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ORIGINAL ARTICLE

Antibacterial properties and human gingival fibroblast cell compatibility of TiO_2/Ag compound coatings and ZnO films on titanium-based material

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Abstract Titanium (Ti)-based materials are widely used in biomedical implant components and are applied successfully in various types of bone-anchored reconstructions. However, in dental implants the Ti materials contact not only bone but also gingival tissues, and are partially exposed to the oral cavity that includes bacteria. This study used titania and silver (TiO_2/Ag) compound coatings and zinc oxide (ZnO) films to enhance the antibacterial activity of the Ti-based implant. The hydrophobicity of each sample was examined by measuring the contact angle. *Streptococcus mutans* and human gingival fibroblast (HGF) was cultured on the coated samples, and the antibacterial effects and cell compatibility were determined using a Syto9 fluorescence staining and MTT methods. For the TiO_2/Ag samples, depositing Ag on the plate at a higher power (which increased the proportion of Ag) increased the contact angle and the hydrophobicity. The bacterial count was lowest for the 50 W TiO_2/Ag sample, which contained 5.9% Ag. The contact angles of the ZnO samples did not show the same tendency. The antibacterial effect was higher on ZnO-coated samples since bacterial count was threefold lower on ZnO samples as compared to control samples (Ti plate). From the MTT assay test, the mean optical density values for TiO_2/Ag-coated samples after 72 h of HGF adhesion were similar to the value obtained from the uncoated Ti. However, biocompatibility was lower on ZnO films than in control samples. Conclusively, the antibacterial activity was higher but the cell compatibility was lower on ZnO films than on TiO_2/Ag coatings.

Keywords TiO_2/Ag compound coatings · ZnO film · Antibacterial activity · Cell compatibility

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Introduction

Titanium (Ti) is commonly used in many types of artificial joints and implants in both dental and orthopedic clinics. Although the biocompatibility of Ti has been confirmed [1, 2], it is still difficult to meet all of the requirements in clinical applications, such as antibacterial activity, hydrophilicity, roughness, and mechanical strength. Some studies of dental implants have indicated that both the quality and quantity of plaque adhesive on the implant surface influences the long-term success of an implant [3, 4]. Since the antimicrobial properties of the Ti surface remain controversial [5, 6], one approach to achieve both better disinfection and biocompatibility is to modify the surface material of the Ti-based implant.

Bacterial attachment plays a significant role in determining the outcome and success of a Ti-based implant [7]. Therefore, modifying the Ti surface by coating with metals or alloys exhibiting antibacterial properties to reduce the

number of bacteria and microbial adhesion seems an efficient way to increase the likelihood of successful clinical treatment. Silver (Ag) is known to be an efficient antibacterial agent because of the specific antimicrobial activity. However, it is believed that Ag has cytotoxicity to human cells at certain concentrations [8]. In addition, the antibacterial activity of Ag has been well-established, and when it is added to hydroxyapatite [9] or Ti [10–12] as a coating material shows antimicrobial potency against bacteria.

Zinc (Zn)-containing materials are also of interest since they have been used for several years in clinical dentistry treatments due to the strong antimicrobial activity that can inhibit the growth of bacteria [13]. In addition, a nanoscale Zn oxide (ZnO) coating has been shown to improve cell function [6]. Even though ZnO has been widely used in orthopedic and dental clinical treatments as an antibacterial agent [14], few studies have investigated the bacterial activity of ZnO used as a coating material for medical implants [15].

In this study, a Ti-based plate was coated with compound materials of Ti and Ag [titania (TiO_2)/Ag] at various ratios to determine the optimal antibacterial activity while maintaining biocompatibility. In addition, ZnO films with different thickness were formed on a Ti-based plate to evaluate their effects on bacterial colonization and their biocompatibility. Hydrophilicity tests were also performed to elucidate the correlation between the antibacterial activity and the hydrophilicity of the experimental samples.

