Doxycycline Release of Dental Implants With Nanotube Surface, Coated With Poly Lactic-Co-Glycolic Acid for Extended pH-controlled Drug Delivery

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When dental implants become infected, the progression of the disease is rapid. Commercially available dental implant surfaces can be easily contaminated, resulting in rapid progression of peri-mucositis and peri-implantitis. The aim of this study was to evaluate, in vitro, the pattern of doxycycline release from by dental implants with titanium nanotube surface (DINS) at different pHs to examine novel drug loading and chemical coating techniques. Nine DINS were loaded with doxycycline and subsequently coated with polylactic-co-glycolic acid (PLGA). High-performance liquid chromatography (HPLC) was used to measure the amounts of released doxycycline in a 30-day period. Cytotoxicity of the DINS was evaluated by an assay using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT). The results showed that the experimental DINS coated with doxycycline and PLGA showed a mean drug release during the experimental period for the groups: pH 7.4 (8.39 μg/mL), pH 6.4 (8.63 μg/mL). The pH 5.4 (15.18 μl/mL) doxycycline release from DINS was faster at pH 5.4 than those at pHs 6.4 and 7.4 ($P = .0031$ and .0034, respectively). This new surface treatment of dental implants with titanium nanotubes and subsequent drug loading demonstrated biocompatibility and sustained doxycycline release over a 30-day period. Additional studies are needed in order to adopt a stable drug release at neutral pH environment while warranting a constant drug release in an acidic pH environment.

Key Words: dental implants, nanotube surface, drug delivery, PLGA, doxycycline

INTRODUCTION

Dental implants are associated with a high complication rate, varying from biological to mechanical.[1] Biological complications can be peri-implant mucositis (a reversible pathological reaction of the peri-implant soft tissues) or peri-implantitis, characterized by progressive destruction of bone around the implant after osseointegration.[2] These biological complications may be caused by bacteria-induced inflammatory changes in the surrounding tissues of a susceptible host; therefore, abnormalities in the tissue around the implant may be the main reason for implant failures.[3]

Usually, bacterial aggregation initiates in the soft tissues around the implant-retained crowns. If this initial bacterial insult is not resolved, the infection may reach the implant-abutment interface.[4,5] The inflammation may progress apically and result in bone loss. This peri-implant bacterial infection process has shown to be similar to the progression involved in periodontal diseases, presenting similar microbiological characteristics.[3,5]

Studies have shown that peri-implantitis occurs in 28%–56% of the implants. Treatment options may be surgical or nonsurgical and involve chemical and physical decontamination processes.[6] The nonsurgical methods of treating peri-implantitis include mechanical instrumentation and the use of various antibacterial agents. The use of different antimicrobial agents is possible but is effective only when applied during the early stages of the disease.[7,8] The use of subgingival disinfecting irrigants and locally applied antibiotics[9]—such as tetracycline fibers[10]—were employed, but neither treatment provided a conclusive therapeutic effect. The systemic administration of

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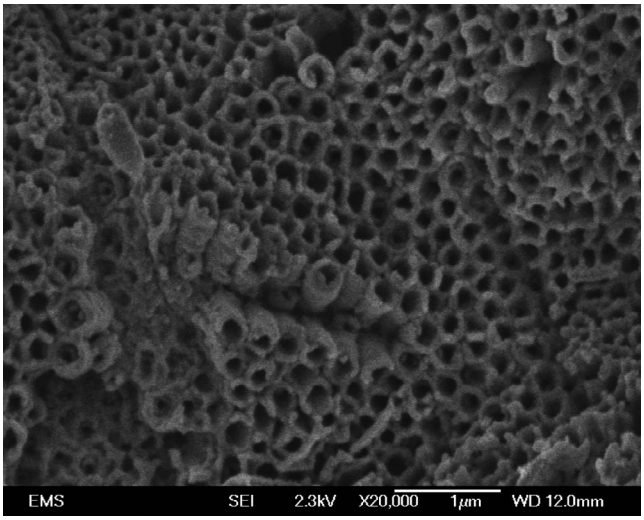


Figure 1. Scanning electron microscopy of a DINS at ×20 000 magnification.

antimicrobial agents was tested in the treatment of peri-implantitis[11]; however, the results were limited due to resistant strains of bacteria and ineffective drug dosages.

