Surface functionalization of titanium substrates with Deoxyribonuclease I inhibit peri-implant bacterial infection

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This study aimed to investigate the effect of Deoxyribonuclease I (DNase I) coating on initial adhesion and biofilm formation of peri-implant bacteria. Titanium (Ti), Ti-polydopamine (Ti-PDOP), Ti-PDOP-DNase I and Ti-PDOP-inactivated DNase I samples were studied. The FE-SEM, EDS and XPS were used to confirm that DNase I was coated onto Ti. The initial adhesion and biofilm formation of Aggregatibacter actinomycetemcomitans (A.a) and Fusobacterium nucleatum (F.n) were observed by CLSM. The osteogenic induction of Ti-PDOP-DNase I on MC3T3-E1 cells was investigated by ALP activity and RT-PCR. The adhesion clearance rate of viable bacteria on the surfaces of Ti-PDOP-DNase I was 91.95% for A.a, and 96.37% for F.n, and the 24 h biofilm formation of the bacteria was significantly inhibited. In addition, on DNase I coating, the mRNA level of osteogenic marker genes (alp, opn, bsp, sp7) and the activity of ALP were both up-regulated. Therefore, DNase I coating could be an alternative approach for preventing implant-related infection.

**Keywords:** Titanium (Ti), Deoxyribonuclease I (DNase I), Dental implant, Adhesion, Biofilm

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**INTRODUCTION**

Titanium and its alloys have been extensively used for medical implant devices, such as dental implants, prosthetic valves, and orthopaedics inner fixing apparatus1,2. Titanium implants have been considered an effective and routine method for restoration of missing teeth3,4. Titanium has many advantages, including excellent mechanical properties, high corrosion resistance and desirable biocompatibility5. However, after implantation, bacteria tend to adhere on the surfaces of titanium-based implants, and then form biofilms, causing implant-related infection6,7. A cross-sectional analysis showed that the prevalence of peri-implant mucositis was 63.4% and that of peri-implantitis was 16.4%. The risk of implant infection of patients with periodontitis was 2.2 times higher than that of healthy people8. So, improving the antibacterial ability of Ti surface is of great benefit to the success of treatment, especially when high risk factors, such as periodontitis, are exist.

Surface modification of implant may be an effective method to improve the anti-infective ability of Ti implants. Various coating strategies have been developed, such as antibiotic coating9-12, silver-zinc coating13 and antimicrobial peptides (AMPs) coating14. Nevertheless, the above approaches are usually accompanied with side effects of cytotoxicity or development of drug-resistant strains15. In recent years, novel coatings strategies based on biofilm-dispersing enzymes have emerged16. It is expected that degradation of components in biofilm extracellular polymeric substances (EPS) could inhibit bacteria adhesion and biofilm formation, and also promote detachment of established three-dimensional biofilm17. Furthermore, after biofilm dispersion, planktonic bacteria could be more susceptible to antimicrobial action or other physicochemical stimuli18. This method can improve the anti-biofilm property of implants without development of resistant strains, and contribute to the balance of the human oral cavity microbiologic environment19. Therefore, a biofilm-dispersing enzyme coating may be ideal anti-infective implant coating.

Deoxyribonuclease I (DNase I) can specifically degrade the important component, the extracellular DNA, in EPS. Studies have shown that DNase I is an effective biofilm-dispersing enzyme20. Utilizing polydopamine composite coating technology, our research team has previously coated DNase I, onto the surface of titanium (Ti), and this method is simple, efficient and biocompatible with easily controlled reaction conditions21,22. We demonstrated that the coating could dramatically inhibit adhere and biofilm formation of Streptococcus mutans (S. mutans) and Staphylococcus aureus (S. aureus)23. However, whether DNase I coating possesses the similar effects on peri-implantitis microorganisms is still unclear.

