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ORIGINAL ARTICLE

Modification of TiO_2 nanotube surfaces by electro-spray deposition of amoxicillin combined with PLGA for bactericidal effects at surgical implantation sites

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Abstract

Objective. To fabricate the antibiotic-releasing coatings on TiO_2 nanotube surfaces for wide applications of implant and bone plate in medical and dental surgery, the optimal deposition time of amoxicillin/PLGA solution simultaneously performing non-toxicity and a high bactericidal effect for preventing early implant failures was found. ***Materials and methods.*** FE-SEM, ESD and FT-IR were used for confirming deposition of amoxicillin/PLGA on the TiO_2 surface. Also, the elution of amoxicillin/PLGA in a TiO_2 nanotube surface was measured by a UV-VIS spectrophotometer. The bactericidal effect of amoxicillin on the TiO_2 nanotube surface was evaluated by using *Staphylococcus aureus* (*S. aureus*). The cytotoxicity and cell proliferation were observed by WST assay using MC3T3-E1 osteoblast cells. ***Results.*** The results indicated that the TiO_2 nanotube surface controlled by electro-spray deposition time with amoxicillin/PLGA solution could provide a high bactericidal effect against *S. aureus* by the bactericidal effect of amoxicillin, as well as good osteoblast cell proliferation at the TiO_2 nanotube surface without toxicity. ***Conclusions.*** This study used electro-spray deposition (ESD) methodology to obtain amoxicillin deposition in nanotube structures of TiO_2 and found the optimal deposition time of amoxicillin/PLGA solution simultaneously performing non-toxicity and a high bactericidal effect for preventing early implant failures.

Key Words: *implant surface coating, electro spray deposition, amoxicillin, PLGA*

Introduction

Numerous dental implant surgeries are performed annually. For the replacement of missing teeth, titanium (Ti) implants have been the first choice of treatment [1]; however, early infection at the implantation site causes implant failure. The major bacterium associated with early implant failure is *Staphylococcus aureus* (*S. aureus*) [2]. The dentist and the surgeon use orally administered antibiotics, especially amoxicillin, to remove these bacteria from around the implantation site [3]. A higher bactericidal effect by amoxicillin at the surgical sites can be achieved by local delivery of amoxicillin.

As a method of local drug delivery, polymer groups that belong to the poly-lactic-co-glycolic-acid (PLGA) are commonly used due to their biocompatibility [4–6]. One of the main concerns with use of PLGA in biomaterials is the unexpected effect of PLGA on osseointegration they biodegraded. However, recent research showed that the hydrolysis of PLGA and its hydrolysate did not interrupt osseointegration compared to that of the non-coated group [5].

A major factor for implant success is osseointegration. Electro-spray deposition (ESD), micro-arch oxidation (MAO), sand blasting with large grit size and acid etching (SLA) have been used to obtain the rapid osseointegration of Ti with surrounding bone. ESD is one of the easiest ways to put nanosized components on the metal at room temperature, while low current anodizing oxidation (LCAO) in hydrogen floride (HF) is useful to obtain a good nanotube structure for getting increased roughness and rapid osseointegration [7,8].

In the present study, a mixture of amoxicillin and PLGA was deposited on the TiO2 nanotube surface

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by ESD. Our designed material was coated with amoxicillin for bactericidal functions in the early period following implantation, while treatment by LCAO promoted good osteoblast attachment with the TiO_2 nanotube structure.

Materials and methods

Surface treatment

The cp Ti was obtained from Ostem Implant (Pusan, Korea). Specimens (r = 0.57 mm, h = 1 mm) were polished using 2000 grit SiC sandpaper and rinsed ultrasonically in acetone, ethanol and distilled water, in that order.

LCAO treatment was performed using a regulated direct current power supply (Genesys 600-2.6, Densei-Lambda, Tokyo, Japan) with a constant voltage mode at 20 V for 60 min using 0.5 wt% HF (Sigma Aldrich, Saint Louis, MO,USA) solution. As soon as LCAO treatment was performed, the specimens were cleaned with distilled water and dried in a vacuum drier 25°C. The samples were heated at 400°C for 3 h, followed by cooling to room temperature.

