Investigation of antimicrobial strategies using various titanium dental implant surfaces to prevent peri-implantitis: *in vitro* models of *Streptococcus* spp.

PhD Thesis

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PUBLICATIONS RELATED TO AND INCLUDED IN THE THESIS

I. **Venkei A**, Ungvári K, Eördegh G, Janovák L, Urbán E, Turzó K: Photocatalytic enhancement of antibacterial effects of photoreactive nanohybrid films in an *in vitro* *Streptococcus mitis* model. *Archives of Oral Biology*. 2020; 117:104837. doi: org/10.1016/j.archoralbio.2020.104837. **IF: 2,635**

II. **Venkei A**, Eördegh G, Turzó K, Urbán E, Ungvári K: A simplified *in vitro* model for investigation of the antimicrobial efficacy of various antiseptic agents to prevent peri-implantitis. *Acta Microbiol Immunol Hung.* 2020; 67 (2):127-132. doi: 10.1556/030.2020.01080. PMID: 32160783. **IF: 2,048**

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I. Niller HH, Masa R, **Venkei A**, Mészáros S, Minárovits J: Pathogenic mechanisms of intracellular bacteria. *Current Opinion in Infectious Diseases*. 2017; 30:309-315 doi: 10.1097/QCO.0000000000000363. **IF: 3,782**

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1. INTRODUCTION

With the expansion of the average human lifespan, materials with specific biomedical applications become more remarkable with the purpose of restoring or reserving the function and form of the human anatomy. In the field of implantology the use of ***titanium*** (Ti) as an implant material has become an integral part of dentistry for oral rehabilitation in partially or totally edentulous patients. Despite the high success rate of dental implants there are some risk factors which can cause complication in the retention of dental implant. The main complication around dental implants begins with inflammation of soft tissues, known as peri-implant mucositis which may progress to ***peri-implantitis*** around the implant. Peri-implantitis affects both soft and hard tissues and is associated with bone resorption. Most studies agree that one of the main etiological factors of peri-implantitis is ***bacterial adhesion and biofilm formation*** on implant surface. Therefore, understanding the etiology, mechanism and treatment protocol of inflammatory processes are important for clinicians involved in implant dentistry.

The oral biofilm is a structurally and functionally well-organized, cooperating community of microorganisms. The formation of biofilm on the surface of the implant is similar to what occurs on the surface of the teeth in the cavity. The acquired pellicle, which is a conditioning film on the clean dental surfaces, promotes the initial adhesion of bacteria to oral surfaces. First the initial colonizers attach to the surface, such as ***Streptococcus mitis*** and ***Streptococcus salivarius***, followed by Gram-positive rods, especially *Actinomyces* species. Then other Gram-positive and Gram-negative bacteria adhere to the early forming Gram-positive biofilm. Strict anaerobic bacteria, such as *Fusobacterium* and *Porphyromonas* species play an important role in the formation of the mature dental biofilm.

The intensified use of antibiotics may result in the development of bacterial resistance and the spread of multidrog resistant species that are difficult to eradicate. Besides this, it has been recognized that the microorganisms growing in the biofilms are more resistant to antimicrobial agents. Therefore, intensive antimicrobial strategies are needed in the fight against biofilm forming bacteria in the field of medicine.

In dentistry Ti is one of the most widely used materials for dental implants due to its good mechanical strength, biocompatibility, and corrosion resistance. Various surface modification methods have been used to improve its biological function. In the last decade, ***coatings*** have been using to modify the surface structure of implants and to create new surfaces to inhibit biofilm formation and provide the osseointegration around implant.

Titanium-dioxide (TiO_2) is one of the best photocatalysts for antimicrobial coatings. The mechanism of TiO_2 ***photocatalysis*** has been discovered by Fujishima and Honda in 1972, and since then, it is used in many fields of industry and biomedicine. TiO_2 is one of the broad-spectrum bactericides with self-sterilizing effects and it could reduce the number of adhered microorganisms via the photo-generated reactive oxygen species (ROS) formation. ROS could damage the bacterial cells, inhibit the microbial growth by direct interaction with the cell membrane or it could diffuse easily into the cell and distract the enzymatic function of microbes.