Materials and methods

Preparation of TiO_2/Ag compound coatings

Various TiO_2/Ag compound coatings were deposited onto pure Ti plate samples (15×15 mm) (biograde 2, Uniti Titanium, Moon Township, PA, USA) using an unbalanced magnetron-based sputtering process (Fig. 1). Before depositing TiO_2 and Ag, different grades of sandpapers were used to burnish and polish the Ti plates, and then the plates were cleaned ultrasonically in pure water and dried. As shown in Fig. 1, pure Ag and Ti targets (99.99 at.%) were arranged on the same side of the chamber to deposit the coatings. The deposition power of the Ti cathode was fixed at 100 W. The use of different powers (20, 30, 40, and 50 W) on the Ag cathode deposited TiO_2/Ag compound coatings with different proportions of Ag onto the Ti plates (Fig. 2a). The pure Ti plate samples were mounted on a substrate holder at 80 mm from the target. Argon and reactive gas (O_2) were introduced via a duct around the target to deposit the TiO_2/Ag compound coatings at a deposition pressure of 0.8 Pa. The deposition temperature was 200±20°C.

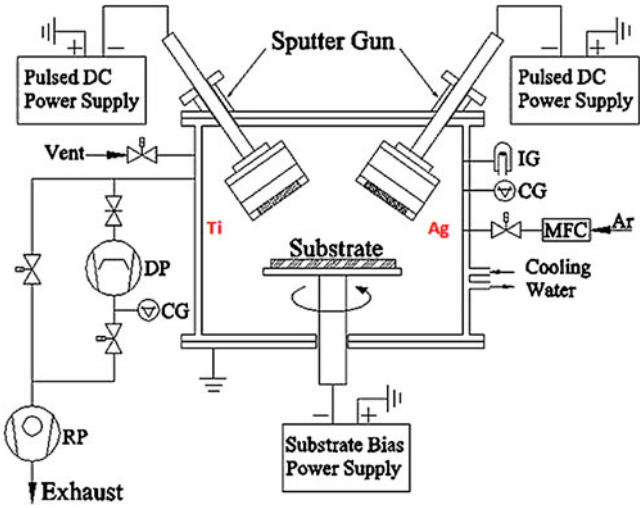

Fig. 1 Flow chart of the experimental processes

Preparation of ZnO coatings

An electroplating method was used to deposit ZnO coatings onto the pure Ti plates (15×15 mm) as experimental samples. Before the electroplating, ZnO seeds were spin-coated onto the Ti-based plates, and the plates were then

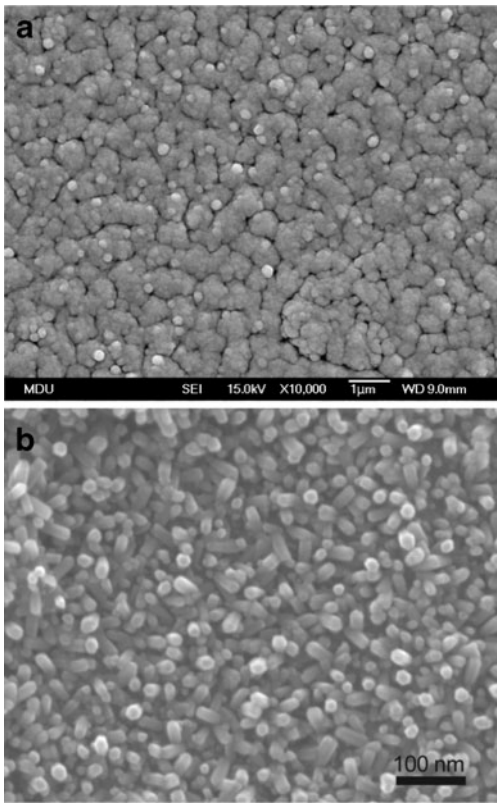

Fig. 2 SEM images of TiO_2/Ag samples coated at 50 W on the Ag cathode (**a**) and ZnO films electroplated at 2.5 V (**b**). On the Ti-based plates, the Ag nanoparticles are embedded into the TiO_2 film and ZnO nanorods with a standardized diameter have grown

placed in an oven to dry for 1 h at 300°C. A solution consisting of 10 ml of 0.1 M Zn nitrate and 10 ml of 0.1 M HMT in 80 ml of deionized water was heated to 90°C to form the ZnO electrolyzed solution that was used to electroplate ZnO coatings with different thickness onto the Ti plates at of 2.0, 2.25, and 2.5 V (Fig. 2b). The microstructures of the ZnO and TiO_2/Ag coatings were characterized using an X-ray diffractometer (XRD) with CuKα radiation and a glancing angle of 2°. The surface morphology, particle size, and chemical content of TiO_2 were determined by scanning electron microscopy (SEM; JEOL JSM-7000F) equipped with an energy dispersive spectroscope (EDS).