Poly-lactic-co-glycolic-acid (PLGA, 0.05 wt%, 50:50) (Sigma Aldrich) and amoxicillin (0.025 wt%) (Sigma Aldrich) in dichloromethane (Sigma Aldrich) were combined in a 50 ml vial and deposited by an Electro Spray Machine (ESP200R, NanoNC, Seoul, Korea) for 30 s, 1 min and 2 min at a rate of 500 μl/min under 20 kV. Each specimen was sterilized by ethylene oxide (EO) gas.

Surface characterization

Specimens prepared by anodizing oxidation using low current were characterized by field emission scanning electron microscopy (FE-SEM, JSM-6700F, JEOL, Tokyo, Japan) and energy dispersive X-ray spectrometry (EDS, INCA energy 7421, Oxford, UK). To observe the surface morphology, magnifications of 100,000 × were selected and the specific components were detected by EDS.

Fourier transform infrared spectrometry (FT-IR, Vertex70, Bruker, Germany) was used for detecting amoxicillin and PLGA attached on the LCAO surface. FT-IR shows molecular bonding using the absorbance and transmittance of the reflected infrared spectrum. An attenuated total reflectance (ATR) device was used for detecting molecular bonding of the deposited polymer layer which was only a few micrometers thick. The specimen was placed on the crystal surface of the ATR and was measured by FT-IR. Both background and samples were measured and in each specimen 32 scans were performed at 4 cm^{-1} resolution. The results of samples were corrected with background scans and peaks with over 5% variation were detected. Each peak was compared with peaks in the spectra of authentic samples of amoxicillin and PLGA.

Detection of amoxicillin elution using a UV-VIS spectrophotometer

Treated specimens of nanotubes modified with amoxicillin and PLGA were placed in the wells of a 24-well plate. Each well contained 1 ml of Alpha modified Minimum Essential Medium (Alpha MEM, Welgene, Daegu, Korea) containing 1% penicillin–streptomycin (Pen Strep, Gibco, Grand Island, NY) and of 10% fetal bovine serum (FBS, Gibco Grand Island, NY), homemade Alpha MEM, with 10^4 MC3T3-E1 cells. The solution was collected every third day and fresh solution was then added. An aliquot (100 μl) of the collected solution was analyzed by measuring the UV-VIS absorbance at a certain wavelength showing maximum absorbance, and this wavelength was compared with the wavelength of amoxicillin in distilled water [9]. All readings were corrected for the background absorbance of Alpha-MEM. Eluted amoxicillin was quantified by comparison with a standard curve generated from authentic amoxicillin over the concentration range of 0.003,15 wt% to 0.025 wt%. The study was continued until no absorbance was detected in the specimen.

Evaluation of bactericidal activity

S. aureus (American Type Culture Collection 6538) were cultured aerobically in a conical tube containing 100 ml medium 0.8 wt% Nutrient Broth (Difco, Le Pont de Claix, France) and 1.5 wt% Agar (Bacto, Le Pont de Claix, France) in distilled water) at 37°C. The initial concentration of bacteria was adjusted to 10^7–10^8 colony-forming units (CFU)/ml by dilution with the medium. Aliquots of this bacterial solution (100 μl) were pipetted onto each specimen in 24 wells. After 4 h, an additional 900 μl of medium was added to each specimen, fully immersing the specimen. After 24 h, the specimens were moved to a 10 ml vial containing 1 ml of new medium. The sample was then fully ultrasonicated for 5 min in order to disperse the bacteria on the surface of the specimen. Aliquots (100 μl) of each solution were plated onto Bacto-Agar plates. Plates were incubated for 24 h at 37°C to determine the number of viable *S. aureus* colony units.

Evaluation of cytotoxicity

The water-soluble tetrazolium salt (WST) assay is a new cell viability assay that measures cell cytotoxicity, proliferation and viability. WST produces water-soluble yellow formazan when the WST solution reacts with viable cells. MC3T3-E1 cells (CRL-2593, ATCC) which are preosteoblasts from mouse calvaria were used in the WST evaluation. An aliquot (0.10 ml) of a solution of cells (10^4 cells/ml in homemade Alpha MEM above) and 0.10 ml homemade Alpha MEM above were placed into each well of a 96-well plate and cells were incubated in a humidified 5% CO_2 incubator at 37°C for 24 h.

