test was employed using Graphpad Prism version 7.0 software to determine statistical significance of relevant differences. A value of p < 0.05 was considered statistically significant.

3.3. Results
3.3.1. Characterization of PDA-NPs and PDA-NP coated titanium surfaces.
PDA-NPs had an average diameter of 52 nm, with a diameter range between 38-79 nm over 5%-95% of the distribution (Figure 1A, panel 1 and Figure 1B). The PDA-NP coated titanium surface showed clearly different from the uncoated titanium surface (Figure 1A, compare panels 2 and 3) and had a thickness of 15.7 μm (Figure 1A, panel 4). XPS spectra of uncoated and coated titanium surfaces (Figure 1C), demonstrated titanium and oxygen in a ratio of 1:2 (Figure 1D), indicating the presence of an oxide skin on the titanium. XPS furthermore confirmed the presence of PDA on the titanium surfaces (Figure 1D), as concluded from the decrease in titanium and oxygen presence, concurrent with an increased presence of nitrogen as compared with uncoated titanium. Nitrogen and oxygen on PDA-NP coated titanium occurred in equal percentages.
3.3.2. Heat generation by PDA-NP coatings on titanium

Previously, we have demonstrated that the light-to-heat conversion efficiency of our PDA-NPs amounted 21% [31]. NIR-irradiation of uncoated titanium samples immersed in different volumes of DMEM-HG medium yielded a minor light-to-heat conversion (Figure 2A) of up to 16 °C after 10 min in presence of 10 μL DMEM-HG medium. Under identical conditions, PDA-NP (200 μg/cm²) coated titanium gave an increase of 36 °C (Figure 2B). Dissipation of the heat generated by PDA-NPs on titanium samples when immersed in larger fluid volumes yielded smaller temperature increases. Photothermal effects in water or PBS were similar as observed in medium (Figure S1).
3.3.3. Integration of PDA-NP coated titanium surfaces by HGFs in mono-culture. HGFs spread and adhered well on uncoated titanium (Figure 3A) as well as on PDA-NP coated titanium surfaces. Cell surface coverage (Figure 3B) as a main parameter to quantify tissue integration of an implant surface, and cell number (Figure 3C) were statistically similar for all PDA-NP coated surfaces, regardless of PDA-NP surface concentration.

Figure 2. Photothermal effects of PDA-NP coated (200 μg/cm²) titanium samples. (A) Temperature of titanium as a function of NIR-irradiation time at 808 nm (1 W/cm²) with different volumes of DMEM-HG medium above an uncoated titanium sample. (B) Same as (A), but now for PDA-NP coated titanium samples.
Figure 3. Interaction of HGFs with PDA-NP coated titanium surfaces in mono-culture. (A) Schematics of the mono-culture experiments carried out (A1) and a fluorescence image (A2) of DAPI/TRITC-stained HGFs on uncoated titanium, showing red-fluorescent skeleton and blue-fluorescent nucleus staining. Scale bar represents 100 μm. (B) Surface coverage by adhering HGFs after 72 h of growth on titanium surfaces with different surface concentrations of PDA-NPs (0 μg/cm² indicates absence of PDA-NPs). (C) Number of adhering HGFs per unit surface area after 72 h of growth on titanium surfaces with different surface concentrations of PDA-NPs. Error bars denote SEM over three experiments with separately cultured cells. There are no statistically significant differences in cell surface coverage or cell numbers at different PDA-NP concentrations (p > 0.05).
3.3.4. Tissue integration versus bacterial killing upon NIR-irradiation of PDA-NP coated titanium surfaces in mono-cultures.

NIR-irradiation of PDA-NP coated titanium surfaces should yield a temperature increase that is high enough to kill infecting bacteria and at the same time maintains tissue integration. Therefore, explorative experiments were first done at different NIR-irradiation times at an intermediate PDA-NP surface concentration ($200 \, \mu g/cm^2$), immersing the titanium samples in different fluid volumes. In this exploratory phase, bacterial killing and tissue integration were separately assessed in mono-cultures. Surface coverage by HGFs decreased as a function of increasing NIR-irradiation time, particularly when samples were immersed in small fluid volumes (Figure 4). Oppositely, staphylococcal killing increased as a function of increasing NIR-irradiation time, particularly when immersed in smaller fluid volumes (see also Figure 4 and Table S1 for numerical details). Subsequently, these graphs were employed to derive NIR-irradiation times that yielded acceptable tissue coverage and bacterial killing. A surface coverage by tissue cells of minimally 40% has been demonstrated in the past to allow tissue cells to win the race for the surface from contaminating bacteria [22]. Hence, acceptable NIR-irradiation times should leave at least 40% surface coverage by tissue cells. Analogously, antimicrobials with potential clinical efficacy, should minimally demonstrate 99.9% (or 3 log-units) bacterial killing [42]. This yielded a second criterion for acceptable NIR-irradiation times. Based on these criteria, this exploratory study showed a narrow window of possible NIR-irradiation times of around 3 min (Figure 4) for samples immersed in 10 or 50 μl that were used for further experiments to more precisely determine the window of possible NIR-irradiation times in co-culture studies. Based on Figure 2, these conditions would yield a temperature increase to 56 °C and 51 °C for immersion volumes of 10 or 50 μl, respectively.
Figure 4. Surface coverage by HGFs and killing of *S. aureus* ATCC12600 upon NIR-irradiation (1 W/cm², 808 nm) of PDA-NP (200 μg/cm²) coated titanium surfaces in monocultures as a function of irradiation time.