The silver nanoparticles (AgNPs) have the ability to boost the photoactive properties of TiO_2 in the visible-light range due to surface plasmon resonance (SPR) mechanism. Surface plasmon resonance is a phenomenon originated from the collective oscillation of conduction electrons of NPs upon interacting with electromagnetic radiation. As a result, on AgNP-modified TiO_2 surfaces the Ag component extended the absorption spectrum of the semiconductor TiO_2, permitting the activation of TiO_2- photocatalysis by visible light.

Moreover, Ag has antibacterial effect, so these properties could make the ***Ag doped TiO_2 photocatalyst*** a promising coating for Ti implants.

Nowadays, more and more people are choosing to replace missing teeth with dental implants, as a result peri-implantitis is considered to be a remarkable and growing problem in dentistry.

The primary treatment goals of peri-implantitis are to eliminate inflammation and arrest disease progression. For the ***chemical decontamination*** of implant surfaces several agents have been suggested; however, there is a lack of consensus regarding the techniques/ antiseptic agents to be used for decontamination. Currently ***chlorhexidine-digluconate*** (CHX) is the „gold standard” agent in implantology but besides it, ***povidone-iodine*** (PI) and ***chlorine dioxide*** (CD) are also a widely-used antiseptic in dental practice.

Based on literature data several disinfectants have been tested with varying results. However, the most suitable chemical agent for disinfecting the peri-implant region has not yet been found because of the lack of comprehensive *in vitro* and *in vivo* experiments.

2. AIM OF THE THESIS

The treatment of peri-implantitis is a very complex and complicated process. For this reason, the present doctoral work focuses on basic *in vitro* models with relevant pioneer colonizer bacteria such as *S. mitis* and *S. salivarius*. I tested the antibacterial effect of newly developed photoreactive nanohybrid films containing TiO_2 and plasmonic Ag-TiO_2 on Ti based implant surface to find a new opportunity for the conservative treatment or even prevention of peri-implant infections. Furthermore, since in the treatment protocol of peri-implantitis disinfection of implant surfaces is also crucial, I compared the *in vitro* bacterial killing effect of three disinfectant agents (CHX, PI, CD) on *Streptococcus* species mono-bacterial biofilm models on Ti discs surfaces.

The points to be examined for nanohybrid surfaces were the following:

- *Investigation of the newly developed photocatalyst containing polymer based hybrid layer.*
- *Determination of the anti-adhesive effect of nanohybrid films without visible light illumination by 3- (4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay.*
- *Evaluation of the visible light induced photocatalysis on adhered bacterial cells on different surfaces compared with dark controls by MTT and protein assay.*
- *Further goal was to determinate the effective illumination time that can be short enough in peri-implant treatment process.*
- *To investigate whether the reduced Ag content of the nanohybrid film can be effective enough in killing pioneer colonizer bacteria.*

The points to be examined for antiseptic agents were the following:

- *Comparison of the antibacterial effect of three different decontamination solutions using monobacterial streptococci models.*
- *Investigation of the response of pioneer colonizer streptococci to antiseptic treatment in distinct laboratory conditions.*

3. MATERIALS AND METHODS

3.1 Antibacterial property of nanocomposite surfaces

- The ***CP4 pure sandblasted and acid etched titanium discs*** (1.5 mm thick and 9 mm in diameter) were uniformly cleaned and sterilized; than the discs were coated with polymer-based photocatalytic composite thin film: TiO_2 and Ag-TiO_2 (with 0.001 wt% plasmonic Ag content). The coated samples were photo bleached by UV-C irradiation before the experiments to provide the partially photodegradation of polymer component of the nanohybrid film, so the surface ratio of uncoated photocatalyst nanoparticles increased on the surface of the film.
- For investigation of the polymer based composite layers ***scanning electron microscope*** was used.
- The photocatalytic properties of the polymer based composite films were studied using the following methods:
    - ***Chemiluminescence probe method*** was used for the determination of the amount of ROS during irradiation from the reaction of luminol and H_2O_2.
    - The photocatalytic activity of the hybrid layers was also verified through ***bovine serum albumin (BSA) photodegradation tests***.
- For modelling of photocatalytic process, a 15 W low-pressure mercury lamp was used with an UV-visible light source. The spectrum of the UV lamp was determined by a ***grating spectrometer***. For exclusion of UV spectral lines the tissue culture plates with Ti discs were covered with glass plates as a result the weak UV spectral lines of mercury vapor below 320 nm were completely eliminated.
- A clinical isolate of ***S. mitis*** was used in our experiments. The mono-bacterial culture of *S. mitis* was incubated with the control and surface modified Ti discs, placed into 24-well tissue culture plate. In order to investigate the visible light-induced antibacterial activity of nanohybrid films, “illuminated” and “dark” sample groups were tested. In the “illuminated” group the discs with adhered *S. mitis* bacteria were illuminated for 5, 10, or 15 min under standardized conditions, while in the “dark” group the discs were kept in the dark. Non coated, sandblasted and acid etched Ti discs were used as controls of nanohybrid surface modifications. In order to follow the growth of *S. mitis* on the various surfaces under dark and illuminated conditions ***MTT and protein assay*** were used.

