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Concept Study for Treatment of Biofilm on Bone Tissue by Blue Light Irradiation

https://doi.org/10.1515/cdbme-2025-0175

Abstract: Implant-associated infections pose a significant challenge in modern medicine, particularly due to the formation of biofilms that lead to a high tolerance of the bacteria against antimicrobial treatments [1]. This study investigates an alternative approach for the prevention and treatment of biofilms on implants and adjacent bone tissue by targeted blue light irradiation with a wavelength of 405 nm.

The irradiation experiments were carried out with biofilms of *Escherichia coli* (*E. coli*) cultivated on bovine bone samples. The effectiveness of the irradiation was investigated both regarding the biofilm using crystal violet (CV) staining as well as specifically against the embedded bacteria by determining the colony forming units (CFU).

The results suggest that irradiation with 405 nm at an irradiation dose of 5.4 J/cm^2 initially leads to no significant reduction in biofilm mass. After irradiation with a dose of 10.8 J/cm^2, a significant biofilm reduction is observed. However, after prolonged irradiation yielding an irradiation dose of 54 J/cm^2, renewed biofilm formation is detected, indicating a limited penetration depth of the light and possibly adaptive mechanisms of the bacteria.

The data reveal that while the irradiation with 405 nm light with the applied radiant exposure was able to partly eradicate biofilms, it does not guarantee reliable eradication of the embedded bacteria. Nevertheless, this method could attain more effective results when applied with higher radiant exposures or in combination with existing treatment approaches.

Keywords: 405 nm, biofilm, *E. coli*, implant-associated infections, irradiation, light therapy

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1 Introduction

Over the last 50 years, medical devices gained increasing importance. The growing number of the elderly population, which comes with an increase in degenerative diseases, makes especially the use of osteosynthetic and endoprosthetic devices more and more important [2]. Additionally, to the primary care, about 32,500 endoprosthetic procedures on hips and knees took place due to follow-up operations. Around 15 % of these operations were due to infections with increasing incidence [3]. Such implant-associated infections can result in disturbed wound healing, implant loosening, osteomyelitis and pseudarthrosis [3-5]. These consequences lead to repeated or prolonged hospitalisations and the need for intensive rehabilitation, placing a significant burden on the healthcare system [3].

Most implant-associated infections are not caused by microorganisms in their planktonic form but accumulated in a biofilm [3]. A biofilm is an organized aggregation of cells embedded in an exopolysaccharide matrix mixed with extracellular products [5, 6]. It has a 3-dimensional structure where its volume consists of 15 % microcolonies of different species of microbial cells and 85 % matrix material. The matrix material consists of substances like proteins, DNA and polysaccharides, which are produced by the different microorganisms [1, 6]. The extracellular polymeric substance serves as a protective barrier shielding the inner laying bacteria from the environment both from external forces like shear stress from fluid flow, defence mechanisms of the host as well as the access of antimicrobial agents into the biofilm [1, 6, 7].

Currently, implant-associated infections are being treated using antimicrobial therapy in combination with surgical approaches [8]. These methods come with problems and limitations, calling for an improved therapy solution. The approach which is investigated in this research uses the direct photoinactivation of microorganisms without the need for an exogenous photosensitizer. In this case contrary to usual photodynamic approaches, the light itself is able to excite intracellular porphyrins which act as endogenous photosensitizers [9, 10]. The occurring photon absorption

leads to an energy transfer facilitating the production of reactive oxygen species, mostly singlet oxygen, which are highly cytotoxic. The oxidative stress on the bacterial cells can lead to DNA or RNA damage, apoptosis and protein oxidation [11]. While in theory, almost all wavelengths are applicable for the photoinactivation, light in the range of 405-470 nm exhibits the highest effectivity [9, 12]. One of the advantages of photoinactivation is that as of current knowledge bacterial species cannot develop a resistance to the treatment [13]. Additionally, the procedure is less harmful to mammalian cells compared to other photodynamic approaches [14, 15].

Understanding the potential of light therapy as an alternative therapy approach is crucial for effective future treatment of implant-associated infections in times of their rising incidence.

2 Methodology

The choice of 405 nm wavelength was based on its ability to avoid significant damage to mammalian cells, making it a safer alternative for potential in vivo applications [16]. Previous studies have shown that photoinactivation can be achieved using light with a wavelength of 405 nm which possesses a good inherent antimicrobial effect [12, 14, 17].

The bacterial strain used in this study was *E. coli* DSM 498, a well-characterized biofilm-forming strain [18]. Bacterial cultures were grown in Lysogeny broth (LB) (5 g NaCl per litre for enhanced biofilm production) at 37 °C with continuous shaking at 170 rpm (revolutions per minute) [19].

For biofilm formation, overnight cultures were diluted 1:100 in fresh LB medium and transferred to a 24-well plate containing bovine bone samples which were acquired from a butcher. The samples were incubated statically at 37 °C for 53 hours, allowing the development of a more mature biofilm.