Peri-implantitis represents the diversity of microbiota including red complex (Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola), orange complex (F.n and Prevotella intermedia), and other microorganisms (A.a, Campylobacter rectus, and so on)24-26. So far, there is no single bacterium to have
been confirmed with implant failure, but it has been found that the predominately gram-positive aerobic and facultative anaerobic bacteria shift towards gram-negative anaerobic microorganisms. Moreover, studies have shown that peri-implantitis and periodontitis own the overall similarities of microbiota compositions, but the microorganisms of peri-implantitis are different from healthy implants. A.a and F.n are the major pathogenic bacteria of aggressive periodontitis (AGP) and chronic periodontitis (CP), which were also bacteria most commonly isolated from peri-implantitis pockets. And F.n is a bridging bacterium that can accelerate the adhesion and aggregation of other pathogenic bacteria to the biofilm, which was found in higher proportion of microbiota around peri-implantitis implants compared with healthy implants in the same patient. Hence, A.a and F.n were very important pathogens in peri-implant plque. Inhibition of A.a and F.n colonization and growth on Ti substrate is of great significance to the prevention of peri-implant infection.

Therefore, in order to further evaluate the anti-infective properties of the DNase I coating, this research focused on investigating its effects on initial adhesion and biofilm formation of F.n and A.a on Ti surface, also, in vitro cellular tests were performed to assess its biocompatibility to osteoblast-related cells.

MATERIALS AND METHODS

**Ti substrate preparation**

Pure titanium (Ti) plates (10×10×1 mm) (Shanxi, China), grade 2, were polished with a series of SiC sandpapers (800, 1000, 1500, 2000 grit), ultrasonically cleaned for 20 min in acetone, anhydrous ethanol and deionized water. Then the slices were sterilized at 121°C, 0.12 MPa, for 30 min in acetone, anhydrous ethanol and deionized water. The substrates were denoted by Ti, and ready for polydopamine coating.

**Surface modification with DNase I**

According to the previous experimental method, briefly, Ti substrates were immersed in dopamine (4 mg/mL, H8502-5G, Sigma-Aldrich, San Francisco, CA, USA) for 24 h at room temperature to obtain the polydopamine-coated samples (Ti-PDOP). Then Ti-PDOP substrates were immersed in DNase I (25 units/mL, Sigma-Aldrich) or the inactivated DNase I solution for 6 h at room temperature to obtain the DNase I-polydopamine-coated samples, denoted by Ti-PDOP-DNase I and Ti-PDOP-inactivated DNase I, respectively.

**Surface characterization**

Surface morphologies of Ti, Ti-PDOP and Ti-PDOP-DNase I were observed using FE-SEM (JEOL, Tokyo, Japan) equipped Oxford EDS detector from UK. Prior to FE-SEM observation, the different samples were dried and sputter coated with gold thin film. The elemental chemical compositions were examined by XPS (Kratos, Shimadzu, Kyoto, Japan) equipped with an X-ray source with an Mg anode maintained at 20 kV with a nominal power rating of 200 W. The XPS full spectrum was obtained at Ti 2p, C 1s, O 1s, and N 1s. And the component percentage of characteristic chemical bonds was analyzed by software XPS PEAK4.1.

**Bacteria culture**

Lyophilized A.a (ATCC 43717, provided by Guangdong Culture Collection Center, China) was dissolved and inoculated in a culture bag with AnaeroPackTM-CO2 (MGC, Tokyo, Japan) and incubated for 48 h (5% CO2, 10% O2, 85% N2, 37°C). F.n (ATCC 25586) stored at −80°C was inoculated onto blood agar plates and incubated in an anaerobic system for 48 h (80% N2, 20% CO2, 37°C). After pre-cultures, bacteria were harvested and diluted to a final density of 107 cells/mL with BHI fluid medium.