The quantitation of extraction was monitored by ISO 10993-12. Aliquots (0.02 ml) of the solution containing eluted amoxicillin and PLGA were added to each of the 96 wells. After 24 h, 100 µl of WST solution was added to each well and the cells were incubated at 37°C for 3 h to allow the formation of the formazan crystals. The absorbance at 450 nm of each well was measured with a Micro Elisa reader and the percentage viability was calculated by optical density (OD) values.

Evaluation of cell attachment and proliferation

MC3T3-E1 cells were used for evaluating short-term cell attachment and proliferation for 3 days using WST assay. MC3T3-E1 cells (10^4) in 100 µl homemade Alpha MEM above were pipetted onto the specimen surface and, after 4 h, 900 µl medium was added in each well with the specimen. The specimen was washed once with phosphate-buffered saline (PBS, Gibco) and was moved to a new 1 ml homemade Alpha MEM above and 100 µl WST solution was added after 3 days. OD was measured after 3 h.

Statistical analysis

One-way ANOVA and Tukey's test were used for analyzing differences among groups. The significance level was set at 95%.

Results

FE-SEM and EDS result

FE-SEM images showed that the LCAO cp Ti formed uniform TiO_2 nanotubes ~ 100 nm in size on the surface (Figure 1A). Similar images show the deposition of PLGA and amoxicillin by ESD in samples treated with ESD for 30 s, 1 min or 2 min (Figure 1B–D). Evaluation of a cp Ti sample treated by ESD of amoxicillin and PLGA for 1 min was performed by energy dispersive X-ray spectrometry (EDS) with transmission of the X-rays through 1 µm of the specimen surface. Peaks of Ti, O, C and Pt are evident, but none for N. PLGA has C, O elements [10] and amoxicillin has C, O and N [11]. Because amoxicillin was used, there should be a peak for N in this result. The peak of N could be masked by the Ti peak because the L-shell of Ti and K-shell of N were positioned in the same keV in the EDS result. Alternatively, there may be only a few N molecules on the specimen.

Detection of amoxicillin and PLGA using FT-IR

The successful molecular bonding between the experimental cp Ti specimen and either amoxicillin or PLGA by ESD for 2 min was examined by FT-IR spectroscopy. The presence of spectral bands corresponding to C = O (1754 cm^{-1}) and C-H (1452 cm^{-1}, 1424 cm^{-1}) in the FT-IR spectrum (Figure 2B) confirmed the attachment of PLGA. Similarly, in the spectrum for amoxicillin and the Ti nanotubes, bands associated with N-H (1581 cm^{-1}) and C=O of carboxyl group (1685 cm^{-1}) were detected that were characteristic for amoxicillin (Figure 2C) [12]. Thus, particles of amoxicillin and PLGA were attached on the specimen surface.