Samples were immersed in different volumes of DMEM-HG medium and PBS for HGFs and staphylococci, respectively (see schematics). The dotted lines indicate NIR-irradiation times considered acceptable for the maintaining of tissue integration (> 40% cell surface coverage; data in red) and meaningful bacterial killing (> 99.9 %; data in blue). Grey-shading represents the window of acceptable irradiation times, based on both criteria.
Figure 5. Growth of HGF cells on NIR-irradiated (1 W/cm², 808 nm) PDA-NP (200 μg/cm²) coated titanium surfaces in a per-operative contamination model. (A) Schematics of bicultures for mimicking per-operative contamination (A1), in which the implant surface is contaminated with *S. aureus* ATCC12600 (1 x 10³ CFU/cm²) and irradiated before tissue integration by HGFs. Fluorescence images (A2) represent DAPI/TRITC-stained HGF cells grown for 24 h on *S. aureus* contaminated PDA-NP coated titanium surfaces, in the absence (0 min) and presence of 3 min irradiation (samples immersed in 10 μL DMEM-HG medium). Red-fluorescence indicates skeleton-spreading and blue-fluorescence are HGF nuclei. Scale bar represents 100 μm. (B) Surface coverage by adhering HGF on bacterially contaminated PDA-NP coated titanium surfaces in the absence (0 min) and presence of irradiation, while immersed in different DMEM-HG volumes. (C) Same as (B) but now for the number of adhering HGF per unit area. Error bars denote SEM over three experiments with separately cultured cells. *denotes a significant improvement, i.e. a significant difference upon NIR-irradiation (p < 0.05), compared with staphylococcal contamination in the absence of NIR-irradiation. #denotes similarity, i.e. no significant difference in presence of staphylococcal contamination and after NIR-irradiation (p > 0.05), compared with absence of staphylococcal contamination.
3.3.5. Tissue integration of NIR-irradiated PDA-NP coated titanium surfaces upon staphylococcal challenges in bi-cultures and in a 3D-tissue infection-model.

The effect of NIR-irradiation was measured in per- and post-operative infection models, mimicking different stages of healing. In a per-operative contamination model (Figure 5A), the presence of staphylococci adhering in low numbers (1 x 10^3 CFU/cm^2), caused a significant decrease in surface coverage (Figure 5B) and numbers of adhering HGFs (Figure 5C). Photothermal killing of adhering staphylococci prior to tissue integration, yielded significant improvement of tissue integration to the level observed in the absence of staphylococcal contamination (Figures 5B and 5C) when NIR-irradiated for a minimum of 5 min, regardless of fluid volume.