Conventional mechanical methods have shown to be ineffective for complete debridement of the bony defect as well as the contaminated microstructured implant surface.[12] Generally, adjunctive application of systemic or local antibiotics and antiseptics has been recommended.[13] Due to potential antibiotic resistance and often insufficient effect of antimicrobial agents for bacterial eradication, as well as poor results of re-osseointegration following adjunctive application during peri-implantitis non-surgical and surgical therapy,[14] novel approaches are still necessary in the treatment of peri-implant diseases.

Drug delivery systems have been used in medicine to target specific microorganism.[15] An advantage of the local delivery of antibiotics is that the total amount of drug used is considerably lower compared to its concentration after systemic administration. Therefore, side-effects—such as hypersensitivity, gastrointestinal discomfort, nausea, vomiting, pseudomembranous colitis, and others—are less likely to occur when the local drug release systems are used.[15]

Nanotechnology has introduced a surface modification to dental implants by using electrochemical treatment in fluoride containing electrolytes, which results in formation of arrayed vertical titanium dioxide (TiO_2) nanotubes.[16] If these structures occur in appropriate dimensions, they may enable the adherence of mesenchymal stem cells and support growth and regeneration of bone tissue.[16] Furthermore, the increased surface area and format of these nano-modified surfaces may allow loading with bioactive agents or antimicrobials, thus serving as in situ drug delivery systems with significant advantages when compared with a systemic delivery system.[16]

Doxycycline has shown to improve wound healing, increase osteogenic mediators, and reduce collagenase activity.[16] Recently, the benefits of doxycycline as an osteogenic agent were observed in in vivo periradicular surgeries,[16] in the treatment of infra-bony defects,[16] and in the downregulation of osteoclastogenesis in vitro.[16]

Polylactic-co-glycolic acid (PLGA) has been used as a delivery vehicle for almost all types of antibiotics[16] due to its tunable degradation profile and biocompatible degradation products. Several studies have reported the release of antibiotics from PLGA over the span of weeks to months.[16] Antibiotic-loaded PLGA has also been incorporated into tissue engineering scaffolds for the purpose of mitigating infection over 8 weeks in vitro.[16]

To improve the physical properties of implants and overcome the disadvantages of treatments currently available for peri-implantitis, an innovative approach is the modification of the titanium surface with nanotubes, using use its biocompatible advantages and serve as a drug reservoir for local drug delivery. The aim of the study was to evaluate in vitro the pattern of doxycycline release, at different pH levels, by nanosurface dental implants using a proposed loading and surface coating technique, as well as the cytotoxicity behavior. This study was reviewed by an independent statistician.

Materials and Methods

Nanotube treatment of dental implants

Prefabricated dental implants were cleaned by means of sonication in acetone (Fisher Scientific, Waltham, Mass) for 30 minutes, rinsed in deionized water and air-dried. Nanotubes were incorporated perpendicular to the dental implant surfaces using an electrochemical anodizing technique. Anodization was performed under optimized condition that were determined in previous studies. The dental implant with nanotube surface (DINS) were attached to a DC voltage source (Keithley 2400 SourceMeter) as the working electrode, with copper mesh used as the counter-electrode. Both electrodes were immersed in an electrolyte mixture of ethylene glycol (Fisher Scientific), 0.3 wt% NH4F and 10 vol.% deionized water. Preliminary data shows the best settings for anodization of samples is 120 V for 2 hours.[16] The samples anodized in this setting showed a more sustained drug release. A magnetic stirrer was used to agitate the electrolyte as constant DC voltage of 120 V was applied for 2 hours. Anodization was performed at room temperature.