**Bacterial initial adhesion**

For bacterial initial adhesion, Ti, Ti-PDOP, Ti-PDOP-inactivated DNase I and Ti-PDOP-DNase I samples were placed in a 6-well plate (n=3). Prepared bacterial suspension (109 cells/mL) was added to each well. After 60 min, bacterial suspensions were removed and the samples were then washed with PBS. The adhered bacteria were observed using LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) with CLSM according to manufacturer’s instructions. The number of viable bacteria and total bacteria adhering on the surface of the samples were counted using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). The adhesion clearance rate of viable bacteria on Ti-PDOP-DNase I surfaces was calculated according to the equation shown below.

\[
\text{Adhesion clearance rate of viable bacteria} (\%) = \frac{\text{viable bacteria (Ti)-viable bacteria (Ti-PDOP-DNase I)}}{\text{viable bacteria (Ti)}} \times 100\%
\]

**CLSM of biofilm**

Ti, Ti-PDOP, Ti-PDOP-inactivated DNase I and Ti-PDOP-DNase I samples were incubated for 24 h to establish mono-species biofilm. After staining with LIVE/DEAD Bac Light Bacterial Viability Kit, biofilm formation was observed using CLSM and scanned layer-by-layer along z-axis. Subsequently, the fluorescent area of viable bacteria and total bacteria adhering on the surfaces were measured using Image-Pro Plus 6.0 software. The percentages of the occupied area of adhered bacteria from the fluorescent images (fluorescence area in view/total area of field×100%) were calculated. Employing Imaris v7.2.3 software, three-dimensional reconstruction was created with optical cross-sections, and average biofilm thickness on the surfaces of samples was further examined.

**Cell culture**

Mouse osteoblast precursor cell line (MC3T3-E1, provided by Research Institute of Endocrinology, Tianjin Medical University, China) was cultured in α-MEM medium.
supplemented with 5% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2. The third passage of cells was used for subsequent experiments.

**Alkaline phosphatase activity**

Ti, Ti-PDOP and Ti-PDOP-DNase I samples were firstly placed in 24-well cell culture plates. MC3T3-E1 cells were seeded onto different surfaces at an initial density of 2×10^4/cm² and incubated for 2 days. Afterwards, the culture medium was changed to freshly prepared osteogenic medium supplemented with 50 μg/mL ascorbic acid (Klamar, Shanghai, China) and 10 mmol/L sodium β-glycerophosphate (HMH, Beijing, China). After being cultured for 14 days, the cells were collected for the ALP activity analysis using ALP kit (Beyotime, Shanghai, China) according to manufacturer's protocol.

**RT-PCR analysis**

MC3T3-E1 cells were seeded on the surfaces of Ti, Ti-PDOP and Ti-PDOP-DNase I at a density of 2×10^4/cm². After osteoinductive induction for 7 days, total mRNA of cells was extracted using RNAiso Plus kit (TaKaRa, Dalian, China) and reverse-transcribed into complementary DNA (cDNA). Then the expression levels of four osteogenic genes, ALP, osteopontin (OPN), bone sialoprotein (BSP) and special protein 7 (SP7) were analyzed by RT-PCR using custom made forward and reverse primers (Table 1) in respect to β-actin level as controls (n=3). After an initial denaturation at 95°C for 7 min, the PCR was run for 45 cycles (95°C for 5 s, 57°C for 15 s, 72°C for 15 s) and at 72°C for 5 min. The relative expression levels of target genes were calculated by 2^−ΔΔCT method.

**Statistical analysis**

All data were expressed as mean±standard deviation (SD) and statistically analyzed with one-way analysis of variance (ANOVA). The statistical analyses between two different groups were assessed by Student-Newman-Keuls (SNK) test. A value of p<0.05 was considered as statistically different and a value of p<0.01 was considered as statistical significant. All statistical analyses were performed using SPSS 22.0 software.

## RESULTS

**Surface characterization**

The micromorphologies of different surfaces were analyzed using FE-SEM. The results showed that, pristine Ti surface revealed some scratches originating from grinding. Also some clusters of spherical particles appeared on the surfaces of Ti-PDOP and Ti-PDOP-DNase I. Backscattered electron images showed that the molecular weight of the deposited particles was lower than that of Titanium (Fig. 1A). EDS analysis showed that C element signals had appeared at the same area of spherical particles on the surfaces of Ti-PDOP and Ti-PDOP-DNase I. The Ti element signals in this area had decreased significantly and had almost disappeared (Fig. 1B).

