Surface Analysis of Titanium Biological Modification with Glow Discharge

Yu-Chi Chang, DDS;* Sheng-Wei Feng, DDS, MS;* Haw-Ming Huang, PhD;† Nai-Chia Teng, DDS, PhD;*,‡ Che-Tong Lin, DDS, PhD;*,‡ Hsi-Kuei Lin, DDS;*,§ Peter-D Wang, DDS;* Wei-Jen Chang, DDS, PhD*,§

ABSTRACT

Background: Glow discharge plasma (GDP) technology has been used to graft various proteins to the titanium surface, including albumin, type I collagen, but without fibronectin.

Purpose: The aim of this study was to evaluate and analyze the physical properties of fibronectin-grafted titanium surfaces after GDP treatment.

Materials and Methods: Grade II titanium discs after cleaning and autoclaving were considered as original specimens, thus divided into four groups. The groups were different upon two treatments (GDP only and fibronectin grafting after GDP) and two storage temperature (4°C and 25°C). The implant surface morphology was characterized by scanning electron microscopy (SEM), roughness measurement, and wettability evaluation. The concentration relationship of fibronectin was by fluorescein isothiocyanate (FITC) labeling.

Results: SEM images showed that regular planar texture revealed on the surface of GDP-treated group, and irregular-folding protein was found on the fibronectin-grafted discs. Fibronectin-grafted groups had higher hydrophilicity and greater surface roughness than GDP-treated specimens. The storage temperature did not make obvious difference on the surface topography, wettability, and roughness. The number of fibronectin dots on the titanium surface labeling by FITC had positive relationship with the concentration of fibronectin solution used.

Conclusions: Biologically modified titanium surface is more hydrophilic and rougher than GDP-treated ones. GDP treatment combined with fibronectin grafting increased the surface hydrophilicity and surface roughness of titanium discs, which may attribute to the affinity of cell adhesion, migration, proliferation, and differentiation.

KEY WORDS: fibronectin, glow discharge, implant design, surface properties, titanium

INTRODUCTION

Since the 1960s, titanium dental implants have been used for oral rehabilitation. In order to improve the quality and quantity of the bone-implant interface contact, a number of surface treatment methods have been developed. These include the physical or chemical vapor deposition coatings ret, abrasive particle blasting,[1] anodic oxidation,[2] laser treatment, acid etching, and the plasma spraying.[3–8] Surface chemistry modification would influence hydrophilicity, surface property, and/or wettability, thus becoming an important factor for new titanium implant surface development.

Modification of the physiochemical properties of titanium surface makes it possible to improve protein adsorption and optimize cell attachment.[9] Previous research showed that glow discharge plasma (GDP) has been used for creating a number of biofunctional groups, increasing surface wettability,[10,11] and applying functional proteins to the titanium surfaces. To enhance the biocompatibility of titanium surface, GDP technology has been used to graft various proteins to the

*School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan; †Graduate Institute of Biomedical Materials & Tissue Engineering, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan; ‡Dental Department, Taipei Medical University Hospital, Taipei, Taiwan; §Dental Department of Taipei Medical University, Shuang-Ho Hospital, Taipei, Taiwan

Reprint requests: Dr. Wei-Jen Chang, School of Dentistry, College of Oral Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan; e-mail: cweijen1@yahoo.com.tw. Dr. Peter-D Wang, School of Dentistry, College of Oral Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan; e-mail: atekpdw@aol.com

DOI 10.1111/cid.12141

titanium surface, including albumin,[12–14] type I collagen,[15,16] and fibronectin.

It is known that the adhesion of cells to the substrate strongly depends on fibronectin.[17,18] This protein is important in the process of cell growth, migration, and differentiation. Thus, fibronectin has been a primary focus in investigations of implant surface-coating materials. For titanium modification, researchers used low-temperature GDP to produce fibronectin-linked titanium surface.[5] Although their results demonstrated that GDP plus fibronectin treatment produced more cell adhesion and differentiation to the titanium surface, the physiological effect of GDP-treated only titanium samples and GDP plus fibronectin-grafted titanium samples was not evaluated. Accordingly, the knowledge for further applications of such technique is still limited.

The aim of this study was to evaluate the physical properties of GDP and fibronectin-treated titanium. Besides, the relationship between fibronectin solution concentration and the fibronectin concentration on the titanium surface were measured.