Nanotube surface characterization

The average nanotube dimensions were verified using a field emission scanning electron microscopy (JEOL JSM-6320F). To determine dimensions of nanotubes, the DINS were placed on a double-sided conductive carbon tape and attached to an aluminum stub for imaging. Next, *ImageJ* software was used to measure the nanotube dimensions. A scanning electron microscopic image was obtained from a sample after adjustment of the anodization settings and fabrication of the experimental samples. The cylindrical and hollow nature of the TiO_2 nanotubes as confirmed by the SEM (Figure 1) suggests the possibility of serving as carrier for drug and polymer loading. The TiO_2 nanotubes showed an approximate diameter of 100 nm and length of 12 nm. During the 30-day drug release study, SEM morphological evaluation showed no

changes that could have incurred during the experiment, when compared to the pre- and post-drug loading morphologies. The structural stability of the nanotube surface modification suggests that it is a promising dental implant surface for long-term pH-controlled drug delivery.

Sterilization of the DINS

All samples were cleaned in an ultrasonic bath using trichlorethylene as a detergent and then rinsed two times in absolute ethanol. Afterward, a loading technique was used on the experimental DINS.

DINS doxycycline loading and PLGA coating

Nine DINS were loaded with doxycycline by dipping DINS in doxycycline solution in deionized water at a concentration of 50 mg/ml. After loading doxycycline in nanotubes on surface, DINS were further coated using a PLGA solution in dichloromethane at a concentration of 2% weight/volume. During the PLGA coating, triethyl citrate at 5% weight/weight of the PLGA was used as a plasticizer.

Doxycycline loading process

The DINS were submerged in prepared doxycycline solution sonicated (Bransonic 2800 Ultrasonic bath 40kHz, Danbury, Conn) for 10 minutes, maintained under vacuum for 5 minutes, and air-dried at room temperature for 10 minutes in a fume hood. The whole loading process was repeated 3 times to maximize doxycycline loading on the DINS.

Polymer coating process

Doxycycline-loaded DINS were submerged in PLGA solution. Next, wet DINS were dried at room temperature for 7 hours under the hood, then dried under vacuum for 9 hours. The whole polymer coating procedure was repeated 5 times to warrant complete covering doxycycline-loaded DINS with PLGA. The coated DINS were characterized by scanning electron microscopy (SEM; 15 kV, Cambridge 360) for atomic composition, coating thickness, and morphology.

SEM analysis

Two DINS were used for morphological analysis. Implant surface morphology alterations were identified by a SEM (Carl Zeiss EVO 40, Peabody, Mass) at the UTHSC College of Dentistry Laboratory of Bioscience Research, following manufacturer's recommendations. Implants were secured to the STEM sample holders and fully inspected by the SEM at 20.00 kV, at $\times$50 magnification for damage. SEM analysis was conducted to verify thickness of coating layer.

In vitro drug release

Prepared DINS samples were divided in 3 groups and assigned for release media of pHs 5.4, 6.4, and 7.4. In vitro doxycycline release in those buffer solutions lasted for 30 days. Biphthalate and phosphate buffer solutions of pHs 5.4, 6.4, and 7.4 were used as drug release media. During the drug release study, samples for the analysis of released doxycycline were taken at designated time points.