- For statistical analysis after checking the normality and homogenity criterion we compared the data with the appropriate tests (Mann-Whitney U test, Kruskal-Wallis test, independent samples t-test, Welch probe, one-way ANOVA). A probability value of less than 0.05 was considered significant. The means ± SEM (standard error of the mean) were calculated for OD_{550nm} values measured by plate reader based on independent experiments.

3.2 Antibacterial property of various antiseptic agents

- Beside the ***sandblasted and acid etched surfaces***, we used ***polished titanium surfaces***. Before the experiments all samples were cleaned and sterilized.
- Clinical isolates of ***S. mitis*** and ***S. salivarius*** were used in our experiment. We incubated the *S. mitis* for 4.5 h on the surfaces of discs, while we extended the incubation time to 48 h in case of *S. salivarius* where after 24 h incubation we changed the culture medium for fresh glucose bouillon.
- After the incubation we washed out the non-adherent cells from the discs surfaces. Than the attached bacterial cells were treated with 2 ml of three different oral antiseptics: CHX, PI and CD for 5 min.
- For investigation of the antibacterial activity of the three different antiseptic agents on mono-species biofilms ***MTT colorimetric assay*** was applied.
- Statistical analyzes: after test of normality (Shapiro–Wilk test) the comparisons within group were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and T-test was used for comparison of independent samples. The means ± SEM (standard error of the mean) were calculated for OD_{550nm} values measured by plate reader based on independent experiments. Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1 Structural and photocatalytic characterization of the nanocomposite surfaces

According to scanning electron microscopy observation the primer photocatalyst particles formed microscale-aggregates; moreover, in increased magnification, nanostructures were also observed. This structure ensures high porosity and good accessibility which is advantageous for photocatalytic process. Based on scanning electron microscopic images both the carbon of the polymer and the Ti content of the photocatalyst are expressed on the nanocomposite surface. The combined presence of these elements resulted photocatalytic surfaces with good mechanical properties.

The amount of ROS that are produced by the photocatalysts was measured from the chemiluminescence of luminol. The results showed that hybrid layer with 60 wt% photocatalyst content shows saturation curve during the studied time interval (0–360 min). This tendency can be explained with the polymer film photodegradation. Due to the illumination the photocatalyst particles became uncoated and have surfaced with a greater extent, which resulted the saturation of free radicals on the photocatalytic surface.

The photodegradation test results reveal that the surface adsorption of the BSA macromolecules was relatively high after 15 min contact time and after that the photooxidation of protein solution almost reached the 100 % under 4 hours UV irradiation time. Thus, the polymer based hybrid layers demonstrated photocatalytic activity.

4.2 Testing of the antibacterial activity of the modified surfaces using the MTT method

The MTT results confirmed that the relative adherence of *S. mitis* was different depending on the surface type even without illumination. The anti-adhesive effect of the 0.001 wt% Ag-TiO_2 nanohybrid was the most remarkable, however we could not find significant differences among data.

We also observed the antibacterial effect of surface modifications during visible light illumination compared to their dark controls. Significant differences ($p < 0.05$) were noticed at 15 min illumination compared with dark controls in case of all Ti based surfaces. In cases of TiO_2 nanohybrid surfaces and standard Ti disc we measured significant differences ($p < 0.05$) between the 5 min and 15 min illuminated samples by the colorimetric MTT assay. The metabolic activity of *S. mitis* on the Ag-TiO_2 nanohybrid film covered sample (OD_{550nm}=0.118±0.014) illuminated for 15 min was 40 % lower compared with its dark control

(OD_{550nm}=0.196±0.028). 15 min of illumination with visible light increased the antibacterial effect of the Ag-TiO_2 coated sample.