MATERIALS AND METHODS

Titanium Disc Cleaning

Grade II titanium discs (BioTech One Inc., Taipei, Taiwan), with 10 mm diameter and 1 mm thickness, were used in this experiment. Before being treated with GDP, the titanium specimens were ultrasonically cleaned in detergent solution, acetone, and pure distilled water for 15 minutes separately, and then autoclaved in 121°C for 20 minutes. These specimens were divided into four groups, which were pretreated as follows.

Glow Discharge Cleaning Groups

The disc surfaces were subsequently cleaned with argon-based GDP (PJ, AST Products Inc., North Bellericca, MA, USA) for 15 minutes, in argon gas at room temperature, 85 watts (W), 13.56 MHz, and 100 millitorr. Two groups of treated titanium discs were then stored at 4°C and 25°C separately after argon plasma treatment. These titanium discs were defined as GDP-4°C and GDP-25°C.

Glow Discharge Protein Grafting Groups

With glow discharge filled with allylamine (AA) gas, amino groups (NH groups) were adhered onto the titanium surfaces after argon plasma treatment. The two remaining groups of specimens were exposed to the AA gas in the GDP reactor at room temperature, 85 W, 13.56 MHz, and 100 millitorr for 30 minutes. These specimens were then immersed in 3% glutaraldehyde (GA) solution (Merck, NJ, USA) for 30 minutes as soon as they were taken out from the plasma chamber. The GA solution was used to couple amine groups and proteins onto the surface of titanium discs.

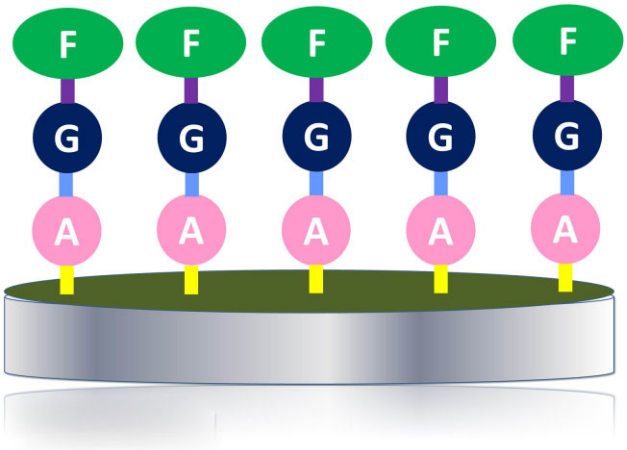

Figure 1 Cartoon of the surface of titanium discs after glow discharge plasma (GDP) and fibronectin modification: A stands for allylamine (AA), G stands for glutaraldehyde (GA), F stands for fibronectin.

After rinsing with 0.1 M phosphate buffered saline (PBS), the titanium specimens were subsequently immersed in 5 μg/ml fibronectin solution (Sigma-Aldrich Co., St. Louis, MO, USA) at 4°C and 25°C for 24 hours to coat fibronectin onto the surface of titanium discs (Figure 1). After the above procedures, the titanium discs were immersed in Tris-phosphate buffer (pH 7.4) for 30 minutes to interrupt the chain reaction between AA and fibronectin. These titanium discs were defined as fibronectin-4°C and fibronectin-25°C.

Surface Analysis

The fibronectin-linked titanium discs were washed with PBS. A thin layer of palladium gold was coated onto the samples using a sputtering apparatus (IB-2, Hitachi, Ltd, Tokyo, Japan). The surface characteristics of the four group samples were observed using scanning electron microscopy (SEM) (Model 2400; Hitachi, Ltd).

Surface wettability was measured by an optical measurement of the advancing contact angle of water between the four groups. For this purpose, a linear goniometer (Digidrop Goniometer, GBX, Romans,

France) was constructed that consisted of a base platform, 35 mm camera, sample holder, and an external lighting system to illuminate the sample. For each measurement, a 4 µl droplet of water (Millipore-Q, Millipore, Bedford, MA, USA; filtered, 20°C) was applied to the test surface, and a picture was taken meanwhile. Developed slides were then projected on a standardized tracing table, the image was traced, and the contact angle was measured. Advancing contact angles for each water droplet were then calculated.