Samples measurement

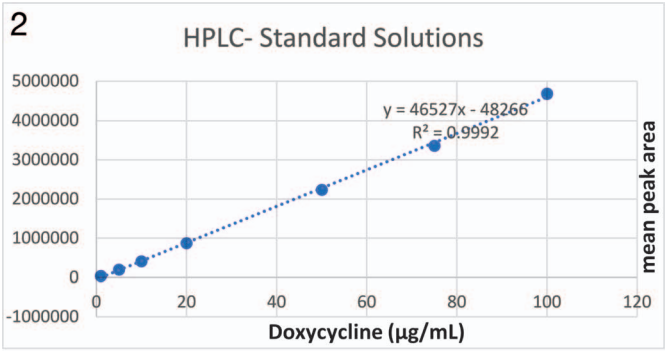
High-performance liquid chromatography (HPLC) assay was performed using the Waters HPLC system with 1525 binary pump, 717plus Autosampler, and 2487 UV detector (Milford, Mass). HPLC analysis was used for drug release study. The chromatographic analysis was conducted at ambient temperature (25°C) on a Waters column (150 $\times$ 4.6 mm, 5.0 μm particle size) with the mobile phase composed of a mixture of water and/0.1% of trifluoroacetic acid (TFA) and a mixture of acetonitrile/0.1% TFA at a volume ratio of 60:40, which was filtered through 0.2 μm membrane filter. The flow rate was 1.0 mL/min with the detector wavelength set at 360nm. Injection volume was 50 μl. The HPLC condition resulted in the retention time of 2.0 minutes for doxycycline.[16] For preparation of standard solution for a calibration curve, 1 mg of doxycycline was accurately weighed and diluted in 1 mL of deionized water to provide the stock solution (1 mg/mL doxycycline solution). Linearity between doxycycline concentration and integrated peak areas was assured in the drug concentration range of 1 μg/mL to 100 μg/mL. Obtained calibration curve showed linearity within the concentration range with a correlation factor of 0.99917 (Figure 2). The sample solutions for HPLC analysis were diluted 2 times with mobile phase and filtered through a fluoropore polytetrafluoroethylene filter membrane (Sigma-Aldrich Corp, St Louis, Mo) syringe-driven filter (0.45 μm) before injection.

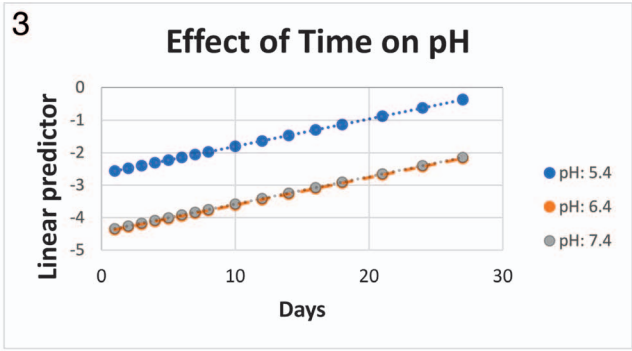
MTT assay

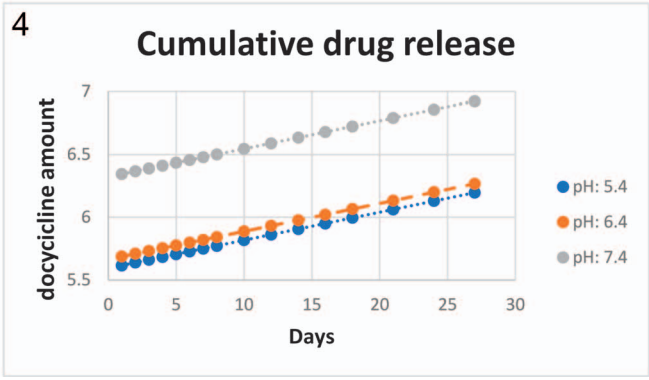
Cytotoxicity of DINS against the gingival fibroblasts was evaluated by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich Corp). Normal gingival fibroblasts were cultured and plated (1×10^5 cells) in a 6-well dish. Three implants from 2 experimental (100 nm diameter DINS, 180 nm diameter DINS) and one control group were placed in individual wells containing attached fibroblasts and incubated for 3 days. At the end of the incubation period, the implants were removed, and cell viability was determined by MTT cell proliferation assay (Table 1).

Data analysis

The significance of differences was obtained by means of SAS system, release 9.3 (2010 SAS Institute, Inc, Cary, NC). The mean, median, minimum, maximum, cumulative percentage average, and standard deviations were calculated for the release study. A generalized linear mixed model of variance analysis was fitted to test the effect of pH and time (days) on the response variables. The adequacy of the model was evaluated through the coefficients of asymmetry and kurtosis, which allowed the evaluation of the adherence of the residues to the Gaussian distribution. Significant pH effects were complemented by the Tukey-Kramer post-hoc test and for the study of the significant effect of time the adjusted parameters of this effect were considered as co-variable of the model (simple linear regression). In all tests, the significance level of 5% was adopted.