The chemical composition of different surfaces was identified by XPS analysis. Using XPS PEAK4.1 software, high-resolution C 1s spectrums of samples were displayed (Table 2). The results showed that only three types of C-based chemical bonds were detected on the surface of Ti, which were C-C/C-H bond (285 eV, 61.61%), C=O bond (288 eV, 10.03%) and C-O bond (286 eV, 28.36%). On the surface of Ti-PDOP, the new peak of C-N bond (12.85%) appeared at 285.6 eV and the C(O)O bond (2.68%) appeared at 290 eV. The proportion of C-H/C-C decreased to 30.67%. Nevertheless, the proportions of C-O (36.52%) and C=O (17.28%) increased. The types of chemical bonds of Ti-PDOP-DNase I surfaces were the same as those of Ti-PDOP surfaces. The proportion of C-N increased to 14.54%, the proportions of C-O (45.00%) and C=O (25.77%) increased more highly, while

| Gene name | Primer (F: forward; R: reverse) |
|-----------|---------------------------------|
| ALP       | F: 5'-GGTTGGGGGTGCCCACGGT-3'    |
|           | R: 5'-CCTTGGACAGAGCCATGTATG-3'  |
| OPN       | F: 5'-ACTCCAATCGTCCCTACAGTC-3'  |
|           | R: 5'-GACTCACCGCTCTTCATGTG-3'   |
| BSP       | F: 5'-AATGGAGACGGCGATAGTTCCG-3' |
|           | R: 5'-GGAAAGTGTGGAGTTCTCTGCC-3'|
| SP7       | F: 5'-TAAACCGGGAAGCACCATCC-3'  |
|           | R: 5'-AAGAAGACGTGGCGTTAGCA-3'   |
| β-actin   | F: 5'-TCAGGTTACTGTCTCGTCT-3'   |
|           | R: 5'-ACCAGAGGCTACAGGGACAG-3'  |
the proportion of C(O)O decreased to 0.64%, and the proportion of C-H/C-C decreased to 14.05%. All results confirmed that the DNase I coating was established successfully.

Bacterial adhesion
It is known that during 1 h after the installation of implants, microorganisms are identified and the complex biofilm is formed subsequently. So, CLSM was used to observe the initial adhesion of bacteria at 60 min. As shown in Fig. 2, bacteria adhering to the surfaces of Ti-PDOP-DNase I were significantly less than those adhering to the surfaces of Ti, Ti-PDOP and Ti-PDOP-inactivated DNase I. Quantitative analysis showed that the number of total bacteria and viable bacteria on the surfaces of Ti-PDOP-DNase I were both dramatically reduced than those of Ti, Ti-PDOP and Ti-PDOP-inactivated DNase I (p<0.01). The adhesion clearance rate of viable bacteria on the surfaces of Ti-PDOP-DNase I was 91.95% for A.a, and 96.37% for F.n. These findings suggest that DNase I coating significantly inhibit the initial adhesion of A.a, and F.n.

Biofilm formation
CLSM images showed that after 24 h A.a were still scattered on the surfaces of Ti-PDOP-DNase I and grew as monolayer. Nevertheless, bacteria started to aggregate and overlap on the surfaces of Ti, Ti-PDOP and Ti-PDOP-inactivated DNase I (Fig. 3A). Quantitative results showed that the bacterial coverage of A.a on the Ti-PDOP-DNase I was extremely lower than that on other control samples (p<0.01) (Fig. 3B). The average biofilm thickness of A.a on the surfaces of Ti was 9.97 μm, whereas on the surfaces of Ti-PDOP-DNase I, the average biofilm thickness was reduced to 6.03 μm (p<0.01) (Fig. 3C).