Surface analyzer (TR200, An-Bomb Instrument Co., Ltd, Tainan, Taiwan) was used to test surface roughness of titanium surface. The measured length (LTH) on sample, was determined in 0.8×5 mm. The amplitude (RAN, the evaluation range) was set in ±40 µm. Ra, the arithmetical mean deviation of the assessed profile, was used to express the collected data points. All data were followed in the International Organization for Standardization (ISO)-4287 standard.

Concentration Analysis

Fluorescein isothiocyanate (FITC) labeling was used for the observation of adsorbed fibronectin on each specimen. According to the guide of manufacturer (Sigma-Aldrich Co.), the ideal concentration of fibronectin solution is 0.5~50 µg/ml. In this study, different concentrations of fibronectin solution (0.5, 1, 2, 3, 4, and 5 µg/ml) were prepared and grafted onto the titanium discs. The relationship between different concentrations of fibronectin solution and the total concentration of fibronectin on the titanium surface was evaluated. The total concentration of fibronectin on the titanium surface was measured by fibronectin dot number by FITC labeling.

Statistical Analysis

All data measured are presented as mean ± standard deviation. For all assays, differences between tested groups were tested by Student's *t*-test. A p value lower than .05 was considered statistically significant for all tests.

RESULTS

SEM image of GDP-treated and GDP plus fibronectin-grafted titanium discs reveals different surface topographies (Figure 2). There was a regular planar texture on the surface of GDP-treated titanium discs (Figure 2A). However, irregular-folding protein was found on the surfaces of fibronectin-grafted discs (Figure 2, B and C).

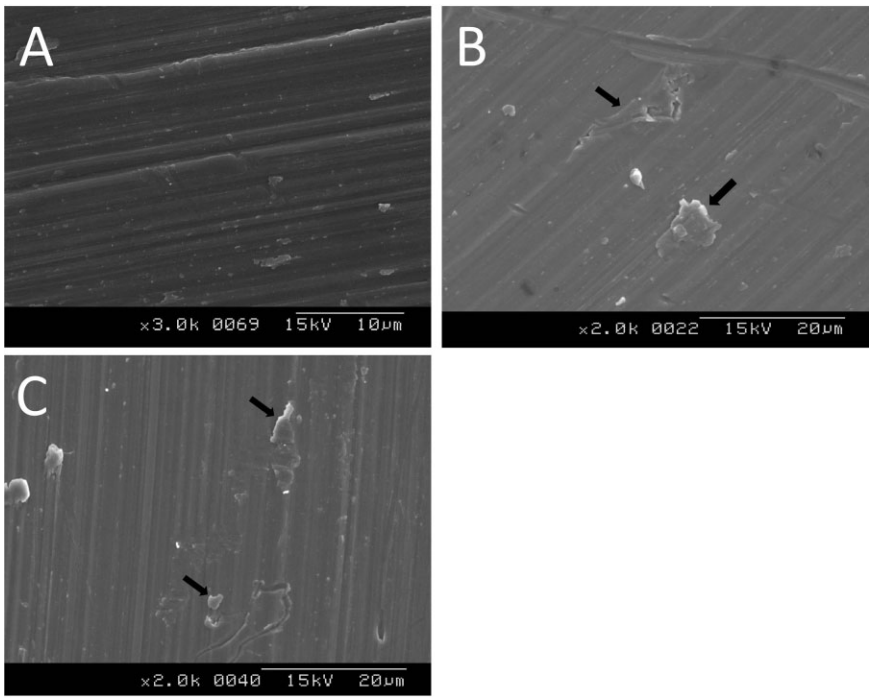

Figure 2 Scanning electron microscopy images of the glow discharge plasma (GDP) (A), fibronectin 4°C (B), and fibronectin 25°C (C) titanium discs. The fibronectin-grafted titanium surfaces appear irregular folding of the protein on the titanium surface (*black arrows*).