Figures 2–4. Figure 2. Calibration curve of the standard solutions used for high-performance liquid chromatography (HPLC) analysis. Integrated areas of HPLC peaks were graphed as a function of doxycycline concentration in standard solutions (x-axis). **Figure 3.** Effect of doxycycline release over time. Note the statistically significant difference at pH 5.4 compared with pHs 6.4 and 7.4. **Figure 4.** Thirty-day cumulative average quantity (µg) of doxycycline released under pH 5.4, 6.4, and 7.4**.**

Results

MTT assay

Cytotoxicity of DINS against the gingival fibroblasts was evaluated by means of the MTT assay. Normal gingival fibroblasts were cultured and plated (1×10^5 cells) in a 6-well dish. Three implants were placed in the wells with fibroblasts and incubated for 3 days. At the end of the incubation period, implants were removed, and the cell viability was determined by MTT cell proliferation assay. Absorbance results from the statistical analysis performed using Student *t* test ($P < .05$); were considered statistically significant. Statistical analysis was reviewed by an independent statistician. The two-experimental diameter (100 nm and 180 nm) nanotube loaded implants did not show statistical difference in cell growth when compared with the control group (Table 1).

HPLC assay

The HPLC assay showed a release of doxycycline during a 30-day period, for all 3 experimental groups.

Doxycycline exerts anti-collagenase activity[18] at the local level of 1.2–8.1 µg/mL.[19] The results showed that DINS inserted in solution with pH 5.4 showed a burst of drug release of 112 µg/mL in the first 24 hrs. In the following 2 days, the mean concentration of the drug released from the DINS reduced to 45.45 µg/mL. In the following 8 days, the mean value of the released drug was 13.0 µg/mL. In the following 17 days, the mean drug release value was 5.15 µg/mL. The last 3 days showed a mean value of drug release of 5.15 µg/mL. All the values evaluated individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 5.4 pH solution (Table 2).

When the DINS were inserted into a 6.4 pH solution, there was a burst of drug release of 91.56 µg/mL in the first 24 hrs. In the following 2 days, the mean concentration of the drug released from the DINS reduced to 22.07 µg/mL. In the following 8 days, the mean value of the released drug was 8.17 µg/mL. In the following 17 days, the mean drug release value was 3.11 µg/mL. The last 3 days showed a mean value of drug release of 1.26 µg/mL. Each of the values individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 6.4 pH solution (Table 2).

When the DINS were inserted into a 7.4 pH solution, there was a burst of drug release of 96.55 µg/mL in the first 24 hrs. In

Table 1

MTT assay result showing absence of cytotoxicity in both test DINS and control implants*

DINS	Viable Cells/Well
100 nm diameter DINS	2.9×10^4
180 nm diameter DINS	2.24×10^4
Control	2.45×10^4

*DINS indicates dental implants with titanium nanotube surface; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

Table 2

Daily average quantity (μg) of doxycycline released from 100 nm diameter dental implants with titanium nanotube surface under pH 5.4, 6.4, and 7.4

	pH 7.4	pH 6.4	pH 5.4
Day	Quantity (μg)	Quantity (μg)	Quantity (μg)
1	96.55	91.56	112.44
2	28.21	25.91	59.22
3	25.51	18.23	31.67
4	8.62	9.10	33.16
5	6.90	18.06	19.32
6	6.35	13.18	15.98
7	5.33	7.20	9.68
8	4.43	8.16	11.38
10	4.30	9.65	14.49
12	4.90	11.61	17.56
14	14.62	11.20	21.83
16	21.33	9.82	16.87
18	23.43	8.86	11.90
21	12.94	3.88	6.93
24	11.14	3.78	6.39
27	9.97	3.73	6.12
30	6.03	3.79	4.26

the following 2 days, the mean concentration of the drug released from the DINS reduced to 26.86 μg/mL. In the following 8 days, the mean value of the released drug was 4.49 μg/mL. In the following 17 days, the mean drug release value was 5.78 μg/mL. The last 3 days showed a mean value of drug release of 2.01 μg/mL. All the values evaluated individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 7.4 pH solution (Table 2).