The irradiation was conducted in a controlled setup as illustrated in Figure 1. The samples were exposed to 405 nm light at an irradiance of 3 mW/cm². Exposure durations of 30, 60, and 180 minutes were chosen, corresponding to cumulative radiant exposures of 5.4, 10.8, and 32.4 J/cm², respectively. The irradiance was kept low to prevent thermal damage to the biofilm and surrounding tissue. The maximum radiant exposure was capped at 36 J/cm² to prevent potential cytotoxic effects on osteoblasts, based on previous studies indicating that higher doses could impair bone cell viability [20, 21].

Biofilm formation was confirmed in a preliminary test using crystal violet (CV) staining. The biofilms were stained with a 0.1 % CV solution. After washing and ethanol extraction, the absorption at 570 nm was measured using a microtiter plate reader to quantify biofilm biomass. Higher absorbance corresponded to increased biofilm accumulation, reflecting greater biomass density. Due to the observation of a slight increase in absorption after 180 minutes of irradiation an additional exposure duration of 300 minutes with a cumulative radiant exposure of 54 J/cm² was added to the experiments. Furthermore, a Nikon fluorescence microscope type TE 2000 was used to visualize bacteria stained with the Live/Dead BacLight Bacterial Viability Kit (L7007, Life Technologies), allowing differentiation between viable and non-viable cells to help determine optimal incubation time.

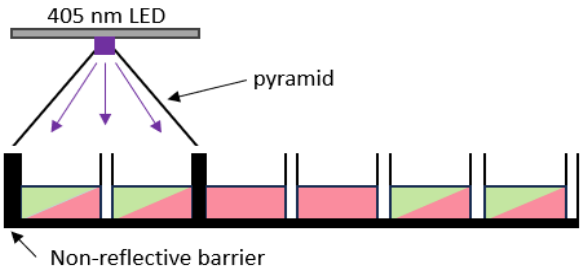

Figure 1: Schematic representation of the irradiation setup for wells in a microtiter plate using a 405 nm LED and a pyramid, enabling simultaneous irradiation of four wells. The added shielding ensures targeted exposure and prevents uncontrolled scattered radiation. Green-red-colouring indicates wells with bacteria suspension that are to be irradiated. Red-colouring indicates wells with bacteria supsension without irradiation.

To evaluate the biofilm on bone after irradiation, it had to be detached from the bone surface. For this purpose, the bone samples were placed in a 50 ml tube containing 2 ml of PBS (phosphate buffered saline) and subjected to two cycles consisting of 1 minute of ultrasonic bath treatment followed by 1 minute of vortexing. In a preliminary experiment the detachment procedure was proven not to cause mechanical damage to the biofilm. After detachment, a dilution series was prepared from the bacterial suspension, which was then plated onto agar plates and incubated for 24 hours to assess bacterial viability by counting colony-forming units (CFU).

3 Results

CV staining results revealed an initial absorbance of 0.6, indicating biofilm presence, while the unirradiated control, which was pure LB medium, remained at 0.1. Absorbance dropped to 0.19 after 30 minutes (5.4 J/cm²) and further to 0.16 after 60 minutes (10.8 J/cm²) of irradiation. After 180 minutes (32.4 J/cm²), a slight increase to 0.17 was observed.

The results from the three-hour irradiation of bone samples are depicted in Figure 2 revealing a similar trend

compared to the CV staining analysis. After 30 minutes of irradiation with a radiant exposure of 5.2 J/cm², the bacterial count decreased by approximately half a log level ($\hat{\mu}_{0h}$ = 1.00·10^8 CFU/ml, $\hat{\sigma}_{0h}$ = 7.34·10^7 CFU/ml; $\hat{\mu}_{0.5h}$ = 2.79·10^7 CFU/ml, $\hat{\sigma}_{0.5h}$ = 1.74·10^7 CFU/ml). After 60 minutes of irradiation, a slight increase in the bacterial count was noted, which became more pronounced after 180 minutes ($\hat{\mu}_{1h}$ = 2.87·10^7 CFU/ml, $\hat{\sigma}_{1h}$ = 2.40·10^7 CFU/ml; $\hat{\mu}_{3h}$ = 6.83·10^7 CFU/ml, $\hat{\sigma}_{3h}$ = 5.50·10^7 CFU/ml). After this irradiation duration, the bacterial count nearly returned to its initial value.

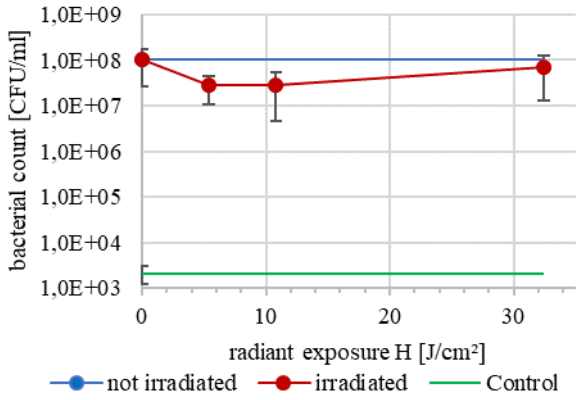

Figure 2: Evaluation of the effect of biofilm irradiation with 405 nm measured in bacterial count at time steps 0, 30, 60 and 180 minutes at an irradiance of 3 mW/cm².