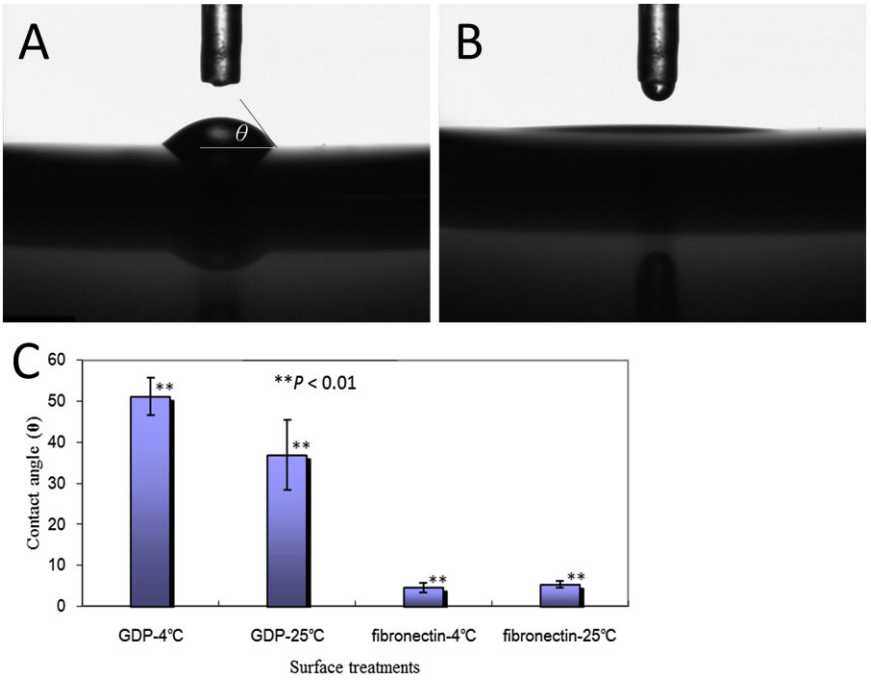

Figure 3 Typical contact angle images of glow discharge plasma (GDP)-treated (A) and fibronectin-grafted (B) samples. Surface wettability is evaluated by measuring the contact angle of each water droplet. The parameter, θ, is defined as the angle formed by a liquid at the sample surface. Quantitative analysis showed surface wettability of the fibronectin-grafted titanium discs was greatly improved when compared to the GDP samples (**$p < .01$).

The storage temperature did not make obvious difference on the surface topography.

Analysis of surface wettability showed that fibronectin grafting treatment significantly improved the surface hydrophilicity of the titanium discs (Figure 3, A and B). Surface wettability is evaluated by measuring the contact angle of each water droplet. If the contact angle becomes smaller, the water droplet becomes flatter; it means the tested surface is more hydrophilic. The mean contact angle of the GDP-4°C and GDP-25°C were $51.1 \pm 4.5°$ and $36.8 \pm 8.5°$ separately (Figure 3C). The mean contact angle of the fibronectin-4°C and fibronectin-25°C discs were significantly reduced to $4.6 \pm 1.1°$ and $5.4 \pm 0.8°$ ($p < .01$).

Fibronectin-grafted titanium specimens had greater surface roughness than GDP-treated specimens (Figure 4). The Ra value of GDP-4°C and GDP-25°C titanium discs were 0.176 ± 0.062 μm and 0.170 ± 0.069 μm, while the roughness value increased to 0.400 μm after fibronectin grafting. Results show that there is significant difference between GDP-treated only group and fibronectin-grafted titanium discs ($p < .01$). However, there is no significant difference between samples storage at different temperature with same treatment.

The relationship between different concentrations of fibronectin solution and the total concentration of fibronectin on the titanium surface was shown in Figure 5. Results of analysis showed that the number of fibronectin dots on the titanium surface labeling by FITC increased with the concentration of fibronectin solution used. Significant differences can be found among each concentration of fibronectin solution used ($p < .01$).

DISCUSSION

GDP treatment is used for cleaning and surface activating by providing a stable, low energy source of ions. It is also effective in the dental implant surface treatment.[19–21] Argon plasma, used in this study, has many benefits. It cleans specimen surface by ion bombardment and physical ablation of contaminants off the surface. Meanwhile, GDP does not react with the surface or alter surface chemistry. In this study, improved surface wettability and greater surface roughness are showed by fibronectin grafting following GDP treatment on titanium surface. The increases of surface wettability and roughness may contribute to the adhesion of cells.