For all loaded DINS evaluated, the drug release at the 3 different pHs (7.4, 6.4, and 5.4) showed drug release above the range shown to induce anti-collagenase activity (Figures 3 and 4).

The released drug concentration was higher when in acid media. The mean released drug concentration in a 30-day period was 13.30 μg/mL, 8.59 μg/mL, and 9.68 μg/mL for the pH groups 5.4, 6.4, and 7.4, respectively (Tables 2 and 3).

In all 3 pH groups, there was an increased discharge of the drug after the first day. The pH 5.4 group showed a higher concentration of the drug released by effect of time on pH. The statistical test Tukey-Kramer was applied to the HPLC assay results. The methodology of this study was reviewed by an independent statistician. This drug released was statistically

Table 3

Shows mean, standard deviation (STD), and number of observations (N Obs) of doxycycline released under pH 5.4 , 6.4, and 7.4 in a 30-day period

	Variable Analysis		
pH	N Obs	Mean	STD
5.4	51	13.30	33.42
6.4	51	8.59	24.08
7.4	51	9.68	26.21

Table 4

Tukey-Kramer statistical test adjusted for multiple comparisons was applied for pH 5.4, 6.4, and 7.4 groups for differences of pH least square means. Values for mean, standard error (STE), and *P* value

pH	pH	STE	*P* Value
5.4	6.4	0.3185	.0031
5.4	7.4	0.3185	.0034
6.4	7.4	0.3185	.9946

significant when comparing the concentration of the released drug in the other 2 groups (Tables 3 and 4). The pH groups 6.4 and 5.4 showed higher drug release in the initial experimental period when compared to group 7.4; however, this was not a statistically significant difference. The pH 7.4 group showed higher cumulative drug released; there was no statistically significant difference between the groups (Tables 3 and 4).