Contact-angle measurement

The static contact angle of the deionized water on each sample at room temperature was measured after the samples had been washed alternately in containers with acetone, ethanol, and deionized water in an ultrasonic cleaner for 30 min each. Drops generated with a micrometric syringe were deposited onto the surface, and their height-to-width ratio was measured and they were photographed immediately using an instrument for measuring contact angles (FTA-125, First Ten Angstroms, Portsmouth, VA, USA). Each contact angle reported here is the mean of at least three independent measurements.

Bacterial strain and cell culture

Streptococcus mutans (ATCC 31383, Bioresource Collection and Research Centre, Hsinchu City, Taiwan) was used as a reference strain. The bacterial strain was recovered from frozen stocks on Brain Heart Infusion agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% sheep blood and 1.5% agar in a microaerophilic atmosphere at 37°C for 1–2 days. An in vitro antibacterial activity of the each coated sample was evaluated with *S. mutans* in log phase.

Human gingival fibroblast tissue was obtained from human gingival connective tissues that were excised from patients during oral surgery extractions and the process was approved by the Ethics Committee of China Medical University Hospital. The human gingival fibroblast cells (HGF) were cultured in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% de-complement FBS (HyClone). Penicillin (100 U/ml) and streptomycin (100 mg/ml) (Invitrogen, Grand Island, NY, USA) was added to the culture medium. The HGF cells were then subjected to cell proliferation assay of each coated sample.

Antimicrobial activity and cell compatibility test

The in vitro antibacterial activities of the coated samples were determined by a fluorescence staining method employing Syto9 (Molecular Probes, Eugene, OR, USA). Briefly, 500 μl of a *S. mutans* suspension (0.5×10^9 colony-forming units/ml) was dropped onto the sample surface. After 6 h of incubation at 37°C at a relative humidity of 38–42% avoiding light exposure, the bacteria were fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) and stained with Syto9 (0.5 μM) at room temperature for 30 min. The antibacterial activity was quantified as the fluorescence detected at 488 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). The mean antibacterial activity was determined from two experiments performed in duplicate and quantified in relative fluorescence intensity.

The proliferation of human gingival fibroblast (HGF) cells was examined with an MTT assay (Sigma-Aldrich, St Louis, MO, USA) after the cells were cultured on uncoated, TiO_2/Ag-coated, and ZnO-coated Ti surfaces. The substance used for the assay was a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt, which turns into a purple formazan product in the presence of viable mitochondria in living cells. Three milliliters of HGF cells were seeded at a density of 2×10^4 cells/ml, and incubated at 37°C in 5% CO_2 for 72 h, at which time proliferation was achieved. The MTT (5 mg/ml) was added into cultured cells and incubated for a further 2 h. The purple formazan was eluted using 100 μl of isopropanol (Sigma-Aldrich). The absorbance of the purple formazan was quantified as the optical density (O.D.) measured at 570 nm by a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with SoftMax Pro 5.2 241 software (Molecular Devices). The mean O.D. was determined from two experiments performed in duplicate. The O.D. of formazan reflected the levels of cell metabolic activity, with higher O.D. values indicating more living cells on the sample and hence better biocompatibility.

Statistical analysis

The statistical analysis of the antibacterial activity and the results of the compatibility test between uncoated pure Ti plates and the TiO_2/Ag-coated samples were determined by Student's *t* test. Differences in data values were considered significant at $P<0.05$.