Similar observations were found for F.n biofilms. At 24 h, no obvious bacterial biofilms were observed on the surfaces, while the bacterial coverage of F.n on the Ti-PDOP-DNase I was significantly lower than that on other groups (p<0.01). However, the bacterial thickness on the four groups was comparable (Fig. 4). These findings suggest that the DNase I coating possesses the ability to inhibit 24 h biofilm formation of A.a, and F.n.

ALP activity
As shown in Fig. 5A, the MC3T3-E1 cells cultured onto Ti-PDOP and Ti-PDOP-DNase I displayed significantly higher (p<0.05) ALP activity than that of native Ti after incubation for 14 days. The ALP activity of cells on Ti-PDOP-DNase I overwhelmed all of the others, while there was no significant statistical difference in ALP activity between Ti-PDOP and Ti-PDOP-DNase I groups (p>0.05). These findings suggest that the ALP activity of the MC3T3-E1 cells is up-regulated.

RT-PCR analysis
To analyze the effect of DNase I coating on expression of several osteogenic genes, RT-PCR was performed on MC3T3-E1 cells on different surfaces incubated for 7 days. The results showed that the gene expression levels of ALP, OPN, BSP and SP7 on Ti-PDOP and Ti-PDOP-DNase I surfaces were significantly higher compared

Table 2  Decomposition of the C 1s photo-electron peaks and their composition ratio of different samples

|                  | C-H/C-C | C-N | C-O  | C=O  | C(O)O |
|------------------|---------|-----|------|------|-------|
| Ti (%)           | 61.61   | 0   | 28.36| 10.03| 0     |
| Ti-PDOP (%)      | 30.67   | 12.85| 36.52|17.28 |2.68   |
| Ti-PDOP-DNase I (%) | 14.05 | 14.54| 45.00|25.77 |0.64   |
Fig. 2 Representative CLSM images of A.a and F.n adhesion at 60 min on various samples (A). The total number of bacteria of adherent A.a (B) and F.n (C) on different samples after incubation for 60 min. Significant difference (**p<0.01).
to those on Ti. Also, the relative expression of the four genes on Ti-PDOP-DNase I displayed highest (p<0.01) among the investigated substrates, which were two to four times more than those on Ti (Fig. 5B). These findings suggest that the mRNA level of osteogenic marker genes in MC3T3-E1 cells is up-regulated.

**DISCUSSION**

Implant-related infection has always been a major challenge for dental implantation in clinical practice. Once infection occurs, it will cause mucosal hemorrhage, alveolar bone loss, and destruction of osseointegration, eventually leading to loosening or loss of implant, and even implant failure. Surface modification on materials is an effective method to improve the anti-infective ability of implants. DNase I functionalized titanium surface can inhibit the colonization and growth of pathogenic bacteria without drug resistance, which is an effective and safer way to prevent infection. And the inhibitory ability of DNase I coating against peri-implant bacteria infection is more important to the
success of implantation.

Bacterial colonization and adhesion on the surfaces are the first and most crucial stage of the biofilm forming[22,39]. At the same time, bacteria are most active during the initial 24 h after implant placement[39]. Therefore, inhibiting initial adhesion and 24 h biofilm formation is of great significance. In this study, high-resolution CLSM coordinated with double fluorescence staining was used to investigate the effect of DNase I coating on initial bacterial adhesion and biofilm formation. Our results showed that DNase I coating could significantly inhibit bacterial adhesion within 60 min, and an adhesion clearance rate of viable bacteria on Ti-PDOP-DNase I surfaces was 91.95% for A.a, and 96.37% for F.n. Ti-PDOP-DNase I significantly prevented the formation of A.a and F.n biofilms in 24 h. Therefore, DNase I coating had great potential for resistance to early infection after implantation. The adhesion and biofilm formation of Ti-PDOP-inactivated DNase I did not differ significantly from that of Ti, indicating that the anti-infective ability of DNase I coating mainly depends on the enzymatic activity. In addition, the proportion of dead bacteria on the Ti-PDOP-DNase I did not increase, confirmed that the DNase I coating’s anti-infective ability acted on the eDNA component of biofilm EPS, without injuring cell bodies. Therefore, we may speculate that this coating will not lead to destruction of oral microecological balance, and the development of resistant strains.