SEM analysis

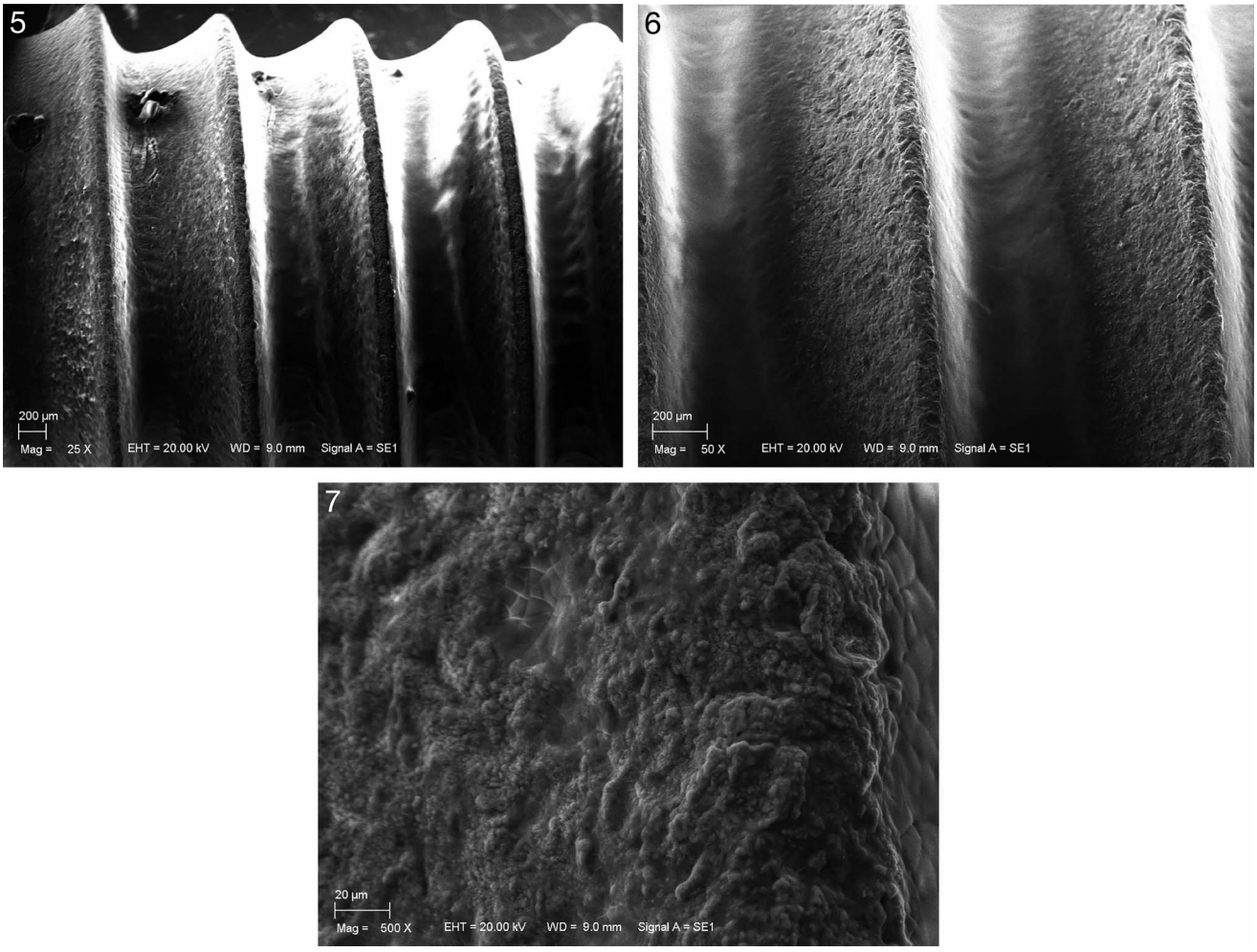
A Carl Zeiss AG – EVO 40 Series scanning electron microscope (Carl Zeiss, Beaverton, Ore) was used to was conducted the SEM analysis. SEM analysis showed presence of and intact the PLGA coating, even after the initial wash with distilled water at ×25 (Figure 5), ×50 (Figure 6), and ×500 (Figure 7) magnifications.

All sample analyses of this study were conducted after thorough calibration of each piece of equipment used.

Discussion

Doxycycline exerts anti-collagenase activity,[18] which is a desirable effect in patients presenting periodontal and/or peri-implant infections. It has been shown that the minimum dose of doxycycline taken systemically at 100 mg/day results in the local level of drug concentration in the crevicular sulcus that ranges from 1.2–8.1 μg/mL.[19] The proposed doxycycline loading and PLGA coating techniques for the experimental DINS is a prolonged drug release up to a 30-day study period. Daily dosages were above the minimum dosage necessary for the drug to exert anti-collagenase activity in the periodontal tissues.[19]

The results of the current study showed that the doxycycline released from the DINS was at a higher concentration at a lower pH, which could have been due to the faster dissolution of the DINS' PLGA-coated layer in an acidic environment,[16] releasing a greater concentration of the doxycycline to the embedding solution. In addition, there was a higher release of the drug at pH 6.4 in the first day of the experimental period. In the present study, the implants immersed in the pH 5.4 solution showed a higher concentration of the released drug as time elapsed. The objective of this proposed loading/coating technique would be to have the drug available in the presence of an infection, here simulated by immersing the DINS in the acidic pH solution. Therefore, this release pattern in an acidic environment is acceptable (Figure 4). The proposed PLGA loaded and coated DINS seem to be promising selective drug-releasing devices. However, in a healthy environment, the normal pH of blood plasma is 7.4.[20]



Figures 5–7. Figure 5. Scanning electron microscopy of a commercially available implant coated with polylactic-co-glycolic acid coating at ×25 magnification. **Figure 6.** Scanning electron microscopy of a commercially available implant coated with polylactic-co-glycolic acid coating at ×50 magnification. **Figure 7.** Scanning electron microscopy of a commercially available implant coated with poly lactic-co-glycolic acid coating at ×500 magnification.