Results and discussion

After the characterization by SEM and EDS chemical analyses, as shown in Table 1, the elemental composition of the TiO_2/Ag coating deposited with 20 W on the Ag cathode was 32.7 at.% of Ti, 65.8 at.% of O, and 1.5 at.% of Ag. The Ag contents were 1.5, 2.3, 4.2, and 5.9 at.% for the TiO_2/Ag coatings deposited with 20, 30, 40, and 50 W on the Ag cathode, respectively. The higher ratio of Ag/

Table 1 Deposition conditions and coating composition of TiO_2/Ag coatings

Samples	Power of Ti cathode (W)	Power of Ag cathode (W)	Coating composition (at.%)		
			Ti	O	Ag
TiO_2/Ag-20 W	100	20	32.7	65.8	1.5
TiO_2/Ag-30 W	100	30	32.6	65.1	2.3
TiO_2/Ag-40 W	100	40	31.6	64.2	4.2
TiO_2/Ag-50 W	100	50	31.5	62.6	5.9

(Ti+Ag) cathode power, the higher Ag content in the TiO_2/Ag coatings was obtained. The XRD analyses indicated that the grain size of TiO_2 in the TiO_2/Ag coatings, at 3.5–5.0 nm, did not change with the Ag proportion. The XRD results (not shown here) revealed the presence of crystalline TiO_2, consisting of anatase and rutile phases, and Ag. The total thickness of the coatings was maintained at 0.9–1.2 μm by using a constant deposition time of 50 min.

The contact angles of the control samples and the TiO_2/Ag samples obtained using the sputtering process at 20, 30, 40, and 50 W on the Ag cathode were 75.28±1.51° (mean± SD), 59.71±2.03°, 66.58±3.8°, 77.62±2.92°, and 82.06± 3.07°, respectively (Fig. 3a). The contact angle increased with the proportion of Ag in the coating, indicating higher hydrophobicity. Also, the TiO_2/Ag samples with the higher Ag content had the lower fluorescence intensity (Fig. 4a), which meant the fewer adhering bacterial counts, and it showed the significant short-term antibacterial effect. This apparent reduction in antibacterial activity with a higher Ag proportion shows that the use of compound coatings containing Ag is very effective at improving the antimicrobial properties of Ti-based materials. The use of a surface film containing Ag can provide direct and immediate antibacterial actions to suppress microbial division and thereby reduce the amount of bacteria [16]. The antibacterial action of Ag could be attributable to Ag^+ ions (1) uncoupling respiratory electrons transported from oxidative phosphorylation and inhibiting the respiratory chain enzymes, (2) interfering with the membrane permeability to protons and phosphate [17] and (3) raising the silver-ion-mediated reactive oxygen species (ROS) generation to inhibit the bacterial ability [18]. In this way Ag^+ can markedly inhibit bacterial growth [19] and also damage existing bacteria [16].

The adherence of oral microorganisms to restorative materials is regarded as a critical step in causing implant failure and leading to soft tissue inflammation. The adherence of bacteria to a solid surface is probably influenced by hydrophobic interactions, van der Waals forces, electrostatic interactions, and hydrodynamic forces [20]. We found that the number of adhering bacteria was inversely proportional to the hydrophilicity, which is consistent with the results of other researches [10, 11].

The biocompatibility results (Fig. 5a) indicate no significant difference between control samples (Ti surface) and TiO_2/Ag coating. Even though Ag may damage the cell envelope [16], it is believed that cellular behaviors including proliferation, adhesion, and spreading are improved by the oxide layer of Ti [21]. Therefore, the combination of antibacterial properties (from Ag) and biocompatibility (from TiO_2) of the TiO_2/Ag compound coating may be advantageous for medical use. For the clinical application, since the species of microbe and cell we chose are *S. mutans* which existed in the oral