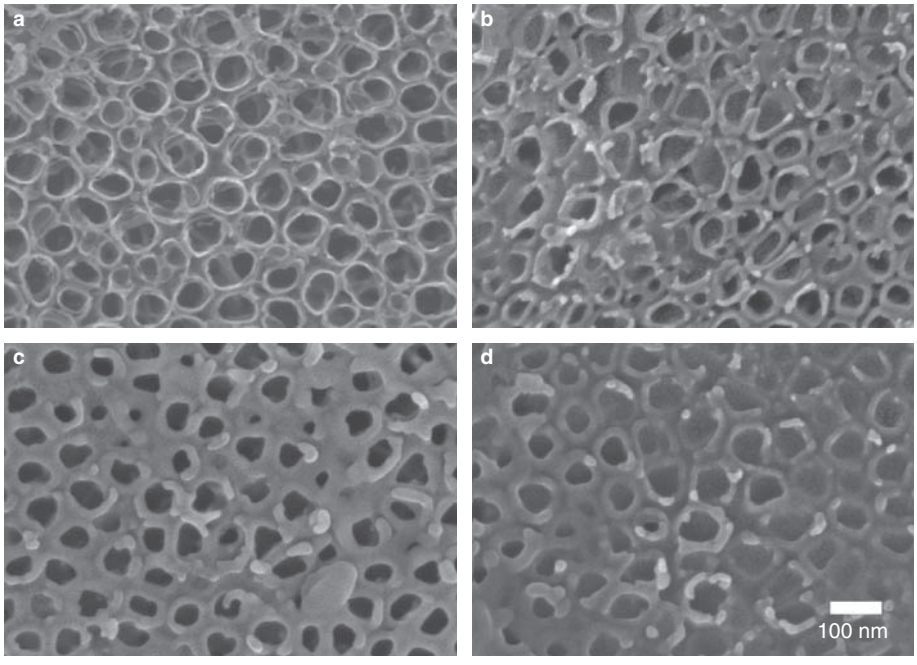

Figure 1. FE-SEM morphology: (a) Low current anodic oxidation (LCAO) of cp Ti. (b) LCAO + during 30 second electro-spray deposition of amoxicillin and PLGA. (c) LCAO + 1 minute deposition. (d) LCAO + 2 minutes.

Detection of amoxicillin elution using a UV-VIS spectrophotometer

The highest absorbance value was ~ 0.17 and the highest absorbance peak was detected in 294.4 nm. The serial absorbance of amoxicillin concentrations from 0.025 wt% amoxicillin to 0.003 wt% were measured (Figure 3). The graph was proportional and the PLGA concentration didn't affect the amoxicillin absorbance graph. The early 3 days amoxicillin elution of the 2 min group was 0.015 wt%, that of the 1 min group was 0.008 wt% and that of the 30 s group was 0.007 wt% on average (Figure 4). Those concentrations were calculated based on a proportional graph (Figure 3). Amoxicillin concentrations from non-treated cp Ti and treated specimens after 12 days were shown in 0.003 wt% amoxicillin concentrations (Figure 4).

Figure 3 shows the UV-VIS absorption spectrum for amoxicillin, with the major absorbance peak at 294.4 nm. The wavelength of 294.4 nm was different with the 272 nm used for detecting amoxicillin concentration in distilled water [9]. The absorbance value of an amoxicillin solution of 0.025wt% was ~ 0.17. The PLGA