4.3 Examination of the different nanohybrid coatings photocatalytic activity

We compared the antibacterial effect of nanohybrid surfaces after illumination for different time with the standard Ti discs (without illumination). We determined significant differences between the control Ti discs and both types of nanohybrid coatings in cases at 10 and 15 min visible light illumination. The TiO_2 nanohybrid coatings eliminated significantly more metabolically active bacterial cells after 10 min (OD_{550nm}=0.191±0.015) and 15 min (OD_{550nm}=0.171±0.013) illumination compared with control Ti discs (OD_{550nm}=0.260±0.028). We also found significant difference between the 10 min illuminated Ag-TiO_2 nanohybrid film (OD_{550nm}=0.136±0.020) and the control Ti discs (OD_{550nm}=0.260±0.028). Furthermore, in our comparisons the 15 min illuminated Ag-TiO_2 nanohybrid film (OD_{550nm}=0.118±0.014) had the most remarkable antibacterial effect since it was significantly better compared with the control Ti discs (OD_{550nm}=0.260±0.028). These findings confirmed that the Ag-TiO_2 coated surface had remarkable antibacterial effect, which is obviously due to the Ag-enhanced photocatalytical activity.

4.4 Investigation of the antibacterial effect of different surfaces on the total bacterial protein content

According to protein assay the amounts of proteins on both nanohybrid surfaces were lower than on the control Ti after 15 min illumination; however, we could not detect significant differences. It can also be stated that no significant changes were observed in protein content reduction on Ag-TiO_2 surface, but this coating inhibited *S. mitis* attachment and showed bactericid effect most of all.

4.5 Disinfectant efficacy of oral antiseptics on S. mitis biofilm using MTT assay

Evaluation of the three disinfectants antibacterial activity against the pioneer colonizer indicated that among antiseptics the PI and the CD showed significant differences both on the polished ($p = 0.0005$) and the sand blasted, acid etched ($p = 0.0004$) Ti surfaces compared with the untreated control Ti discs after the 5 min treatment time. In our work we converted the MTT data to percent values and mentioned them in this way in the dissertation. In this context the attachment to the control Ti surface was considered 100 % (highest OD value) and the number

of metabolically active cells on the surfaces was expressed in relative percentages in the results section.

According to our MTT results all antiseptic decreased the cell metabolic activity in biofilm on sand blasted, acid etched and polished surfaces compared with control discs. However, the PI and CD showed significant cell reduction on both surfaces ($p < 0.05$). The PI was the most effective antiseptic against the *S. mitis* cells incubated for 4.5 h, since it decreased the number of active cells with 37 % ($OD_{550nm} = 0.043 \pm 0.001$) on polished surface compared with the control disc ($OD_{550nm} = 0.068 \pm 0.008$) after 5 min treatment time ($p = 0.0012$). We also observed a similar tendency with regard to the sand blasted, acid etched surfaces. The decrease of the metabolically active cells was 33 % ($OD_{550nm} = 0.044 \pm 0.001$) after rinsing with PI compared with the untreated control Ti discs ($OD_{550nm} = 0.065 \pm 0.007$) ($p = 0.0007$).

4.6 Disinfectant efficacy of dental antiseptics on S. salivarius biofilm using MTT assay

Our results suggest that all tested agents significantly decreased the amount of metabolically active cells in *S. salivarius* biofilm on polished surfaces compared with the untreated Ti surfaces *in vitro* ($p < 0.0001$). The most remarkable antibacterial activity was attributed to PI, which eliminated 65 % ($OD = 0.048 \pm 0.003$) of streptococci cells on polished surface after 5 min treatment time ($p = 0.0002$). However, the CD also eliminated a considerable percent of the biofilm (60 %) ($OD = 0.056 \pm 0.001$) compared with the control polished discs ($OD = 0.139 \pm 0.01$, $p = 0.0002$). Comparing the three agents significant differences could be observed between the PI and CHX ($p = 0.0002$) and in this respect between the CD and CHX ($p = 0.0006$) as well.