“Race for the surface” is an important theory in implantology. It suggests that there is a race between human tissue cells attachment and bacterial adhesion to implant surfaces. If bacteria attach to the surface first, it will be very difficult for tissue cells to replace bacteria and form a stable combination. In contrast, inhibition of bacterial adhesion will benefit the combination of cell-implant interface[18]. We have shown that Ti-PDOP-DNase I, the DNase I coating, could significantly prevent early biofilm formation of S. mutans, S. aureus[20], A.a and F.n, which are closely related to implant surface infection. According to our results, more than 90% of the A.a and F.n failed to adhere on the surfaces of Ti-PDOP-DNase I, which means that more cells have a chance to attach to the surface. Besides, surface hydrophilicity has a significant effect on the attachment and vitality of cells on Ti material[40]. PDOP coating, which is hydrophilic, could significantly promote cell attachment and proliferation on the implants[41]. Our results showed that the bacterial biomass on Ti-PDOP surfaces was larger than that on Ti surfaces, which might be related to the adhesion promoting characteristics of dopamine[20]. And our previous studies have shown that the hydrophilicity of DNase I coating is higher than that of polydopamine coating, which is also beneficial to adhesion, spreading and proliferation of MC3T3-E1[22]. These properties of DNase I coating may lead to a better combination of cells and Ti implant.

In the early healing stage of osseointegration, bone marrow mesenchymal stem cells (MSCs) or immature osteoblasts are possible initial colonizers. After proliferation and differentiation, the initial osteoblast lineage cells transform into osteoblasts and contributed in osteogenesis[42]. Therefore, effective osteogenic differentiation is a critical factor in good osseointegration. It is reported that the lipopolysaccharides produced by periodontal pathogens, such as those from A.a and F.n, had significant influence on the osteogenic differential ability of stem cells, and the effects varied with the concentration[43,44]. Whether DNase I coating had negative effects on cell osteogenic differentiation is still unclear. In this study, MC3T3-E1 was used as the preosteoblast model in vitro to investigate the effect of DNase I coating on osteogenic differentiation. ALP is a recognized marker for the differentiation of osteoblasts at an early stage, whose expression level peaks at matrix maturation phase[45]. The results of ALP activity in this study showed that the ALP activity of cells on Ti-PDOP-DNase I surfaces increased significantly compared with that on Ti surfaces. The results of RT-PCR showed that the mRNA levels of osteogenic genes ALP, OPN, BSP and SP7 on Ti-PDOP-DNase I surfaces were all up-regulated compared to those on Ti. All results indicated that the novel DNase I coating Ti-PDOP-DNase I had no adverse effect on osteoblast function while improving the anti-infective ability of implants. Furthermore, it might even increase the expression of osteoblast markers and have the potential to promote early osteoblastic differentiation. Given that high-density cell adhesion has a positive effect on cell aggregation and differentiation, we speculate that the reason may related to increased cell adhesion density and cell activity, but the exact mechanism needs further investigation[46].

CONCLUSIONS

In conclusion, the Ti-PDOP-DNase I coating could significantly prevent adhesion and 24 h biofilm formation of peri-implant pathogen A.a and F.n in vitro. And DNase I functionalized Ti substances had no adverse effect on osteoblast function. Thus, the biocompatible DNase I coating could be an alternative approach for preventing implant-related infection and improving achievement ratio of dental implantation.

However, in clinical practice, biofilms causing implant-related infection consist of mixed strains rather than a single strain. More efforts are needed to explore the multi-species infection. In addition, the cell model utilized in this study is primary, further in vitro and in vivo analyses are necessary.

ACKNOWLEDGMENTS

This work was supported by Science Foundation of Tianjin Medical University Stomatological Hospital (grant no. 2015YKY02).

We are grateful to Bette Smith, ELICOS teacher of La Trobe Melbourne (Navitas Ltd.), Australia for the proofreading of the manuscript.
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