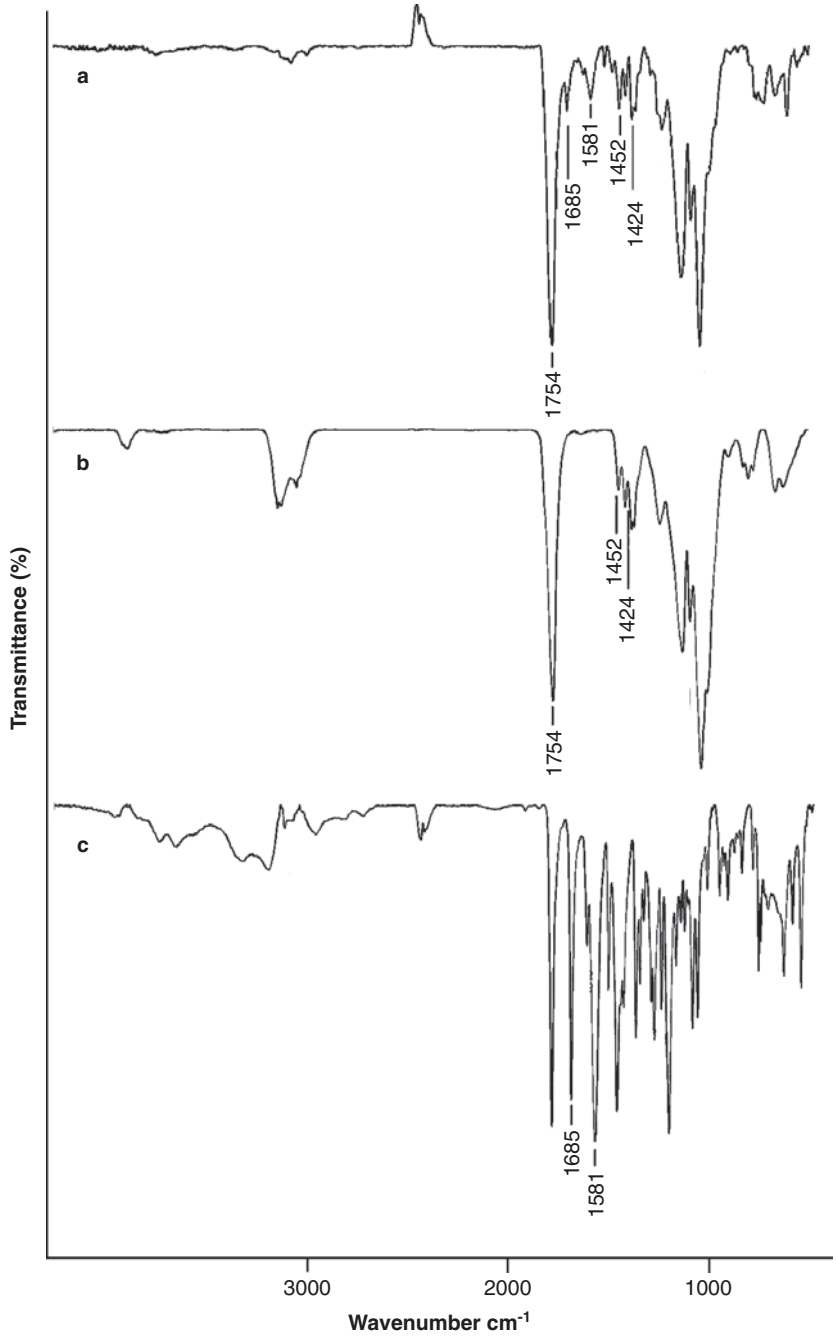

Figure 2. FT-IR spectra: (a) the sample of cp Ti nanotubes (b) cp Ti and PLGA and (c) cp Ti and amoxicillin. All samples were treated by electro spray deposition for 2 min as described in the Material and methods section.

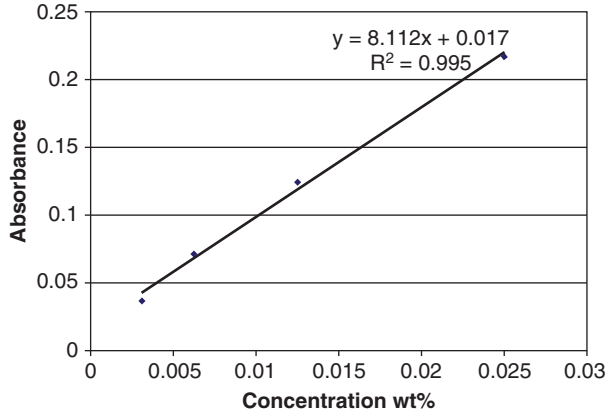

Figure 3. Proportional graph of serial amoxicillin concentrations in medium solution

concentration did not affect the amoxicillin absorption. Elution of amoxicillin after 3 days in the 2 min group was 0.015 wt%, that of the 1 min group was 0.008 wt% and that of the 30 s group was 0.007 wt% on average (Figure 4). Amoxicillin concentrations eluted from treated samples of Ti nanotubes after 12 days were shown to contain no amoxicillin concentrations (Figure 4).

Evaluation of bactericidal activity

Significantly fewer bacterial colony forming units (CFUs) appeared in samples containing amoxicillin-modified Ti nanotubes prepared with deposition times of 30 s and 2 min than were detected in the cp Ti control sample ($p < 0.05$, Figure 5). However, there was no significant difference between the 1 min group and cp Ti ($p > 0.05$). The samples treated with a deposition time of 2 min formed significantly fewer CFUs than samples treated for only 30 s or for 1 min ($p < 0.05$).

Evaluation of cytotoxicity

Extracts prepared from amoxicillin-modified Ti nanotubes or the unmodified cp Ti nanotubes were

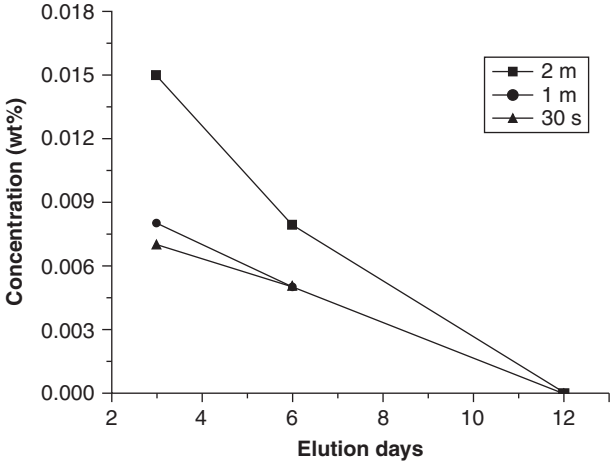

Figure 4. Eluted amoxicillin concentrations: According to different electro spray deposition time after 3days, 6days and 12 days.