In an early post-operative contamination model, in which an HGF layer is formed but not yet sealed with a layer of protecting keratinocytes, tissue integration was also entirely lost upon a S. aureus challenge (Figure 6A). NIR-irradiation improved tissue integration upon a staphylococcal challenge when applied for 3 min (Figures 6B and 6C), except in the largest immersion fluid volume (100 μL). A shorter irradiation time was insufficient, because it allowed survival of staphylococci, while longer irradiation times caused collateral damage to the HGFs integrating the surface.
Figure 6. Growth of HGF cells on NIR-irradiated (1 W/cm², 808 nm) PDA-NP (200 μg/cm²) coated titanium surfaces after a challenge by *S. aureus* ATCC12600 in an early post-operative contamination model in absence of a keratinocyte seal. (A) Schematics of the early post-operative contamination model (A1), in which an HGF layer on an implant surface in absence of a protective keratinocyte seal, is challenged with *S. aureus* ATCC12600 (1 x 10³ CFU/cm²) and irradiated, followed by 24 h of further growth of the HGF layer. Fluorescence images (A2) of DAPI/TRITC-stained HGF cells further grown after an *S. aureus* challenge, in the absence (0 min) and presence of 3 min irradiation (samples immersed in 10 μL DMEM-HG medium). Red-fluorescence indicates skeleton-spreading and blue-fluorescence are HGF nuclei. Scale bar represents 100 μm. (B) Surface coverage by adhering HGF on PDA-NP coated titanium surfaces after a staphylococcal challenge in the absence (0 min) and presence of irradiation, while immersed in different DMEM-HG volumes. (C) Same as (B) but now for the number of adhering HGF per unit area. Error bars denote SEM over three experiments with separately cultured cells. *denotes a significant improvement, i.e. a significant difference upon NIR-irradiation (p < 0.05), compared with staphylococcal contamination in the absence of NIR-irradiation. # denotes similarity, i.e. no significant difference in presence of staphylococcal contamination and after NIR-irradiation (p > 0.05), compared with absence of staphylococcal contamination.
Figure 7. Growth of HGF cells on NIR-irradiated (1 W/cm², 808 nm) PDA-NP (200 μg/cm²) coated titanium surfaces after a challenge by *S. aureus* ATCC12600 in a late post-operative infection-model in which a protective keratinocyte seal is present. (A) Schematics of the late post-operative infection-model (A1), in which an HGF layer on an implant surface in presence of a protective keratinocyte seal, is challenged with *S. aureus* ATCC12600 (1 x 10³ CFU/cm²) and irradiated, followed by 24 h of further growth of the HGF layer. Fluorescence images (A2) of DAPI/TRITC-stained HGF cells further grown after an *S. aureus* challenge, in the absence (0 min) and in presence of irradiation (samples immersed in 10 μL DMEM-HG medium). Red-fluorescence indicates skeleton-spreading and blue-fluorescence are HGF nuclei. Scale bar represents 100 μm. (B) Surface coverage by adhering HGF on PDA-NP coated titanium surfaces after a staphylococcal challenge in the absence (0 min) and presence of NIR irradiation, while immersed in different DMEM-HG volumes. (C) Same as (B) but now for the number of adhering HGF per unit area. Error bars denote SEM over three experiments with separately cultured cells. * denotes a significant decrease upon NIR-irradiation (p < 0.05), compared with staphylococcal contamination in the absence of NIR-irradiation. # denotes similarity, i.e. no significant difference in presence of staphylococcal contamination and after NIR-irradiation (p > 0.05), compared with absence of staphylococcal contamination.

In a late post-operative infection model, in which HGFs are protected by a keratinocyte seal, a staphylococcal challenge was far less harmful to tissue integration of the titanium surface than in absence of the protective keratinocyte seal (compare Figures 6 and 7). NIR-irradiation for 10
min maintained surface coverage (Figure 7B) and restored cell number (Figure 7C) to the level observed in the absence of a staphylococcal challenge except in the largest immersion fluid volume (100 μL). NIR-irradiation only showed advantageous upon relatively long irradiation times (10 min), due to heat-dissipation in the additional volume of the keratinocyte seal.

3.4. Discussion
In this chapter, we show that both the volume of immersing body fluids as well as the volume of tissue surrounding an infectious biofilm can absorb heat to diminish photothermal killing of bacteria by NIR-irradiated nanoparticles. Moreover, this chapter is the first to show collateral thermal damage to tissue covering an implant surface coated with photothermal nanoparticles. Importantly, this chapter bases its conclusions on the surface coverage of an implant material as the “crown” parameter in the race for the surface between tissue integration and bacterial colonization [43]. Cell surface coverage is determined by cell spreading and the number of cells per unit area. Cells mostly round up under a bacterial challenge and only detach when they “realize” that the race cannot be won. Thus, when the spreading of an individual cell is less due to bacterial presence, but the total number of cells on a surface stays the same, the cell surface coverage will decrease (see several of the scenarios depicted in Figures 6 and 7).

In different in vitro models, it is demonstrated that the merits of photothermal bacterial killing without collateral damage to surrounding tissue leaves only a narrow NIR-irradiation time window. Furthermore, merits heavily depend on whether photothermal treatment is applied as a prophylactic measure in the per-operative phase or as a therapeutic measure in the post-operative phase. In an early per-operative scenario, photothermal treatment only aims to kill bacteria that may have contaminated the implant surface during surgery and collateral tissue damage due to dissipating heat is not important. Bacterial challenges can also arise however, once healing, bone anchoring and the formation of a soft-tissue seal as around dental implants, has commenced (early post-operative scenario). Particularly in a bacteria-laden environment as the oral cavity, bacterial challenges during healing are impossible to avoid. Also, once healing is completed and a protective soft-tissue seal has been formed with a keratinocyte layer covering fibroblasts (late post-operative scenario), bacterial challenges can be detrimental to an implant. In the latter two cases, we here show that NIR-irradiation of implant surfaces coated with photothermal nanoparticles can have beneficial effects on tissue coverage, provided NIR-irradiation times are carefully chosen and do not cause collateral photothermal damage to the tissue cells in the soft-tissue seal.