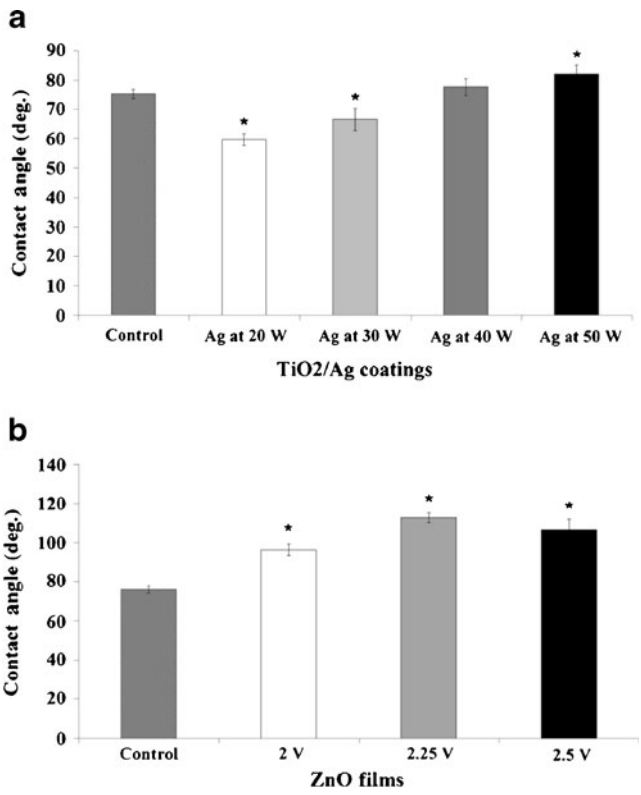

Fig. 3 Contact-angle values of ZnO-coated (**a**) and TiO_2/Ag-coated (**b**) samples. Data are mean and SD values. The asterisk mark, represents $P<0.05$ which meant a significant different relative to control samples

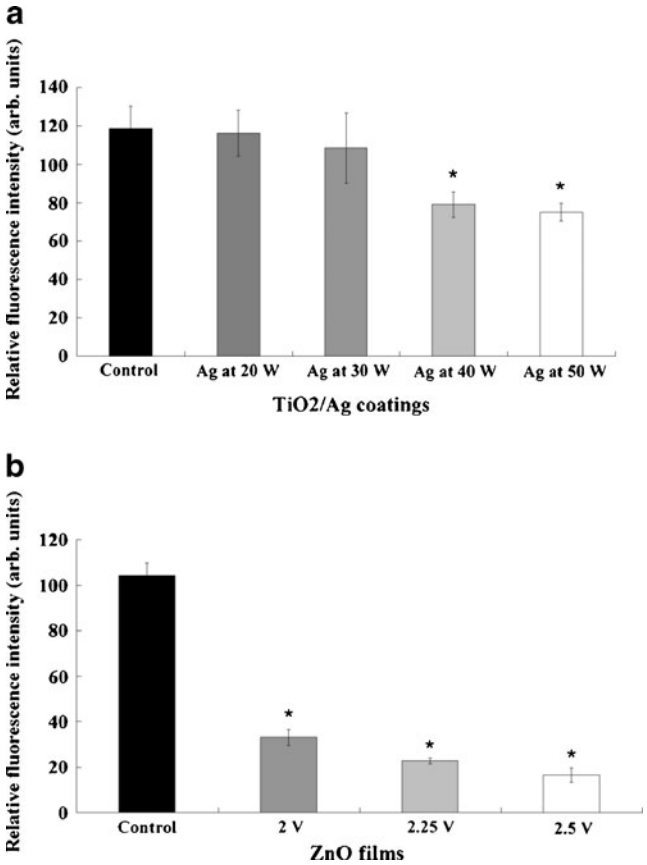

Fig. 4 Antibacterial activity (in relative fluorescence units) of ZnO-coated (**a**) and TiO_2/Ag-coated (**b**) samples. The control samples in both groups were the same size as the other samples in the group. However, the ZnO-group samples had larger surface areas than the TiO_2/Ag-group samples, making the relative fluorescence much higher in the former samples. The values are means and standard deviations (S.D.) of three independent experiments performed in duplicate. Statistical significance was evaluated using the Student's *t* test (asterisk, $P<0.05$)

environment and human gingival fibroblast cells, this research may apply to the dental use of Ti implants. Because the components of dental implant contact not only bone but also the gingival tissue, and are partially exposed to the oral cavity that includes bacteria, making the likelihood of the success of a dental implant improve if a coating material with both antimicrobial properties and biocompatibility—for example: TiO_2/Ag compound coating can be used in the manufacture of dental implants.

The contact angles of the control samples and the ZnO samples electroplated at 2.0, 2.25, and 2.5 V were 76.05±1.87°, 96.38±2.92°, 112.78±2.48°, and 106.65±5.32°, respectively (Fig. 3b). Fluorescence staining indicated that the antibacterial activity was highest for the 2.5 V ZnO sample, with this sample also exhibiting the lowest bacterial adhesion (Fig. 4b). Moreover, the bacterial counts on the material surfaces were significantly lower for the ZnO-coated samples than for the control samples, which indicate that the ZnO coatings increased the antibacterial activity of the Ti plate.