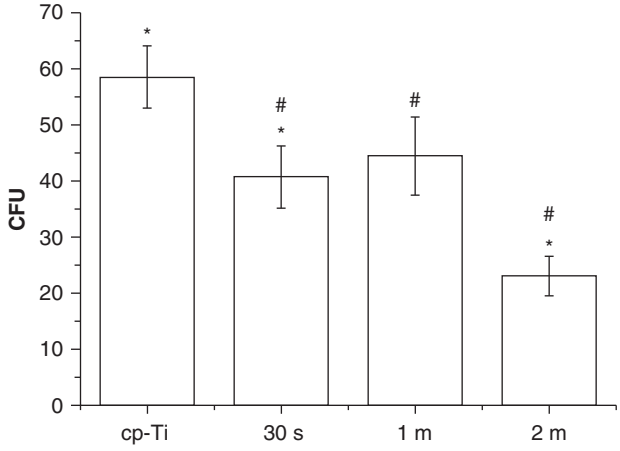

Figure 5. 10 to the seventh power CFUs in 24 h incubations containing S. aureus in the bactericidal activity test: [*: level of significance between cp Ti and the 30s or 2m groups ($p < 0.05$); #: level of significance between 2m and the 30s or 1m groups ($p < 0.05$)] Values represent the average of 5 observations.

not cytotoxic to samples of MC3T3-E1 pre-osteoblast cells (One-way ANOVA and Tukey's test, $p > 0.05$). We noted an increasing trend in the number of viable cells in the 30 s, 1 min and 2 min groups as compared to the negative cp Ti control, but the differences were not significant compared to cp Ti cell viability. The value of cell viability of all experimental groups was over 100%. Thus, the amoxicillin and PLGA complex was not cytotoxic toward MC3T3-E1 cells.

Evaluation of cell attachment and proliferation

In a study following the growth of MC373-E1 cells over 3 days, the cell attachment and proliferation were significantly greater in the amoxicillin-modified nanotube preparation that was treated by electro deposition for 2 min on the LCAO surface (Figure 6).

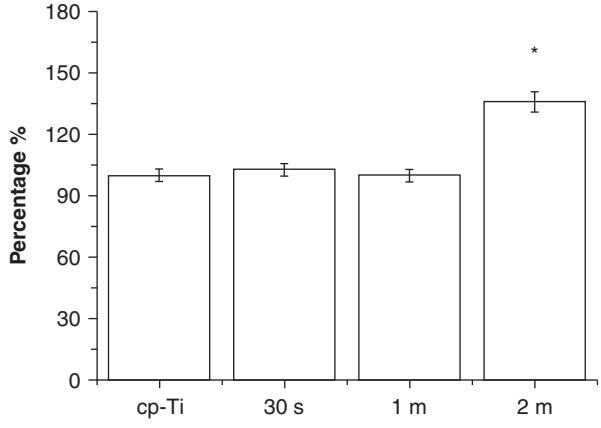

Figure 6. The percentage of attached cell proliferation after 3 days exposure to the surface: After the amoxicillin-modified nanotubes were prepared by 30 s, 1 min, or 2 min of electro spray deposition, cell viability was assayed by WST. (One-way ANOVA and Tukey's test, $p < 0.05$, cp Ti OD value= 0.3). Each study represents the mean and standard deviation of 5 observations.

Discussion

Methods for coating implants to improve bone production have been developed. The attachment of osteoblasts onto the implant was the conclusive factor to improve the bone formation during the early period of implantation [13]. LCAO is one of the coating methods to improve osteoblast attachment on Ti surfaces by formation of a TiO_2 nanotube structure. In the present study, FE-SEM results showed that there were TiO_2 nanotube structures on the Ti surface (Figure 1A). Furthermore, in the preparation of amoxicillin-modified Ti nanotubes, 2 min of electro-spray deposition of the drug and polymer afforded better attachment with cultured MC373-E1 pre-osteoblast cells (Figure 6).