Due to the results of the CV staining experiment further tests with a duration of 300 minutes were conducted to analyse the regrowth of the biofilm, with the results visualised in Figure 3. The bacterial count returned to its original baseline value ($\hat{\mu}_{0h}$ = 2.56·10^7 CFU/ml, $\hat{\sigma}_{0h}$ = 1.98·10^7 CFU/ml; $\hat{\mu}_{5h}$ = 2.58·10^7 CFU/ml, $\hat{\sigma}_{5h}$ = 2,01·10^7 CFU/ml).

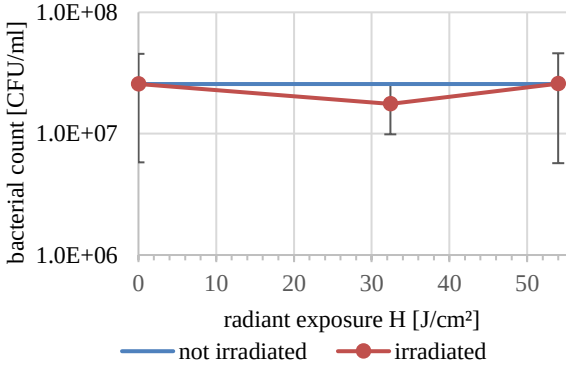

Figure 3: Evaluation of the effect of biofilm irradiation with 405 nm measured in bacterial count at time steps 0, 180 and 300 minutes at an irradiance of 3 mW/cm².

A t-test (p = 0.05, n = 3) was performed to compare irradiated and non-irradiated biofilms at each time point. After 5.4 J/cm², a decrease in bacterial count was observed, but the difference remained statistically insignificant (p = 0.0787). A significant reduction occurred after 10.8 J/cm² (p = 0.0099), indicating an observable effect of irradiation. However, at 32.4 J/cm², statistical significance was lost (p = 0.4319). Upon further investigation at 54 J/cm², the loss of significance persisted (p = 0.97296).

4 Conclusion

This study investigates the reduction of implant-associated biofilms using blue light irradiation. The results show that the irradiation only reduces bacterial load significantly at a radiant exposure of 10.8 J/cm² (p = 0.0099). A reducing effect was not sustained, as bacterial regrowth was observed at 32.4 J/cm² (p = 0.4319).

This could be due to the biofilm absorbing a large portion of the radiant exposure, allowing only a small fraction of the initial intensity to reach the bacteria. Initially, the matrix could be thin enough to let sufficient irradiation through to the inner laying bacteria leading to their inactivation which explains the observed decrease in bacterial count in the first 30 minutes of irradiation. As a result, these inactivated bacteria could contribute to the thickness of the matrix leading to impeded penetration of further irradiation which could explain the anew increase in bacterial count after 30 minutes. Additionally, the limited penetration depth of the 405 nm light further restricts its effectiveness, enabling deeper bacterial layers to survive and repopulate. These effects could explain why the bacterial count returned to the baseline after 54 J/cm².

A comparison with existing literature does not explain the return of the bacterial count to the baseline but suggests that higher irradiance and prolonged exposure times are necessary for significant biofilm elimination. McKenzie et al. demonstrated biofilm formation within a stable growth window of 48 to 72 hours, showing that irradiation with 140 mW/cm² for 60 minutes was necessary to achieve complete bacterial load reduction [22]. Halstead et al. found that the irradiation of *E. coli* biofilms with 405 nm light with an average radiant exposure of 409.5 J/cm^2 led to a decrease in biofilm seeding of 35.3 % compared to a nonirradiated control [23]. These studies highlight that higher irradiance and extended exposure times may be required for significant biofilm elimination [22]. Similarly, Vollmerhausen et al. reported that the inactivation of a 48-hour biofilm required a cumulative dose of 504 J/cm² [24]. Notably, Halstead et al. observed an increase in *E. coli* biofilm biomass for all their

investigated wavelengths in the violet-blue spectrum. This led them to assume that these results support the findings of other researchers, who observed that suboptimal irradiation doses of blue light can promote biofilm growth [23]. This suggests that bacterial adaptation and light intensity play critical roles in biofilm response to irradiation [24].

In this study, radiant exposure was limited to 36 J/cm² to prevent osteoblast damage. However, given these findings, increasing irradiation doses will be necessary to achieve complete biofilm removal, provided that deeper bone structures remain unaffected. The balance between antimicrobial efficacy and potential tissue damage must be carefully evaluated. If deeper bone layers remain intact, higher radiant exposures may present a viable strategy for complete biofilm elimination.

Author Statement

Research funding: The author state no funding involved. Conflict of interest: Authors state no conflict of interest.

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