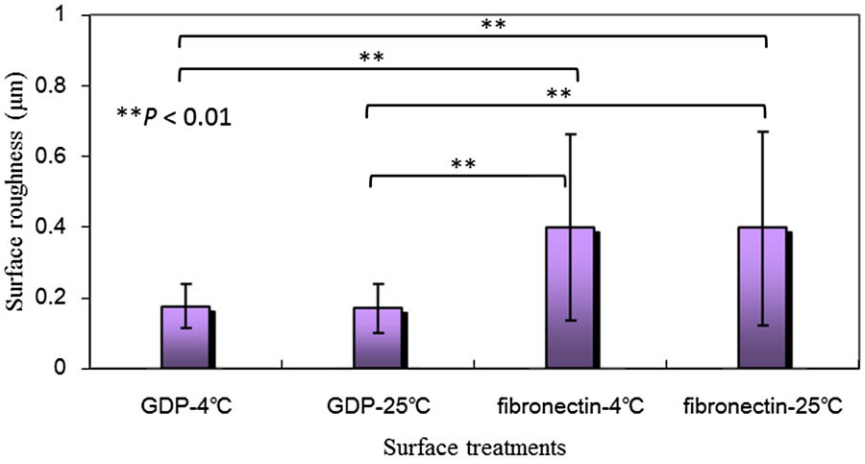

Figure 4 Surface roughness of GDP-treated and fibronectin-grafted titanium discs. Ra, the arithmetic average of absolute values, was used to express the collected data points. Results show that there is a significant difference between GDP-treated only and fibronectin-grafted titanium discs (**$p < .01$). However, there is no significant difference between different temperature-treated ones.

Fibronectin is a high-molecular weight (~440 kDa) extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrin. It has been found that fibronectin plays a major role in the adhesion process of mammalian cells through integrin. This binding activates a signaling cascade that generates cell migration, proliferation, and differentiation.[22] Initial adhesion of cells to implant surfaces and subsequent cell behavior are important factors for biocompatibility. Fibronectin acts as integrin to adhere anchorage-dependent cells (such as fibroblasts or MG-63 cells) onto specific surface. Therefore, fibronectin also plays a role in wound healing.[23] In light of these beneficial effects of fibronectin, it has inevitably become a primary focus in the investigation of implant materials.

AA has been used to modify material surfaces to allow biofunctional protein bonding.[24] GA was used as a cross-linking agent during the surface modification process. It is due to the amine groups exhibiting positive charge, which may attract negatively charged biomolecules, thus providing an ideal connections between biomaterials and cells.[25]

In general, hydrophilic surfaces displayed better affinity for cells. Hydrophilic surface modification of titanium was reported to have a significant influence on differentiation and growth factor production of osteogenic cells.[26] In this study, it was also found that the contact angle of fibronectin-grafted titanium surfaces dramatically decreased, which may increase the adhesion affinity of cells and proteins onto the titanium surfaces. Besides, the storage temperature did not affect the surface wettability, which may benefit the future commercial products fabrication and preservation. However, the irrelevance between temperature and surface wettability may attribute to the short storage period between modification treatment and evaluated tests. Extending storage period of biologically modified titanium to enhance the commercial potential still needs further research.

In vivo after implantation or in vitro in cell culture, tissues, and cells might react differently as they encounter rough or smooth surfaces maintained in biological fluids able to coat them with specifically active proteins. It was reported that cell adhesion and migration might increase with surface roughness.[27] In this study, it was also found that the fibronectin-grafted titanium discs had much rougher surface compared with GDP-treated samples (Figure 4). The difference of surface roughness may affect the wound healing process after implantation.

Analysis of different fibronectin solution concentration revealed that number of fibronectin dots labeling by FITC on the titanium surface increased with the concentration of fibronectin solution. Because fibronectin is an important determinant for cell adhesion and migration, the higher concentration of fibronectin may also contribute to a sooner osseointegration process of dental implant in the early wound healing stage following implantation.