Temperatures above 50 °C generally lead to killing of infectious bacteria due to damage to vital proteins and enzymes [32]. Unfortunately, heat-induced denaturation of tissue cell proteins readily occurs already above 40 °C causing cell injury or death [33]. Relevant for several types of biomaterials implants, such as dental implants and orthopedic implants requiring anchoring in bone, cortical bone necrosis occurs above 47 °C [34]. Gold-nanorod-coated titanium surfaces reached temperatures of 49 °C upon NIR-irradiation for 20 min in a large immersion volume of 1 mL, which maintained viability of osteoblast pre-cursor cells in mono-culture, but killed only 60% of adhering bacteria, also in monoculture [4]. Gold-nanostar-coated glass induced killing of S. aureus biofilms upon NIR-irradiation when immersed in 0.5 mL fluid [14].

PTT was initially applied for tumor treatment [35]. In clinical tumor treatment, heat
dissipation into tissue surrounding a tumor is not an issue because of the relatively large volume of a tumor compared with infection sites [44]. Clinically for instance, the size of prostate tumors could be reduced 49 to 42 cm³ using gold-silica nanogels [36]. *In vitro* however, success depends heavily on the immersion fluid volume in which the heat generated dissipates and tumor cells are photothermally treated. In some studies, immersion fluid volumes are clearly mentioned. A study on colorectal cancer cells treated with copper (II) sulfide nanocrystals [37] explicitly reports NIR-irradiation time (5 min), power density (33 W/cm²) and immersion fluid volume (375 μL). However to our knowledge, many if not most other studies involving PPT on tumor cells do not affirmatively report immersion fluid volumes [38,39]. The flaw of not properly reporting immersion fluid volumes exist also in many papers dealing with PTT for bacterial infection-control, such as in the evaluation of the photothermal killing of P. aeruginosa [40]. Evaluation of a photothermal PDA coating against adhering *S. aureus*, *E. coli* and *C. albicans*, removed the coating with adhering bacteria even out of its immersion fluid and allowed it to dry in air before NIR-irradiation, yielding only 96% killing [41], far less than the 3-4 log-unit reduction in CFU’s required for potential clinical efficacy [42]. Air-drying is entirely alien to the clinical situation, in which coated implants are in direct contact with body fluids or surrounding tissue, that both absorb heat. The omission of not properly reporting immersion fluid volumes or accounting for the presence of surrounding tissue into which heat generated during PTT can dissipates, leaves many bridges to cross before PTT can be clinically applied in infection-control. Use of mono- and bi-culture models, including 3D-tissue infection-models may facilitate easier crossing of these bridges, because its use will not only provide measures of bacterial killing but also of collateral heat-damage to tissues surrounding an infection site.

### 3.5. Conclusion

Photothermal killing of infectious bacteria is generally presented in the literature as a success story without side-conditions. *In vitro*, success can easily be ensured by properly adjusting immersion fluid volumes, but many articles do not clearly report or justify immersion fluid volumes. In this chapter, we present a photothermal PDA-NP-coating for biomaterials implants and show that killing of bacteria contaminating the surface or challenging the protective tissue surrounding an implant, critically depends on the immersion volume in which experiments are done. Moreover, we show that photothermal treatment of a biomaterial-associated infection requires precise timing of the NIR-irradiation in order to maintain tissue integration, which eventually provides the best long-term protection of a biomaterials implant against infection. Exact timing depends on whether photothermal treatment is done as a prophylactic measure in the per-operative phase or therapeutically in the post-operative phase. This chapter clearly demonstrates the importance of the influence of these important side-conditions that need to be taken into account for the clinical translation of photothermal treatment of bacterial infections, and biomaterial-associated ones in particular.
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Supporting Information

Figure S1. Photothermal effects of titanium samples immersed in different fluids. (A) Temperature of PDA-NP coated titanium as a function of NIR irradiation time at 808 nm (1 W/cm²) immersed in 10 μL of fluid above the sample. (B) same as (A), but now for immersion in 300 μL of fluid above the sample.

Table S1. Killing (%) of *S. aureus* ATCC12600 adhering on PDA-NP (200 μg/cm²) coated titanium surfaces immersed in different PBS volumes after different NIR irradiation times (1 W/cm²). Staphylococcal killing was expressed with respect to the number of CFUs observed on samples in absence of NIR irradiation.

| NIR irradiation time (min) | 10 μL | 50 μL | 100 μL | 300 μL | 600 μL |
|---------------------------|-------|-------|--------|--------|--------|
| 0.5                       | 42.1  | 37.5  | 23.4   | 15.6   | 15.6   |
| 1                         | 93.8  | 86.3  | 81.7   | 78.1   | 73.4   |
| 3                         | 99.9  | 99.2  | 96.1   | 84.4   | 75.2   |
| 5                         | 99.9  | 99.9  | 99.9   | 99.9   | 76.6   |
| 10                        | 99.9  | 99.9  | 99.9   | 99.9   | 94.1   |