Another challenge of implant coating methodology is the prevention of infection after implantation surgery. Kronstrom et al. [14] reported that the main reason of implant failure was due to the early infection at the implantation site and the most common bacterium involved in the infection is *S. aureus*. It is frequently found in the oral cavity. These bacteria could attach to the Ti surface well and cause periimplantitis. So, incorporating a bactericidal effect into the coating has been under development for a while.

Three methods have addressed this issue of a bacteriostatic effect. The first method includes the use of antibiotics, the second is using an inorganic material like Ag and the last one is using nitrogen oxide for killing bacteria which are produced in the macrophage. Song et al. [15] used plasma for showing the bactericidal effects of Ag on the Ti surface and Kim et al. [16] examined the use of the chlorhexidine for a bactericidal effect on the Ti surface. Nablo et al. [17] used sol-gel nitrogen oxide. The most common process to obtain a bactericidal effect is using antibiotic drugs. One of the methods using antibiotic drug is ESD and it was easily approached in the experiment and showed regular molecular deposition on the metal surface. The molecules are positively charged and can attach to the negatively charged Ti surface.

In previous studies, the antibiotic itself was used but it did not show good attachment on the metal surface. Thus, poly-lactic-acid (PLA) or PLGA were introduced. As a method of local drug delivery, polymer groups that belong to the poly-lactic-co-glycolic-acid (PLGA) are commonly used due to their biocompatibility [4–6]. One of the main concerns with use of PLGA in biomaterials is the unexpected effect of PLGA on osseointegration they biodegraded. However, recent research showed that the hydrolysis of PLGA and its hydrolysate did not interrupt osseointegration compared to that of the non-coated group [5].

Some authors used these compounds as combining polymers to easily attach some molecules for getting an antibiotic effect [4]. Also, Kim et al. [16] used tetracycline plus PLA as a drug delivery system, but tetracycline was only effective against the Gram (+) bacteria and this bacteriostatic antibiotic just limited the growth of bacteria without killing them. Furthermore, the PLA degradation ratio was too slow to show a rapid biodegradation ratio. In a clinical setting, a facultative anaerobic Gram-positive coccal bacterium like *S. aureus* should be removed at the implantation area. So amoxicillin was used in this experiment and, to improve the degradation period, PLGA was considered as a carrier of the drug. PLGA (50%:50%) was degraded in 2 weeks [18].

The existence of amoxicillin and PLGA on the Ti nanostructures was confirmed by FT-IR. The results of FT-IR showed that there were the same peaks of amoxicillin and PLGA in the treated Ti specimen (Figure 2), while the molecules themselves were shown in the FE-SEM images (Figure 1). The elution of amoxicillin was evaluated by a UV-VIS spectrophotometer and elution of amoxicillin from the treated specimen was sustained over ~ 2 weeks (Figure 4). Also, there was a bactericidal effect of amoxicillin (Figure 5) and 2 min of deposition showed a greater bactericidal effect than any other experimental group (Tukey's test, $p < 0.05$) without cytotoxicity (One-way ANOVA, $p > 0.05$). However, good osseointegration will be expected in only 2 min of deposition (Figure 6). The PLGA 50:50 foams we used in this experiment degraded faster with a significantly shorter half-life of 3.0 weeks [19]. This means that the release of the drug came to an end in 2 weeks and biodegradation of PLGA was also finished in 6 weeks after considering the PLGA biodegradation half-life above. However, it is not clear that this experimental bactericidal effect is enough to prevent early infection and to permit Ti osseointegration to be performed *in vivo*. Future testing will involve *in vivo* studies with these treated specimens being undertaken. We will also examine the elution of amoxicillin at different pH values because pH will be changed at the surgical site. With these future experiments, ideal implant coating methods against infection will be developed.

To conclude, in previous discussions many methods have been proposed to minimize infection problems. The electro-spray deposition method was one of them and it is an easy way to attach the polymer to the metal. We observed that 2 min of deposition of amoxicillin and PLGA in dichloromethane was the optimum time for obtaining both bactericidal effect and good osteoblast attachment on the Ti surfaces without cytotoxicity. Moreover, elution of the drug was achieved in a week and is consistent with clinical needs. In conclusion, rapid osseointegration of Ti by TiO_2 nanotube structures on the surface and prevention against early infection in 2 weeks by elution of amoxicillin can be expected from samples that were subjected to 2 min ESD treatment on the TiO_2 nanotube surface.

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