Compared with the control discs, each of the three antiseptics studied decreased the metabolic activity of *S. salivarius* biofilms on polished surfaces as well as on sand blasted, acid etched surfaces ($p < 0.0001$).

We observed that the PI and CD showed significantly higher antibacterial activity against *S. salivarius* compared with the CHX treatment (PI vs. CHX $p = 0.0007$, CD vs. CHX $p = 0.0212$).

Next we also observed that the metabolic activity of control *S. salivarius* cells was lower on the sand blasted, acid etched Ti surface than on the polished surface ($p = 0.0063$). However, we did not notice such a difference in case of control *S. mitis* biofilms.

5. DISCUSSION

The use of Ti dental implants is becoming a widely accepted method among dentists for replacing missing teeth. However bacterial infection on and around the implant (peri-implantitis) is one of the most common cause of implant failures. Therefore, there is a growing need to develop surfaces to control the spread of implant related infections and find alternative agents, which may kill a broader spectrum of microbial species without any harmful effect on the surrounding human cells.

The methods of my dissertation are simplified *in vitro* basic models using pioneer colonizer *Streptococcus* spp. These models serve, however, as a basis for the investigation of more complex biofilm forming communities.

5.1 Evaluation of the antibacterial effect of nanocomposite surfaces in the light of my results

We investigated the antibacterial potential of two photocatalytic nanocomposite films: TiO_2 and Ag-TiO_2 (with 0.001 wt% plasmonic Ag content) as new coatings on Ti implant surfaces. Basically, first we studied the structural and photocatalytic properties of composite layers with different methods. Our physicochemical findings connecting to nanocomposite surfaces suggest that the new polymer based surfaces showed good structural and photocatalytic properties.

According to our MTT results the AgNP-coated Ti surface showed effective anti-adhesive potency against the pioneer colonizer *S. mitis* under dark conditions, and this property may even make it suitable for the prevention of peri-implantitis. To investigate the photocatalytic effect we applied short illumination time such as 5, 10, 15 min, which can be short enough to be applicable in dental practice.

Based on our results, the number of metabolically active bacteria decreased on all Ti based surfaces under illumination for different time, compared with their dark control, indicating the visible light-driven ROS generation. Surprisingly, the standard sandblasted, acid-etched Ti disc surfaces showed visible light induced antibacterial property, too: 15 min of illumination with visible light resulted in the most remarkable killing, compared to Ti discs kept in the dark. In addition, we found that the metabolic activity of *S. mitis* on the Ag-TiO_2 nanohybrid film covered sample illuminated for 15 min was 40 % lower than its dark control. This time span of illumination is tolerable in the dental practice.

Next we compared the antibacterial effect of photocatalytically activated nanohybrid surfaces after illumination for different time with the standard Ti disc (without illumination). Both types of nanohybrid coatings showed significant antibacterial effect in cases of 10 and 15 min illumination compared with the standard Ti disc. Furthermore, in our comparisons, the 15 min illuminated Ag-TiO_2 nanohybrid film had the most remarkable antibacterial effect since it proved to be significantly better compared with the control Ti discs as well as surpassed the TiO_2 nanohybrid coating in its photocatalytic cell damaging antibacterial activity. These findings demonstrated that the addition of plasmonic properties of noble metal NPs (e.g., AgNPs) enhances the photocatalytic activity of TiO_2 under visible light. Therefore, we established, that plasmonic photocatalysis may play key role in eliminating the development of the bacterial biofilm.

Our measured protein concentrations suggested that the illuminated Ag-TiO_2 nanohybrid surface had efficient bactericidal effect and inhibited bacterial attachment. The observed tendency of decreasing protein concentration in the case of photoreactive coatings is presumably attributed to the photooxidation of the organic macromolecules in spite of the short irradiation time (5-15 min), although our protein assay data could not support the statistically significant differences between the various surfaces demonstrated with the MTT assay.

5.2 Antibacterial efficacy of oral antiseptics on Streptococcus spp.

We created an *in vitro* model to evaluate the antibacterial effect of three widely used antiseptics in dental practice on two test bacteria (*S. mitis*, *S. salivarius*).