Ideally, the DINS should not release the drug at pH 7.4. In the present study, the drug was released at a mean value of 9.68 µl/mL at pH 7.4.

Saliva of patient presenting chronic generalized periodontitis has shown to be chronic generalized periodontitis was 6.85 ± 0.11.[21] At this pH, it is expected for the drug to be released from the DINS. In the present study, the mean value of the released drug from the DINS at pH 6.4 was 8.59 µl/mL, Ideally, the DINS should release the drug at pH 6.4. In the present study, the drug was released at a mean value of 8.59 µg/mL at pH 6.4.

In the inflamed tissues, several mediators are recruited into the interstitial fluid forming an inflammatory exudate. Cytokines present in the exudate recruit leukocytes, which actively pump lactic acid into the exudate, lowering the pH.[22] The high hydrogen ion concentrations of the inflamed tissue have shown to go down to pH 5.4.[23] In the present study, the mean value of the released drug from the DINS at pH 5.4 was 13.30 µl/mL. During all time periods evaluated in this study, the DINS release doxycycline at pH 5.4 above the minimum range indicated for it to exert anti-collagenase activity.[19]

Antibiotics such as doxycycline are commonly used to prevent and treat peri-implantitis.[7] The systemic administration of antibiotics may result in inadequate dosage of the drug at the crevicular sulcus.[24] In addition to doxycycline's anti-collagenase activity,[18] it has shown to exert regenerative potential in guided bone regeneration[25] and osseointegration.[7]

A minimum concentration of approximately 1.4 µg/mL of doxycycline is necessary locally to have beneficial osteogenic effects.[19] The present study has shown that the proposed dental implant nanotube dimensions, loading, and coating techniques were able to sustain a sufficient drug concentration to or above the minimum level to promote anti-collagenase activities sustained for a 30-day period (Figure 4, Table 1). At the proposed acidic environment, the mean concentration of the drug released was 13.30 µm/mL for the period of 30 days.

The doxycycline loaded and coated DINS' biocompatibility is an important factor that needs to be highlighted. The PLGA

applied to the surface of the implant did not alter the tested monocyte's cytotoxic response.

A limitation of this study was possible pH changes that may have occurred during the release of the drug into the PBS solution. The initial pH, within the 24-hour span in which each sample was maintained in the solution, could have changed as the drug had been released into the media. The influence of this pH change was not evaluated in this study.

The present study showed that the proposed loaded/coated doxycycline resulted in a slow and prolonged release of doxycycline for a 30-day period in vitro. Future studies are controlling the initial burst of the drug release to extend the life of the loaded drug. In addition, the drug release should not occur in neutral pH and be released only at lower pH, simulating an inflammatory environment where the drug would ultimately be required. Thus, a local drug delivery system could be the solution to achieving a sufficient local antibacterial effect in search for a dental implant that would allow reduction or control of peri-implantitis infections at the initiation of the process.

Conclusion

The novel dental implant nanotube surface treatment and drug loading/coating protocols showed biocompatibility and a long-term doxycycline release for a 30-day period. Future studies are necessary to stabilize the unloading of the drug from the implants in a controlled manner, maintaining the implants unloaded at neutral pH. This study was reviewed by an independent statistician.

Abbreviations

DINS: dental implants with titanium nanotube surface
HPLC: high-performance liquid chromatography
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
PLGA: polylactic-co-glycolic acid
SEM: scanning electron microscopy
TFA: trifluoroacetic acid
TiO_2: titanium dioxide

Note

The authors declare that there is no conflict of interest.

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