An SEM image of a ZnO sample demonstrated that ZnO grew as nanorod structures (approximately 20 nm in diameter) on the Ti-based plate (Fig. 2b). ZnO nanorods have recently been used to create superhydrophobic cotton fabrics due to their fabrication of rough surfaces [22]. The present study has shown that films of ZnO nanorods also promote the oral antibacterial activity. The antimicrobial mechanism of ZnO is considered to be due to the generation of hydrogen peroxide (H_2O_2) from its surface [14]. H_2O_2 is a strong oxidizing agent that exhibits bactericidal activity, and hence ZnO may directly inhibit the growth and proliferation of bacteria and other microorganisms. According to the results of the present study, Ti-based materials with ZnO films exhibit excellent antibacterial activity and successfully inhibit the proliferation of microorganisms.

The result in MTT assay test (Fig. 5b) revealed that biocompatibility was lower on ZnO films than in control samples. This is consistent with Lee et al. [23] finding that

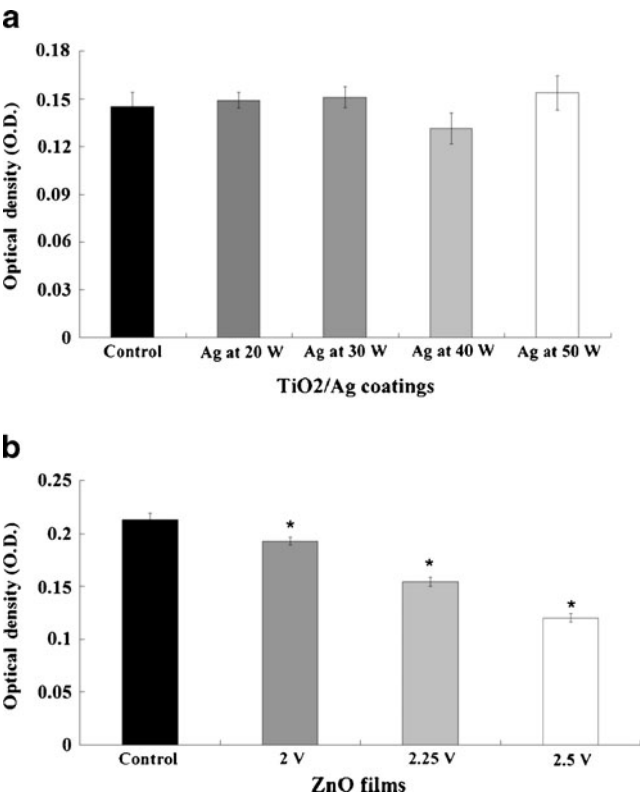

Fig. 5 Cell compatibility test using an MTT assay of HGF cells after 72 h of incubation at 37°C for uncoated (control), TiO_2/Ag-coated, and ZnO-coated Ti surfaces. Data are mean and SD values. The values are means and standard deviations (S.D.) of three independent experiments performed in duplicate. Statistical significance was evaluated using the Student's *t* test (asterisk, $P<0.05$)

the presence of ZnO nanorods greatly altered the viability and adhesion of cells. A reduction in cell viability, adhesion, and spread could therefore reduce cell survival on a surface coated with ZnO nanorods. Since controlling cell performance with biomaterials is important for the success of tissue engineering [24] and implanted drug delivery devices, ZnO films may also have potential as a cell–adhesion–resistant biomaterial [23] for provoking decease in cells for biomedical applications.

Conclusions

The reported results showed that the amount of Ag in the TiO_2/Ag compound coating on Ti-based plates has an effect on the inhibition of bacterial growth. Three ZnO-coated samples also showed an inhibition tendency, with the antibacterial capacity increasing with the amount of electroplated ZnO. From the MTT assay test, the mean optical density values for TiO_2/Ag-coated samples after 72 h of HGF adhesion were similar to the value obtained from the uncoated Ti. However, cell compatibility was lower on ZnO films than in control samples (Ti plate). Conclusively, the antibacterial capacity appears to be higher for ZnO films; however, the cell compatibility was lower for ZnO films than for TiO_2/Ag compound coatings.

Conflict of interest This research was supported by the Government Organization—National Science Council (NSC 98-2815-C-039-049-B) and (NSC 98-2320-B-039-005-MY3), Taiwan. All of the authors have no financial relationship to any private companies and organizations.

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