According to present literature CHX is one of the most well-known and widely used antiseptic agent in dentistry, but based on our results it is showed significant cell reduction only in case of *S. salivarius* after 5 min treatment time.

Based on MTT data PI and CD were the two most effective antiseptic agents against *S. mitis* and *S. salivarius.* Moreover, both agents proved to be significantly better compared with CHX in the elimination of the 48 h biofilm of *S. salivarius*. These results are in concordance with other researchers who established in their work that CD was more effective compared with CHX after 5 min treatment time.

Our work presented that PI was the most effective in the *in vitro* elimination of both streptococci biofilms. However, in case of *S. mitis* we could not observe significant difference between PI and CD. In this context it is worth to mentioning our earlier *in vitro* studies, where we compared the antibacterial effect of PI, CHX and citric acid against *P. gingivalis*. Our results suggested that PI seemed to be superior in this comparison against anaerobic

periodonthopatogenic bacteria, but not significantly. These properties could make it an ideal antiseptic besides CD in dental practice to treat inflammation caused by microoganisms.

5.3 Future possibilities of antibacterial strategies in infection control

The visible light induced antibacterial property of Ag-TiO_2 may open a new potential for the treatment of peri-implant infections, which is more efficient than the currently used medical alternatives. For instance, if primary peri-implantitis develops, the complete elimination of bacteria from the implant surface might be achieved by the visible light illumination of the Ag-TiO_2 surface with a dental curing lamp. The photocatalytically-induced release of ROS from the implant surface may be more effective in bacterial killing than surgical decontamination because it does not damage the surface. The main therapeutic goal in the treatment of implant related infections is the complete removal of bacteria from the surgical site, including the implant surface and the surrounding tissues. The surrounding tissues can be decontaminated by surgical debridement, but actually, it is a challenge to free the implant surface from bacterial attachment. The currently applied surgical decontamination of the implant surfaces does not guarantee the complete elimination of bacteria, and it can also have a negative impact on the osseointegration process. The tested Ag-TiO_2 surface may offer an alternative solution for this problem. Moreover, the visible light induced antibacterial property of the Ag-TiO_2 supplemented with PI or CD rinsing could control the infection at the surgical site and can open a totally new direction in dental practice. However, this assumption is currently theoretical, but it is worth investigating this assumption in the future.

6. SUMMARY AND CONCLUSIONS

Osseointegrated dental implants have become an increasingly popular therapeutic method for the replacement of absent or lost teeth. However, the cases of patients with inflammation around peri-implant tissues has become widespread. Peri-implant inflammation is one of the most common complications of succesful implantation. Most studies agree that one of the main etiological factors of peri-implantitis are bacterial attachment and colonization on biomaterials.

Therefore, novel surface coatings on Ti implant and effective disinfection strategies are necessary for the prevention and treatment of inflammation caused by microorganisms around implantable medical devices.

We examined the direct interaction of new photoreactive composite coatings with an abundant pioneer colonizer in the oral cavity, *S. mitis*, and determined the effective illumination time that was short enough to be used in everyday dental practice.

Using two different methods, we monitored the antibacterial activity of the Ag-TiO_2 nanohybrid coating containing 0.001 wt% Ag, which is physiologically acceptable for human patients

Furthermore, we compared the antimicrobial effect of three antiseptics (CHX, PI, CD) on *Streptococcus* spp. *in vitro* biofilm models adhering to Ti surfaces using MTT assay.

The main conclusions of this thesis are:

- The new polymer based surfaces showed favorable surface properties and good *in vitro* photocatalytic activity due to their physicochemical properties; thus, the nanocomposite materials proved their suitability for the photocatalysis induced bacterial elimination.
- The results of our *in vitro* experiments suggested that the Ag-TiO_2 (with reduced Ag content) containing surface had remarkable anti-adhesive potential under dark condition.
- Ag-TiO_2 showed antibacterial property against *S. mitis* cells after 15 min of visible light illumination, due to the photocatalytic mechanism.
- Our data indicated, that PI and CD had remarkable eliminating property against both streptococci in biofilm after 5 min of treatment time on the Ti surfaces. Compared to CHX, PI and CD proved to be more effective against the biofilms of pioneer colonizers.

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