In conclusion, these results indicated that GDP treatment combined with fibronectin grafting increased the surface hydrophilicity and surface roughness of titanium

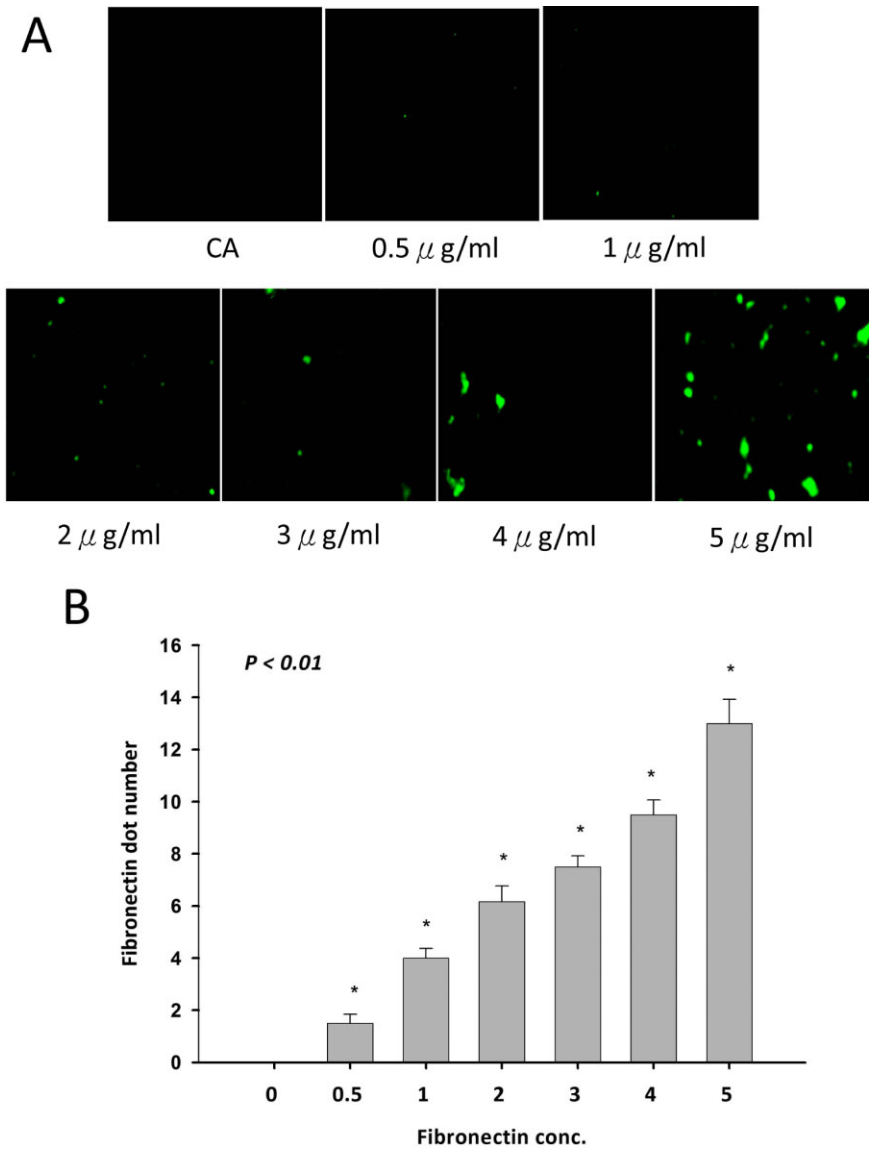

Figure 5 *A*, Fluorescein isothiocyanate (FITC) labeling images of different fibronectin solution concentration and fibronectin dot number on the surface of titanium discs. *B*, Analysis revealed that the number of fibronectin dots on the titanium surface increased with the concentration of fibronectin solution used (*$p < .01$).

discs, which may attribute to the affinity of cell adhesion, migration, proliferation, and differentiation. These results can be a useful reference for further in vitro cell culture study and future endosseous implant design.

REFERENCES

1. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res 1991; 25:889–902.
2. Choi JW, Heo SJ, Koak JY, et al. Biological responses of anodized titanium implants under different current voltages. J Oral Rehabil 2006; 33:889–897.
3. Wennerberg A, Albrektsson T. Suggested guidelines for the topographic evaluation of implant surfaces. Int J Oral Maxillofac Implants 2000; 15:331–344.
4. Suvorova EI, Buffat PA. Pathological mineralization of cardiac valves: causes and mechanism. J Long Term Eff Med Implants 2005; 15:355–368.
5. Shibata Y, Hosaka M, Kawai H, Miyazaki T. Glow discharge plasma treatment of titanium plates enhances adhesion of osteoblast-like cells to the plates through the integrin-mediated mechanism. Int J Oral Maxillofac Implants 2002; 17:771–777.
6. Placko HE, Mishra S, Weimer JJ, Lucas LC. Surface characterization of titanium-based implant materials. Int J Oral Maxillofac Implants 2000; 15:355–363.

7. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez BS, Arvidson K. Determining optimal surface roughness of TiO(2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. Clin Oral Implants Res 2001; 12:515–525.
8. Le Guehennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. Dent Mater 2007; 23:844–854.
9. MacDonald DE, Deo N, Markovic B, Stranick M, Somasundaran P. Adsorption and dissolution behavior of human plasma fibronectin on thermally and chemically modified titanium dioxide particles. Biomaterials 2002; 23:1269–1279.
10. Hesby RM, Haganman CR, Stanford CM. Effects of radiofrequency glow discharge on impression material surface wettability. J Prosthet Dent 1997; 77:414–422.
11. Ozden N, Akaltan F, Suzer S, Akovali G. Time-related wettability characteristic of acrylic resin surfaces treated by glow discharge. J Prosthet Dent 1999; 82:680–684.
12. Ji J, Feng L, Barbosa MA. Stearyl poly(ethylene oxide) grafted surfaces for preferential adsorption of albumin. Biomaterials 2001; 22:3015–3023.
13. McFarland CD, De Filippis C, Jenkins M, et al. Albumin-binding surfaces: in vitro activity. J Biomater Sci Polym Ed 1998; 9:1227–1239.
14. Huang HM, Hsieh SC, Teng NC, Feng SW, Ou KL, Chang WJ. Biological surface modification of titanium surfaces using glow discharge plasma. Med Biol Eng Comput 2011; 49:701–706.
15. Chang WJ, Ou KL, Lee SY, et al. Type I collagen grafting on titanium surfaces using low-temperature glow discharge. Dent Mater J 2008; 27:340–346.
16. Yamamoto H, Shibata Y, Miyazaki T. Anode glow discharge plasma treatment of titanium plates facilitates adsorption of extracellular matrix proteins to the plates. J Dent Res 2005; 84:668–671.
17. Webb K, Caldwell K, Tresco PA. Fibronectin immobilized by a novel surface treatment regulates fibroblast attachment and spreading. Crit Rev Biomed Eng 2000; 28:203–208.
18. McClary KB, Ugarova T, Grainger DW. Modulating fibroblast adhesion, spreading, and proliferation using self-assembled monolayer films of alkylthiolates on gold. J Biomed Mater Res 2000; 50:428–439.
19. Aronsson BO, Lausmaa J, Kasemo B. Glow discharge plasma treatment for surface cleaning and modification of metallic biomaterials. J Biomed Mater Res 1997; 35:49–73.
20. Serro AP, Saramago B. Influence of sterilization on the mineralization of titanium implants induced by incubation in various biological model fluids. Biomaterials 2003; 24: 4749–4760.
21. Kibayashi H, Teraoka F, Fujimoto S, Nakagawa M, Takahashi J. Surface modification of pure titanium by plasma exposure and its bonding to resin. Dent Mater J 2005; 24:53–58.
22. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci 2002; 115:3861–3863.
23. Valenick LV, Hsia HC, Schwarzbauer JE. Fibronectin fragmentation promotes alpha4beta1 integrin-mediated contraction of a fibrin-fibronectin provisional matrix. Exp Cell Res 2005; 309:48–55.
24. Stine R, Cole CL, Ainslie KM, Mulvaney SP, Whitman LJ. Formation of primary amines on silicon nitride surfaces: a direct, plasma-based pathway to functionalization. Langmuir 2007; 23:4400–4404.
25. Nelea V, Luo L, Demers CN, et al. Selective inhibition of type X collagen expression in human mesenchymal stem cell differentiation on polymer substrates surface-modified by glow discharge plasma. J Biomed Mater Res A 2005; 75: 216–223.
26. Schwarz F, Wieland M, Schwartz Z, et al. Potential of chemically modified hydrophilic surface characteristics to support tissue integration of titanium dental implants. J Biomed Mater Res B Appl Biomater 2009; 88:544–557.
27. Lampin M, Warocquier C, Legris C, Degrange M, Sigot-Luizard MF. Correlation between substratum roughness and wettability, cell adhesion, and cell migration. J Biomed Mater Res 1997; 36:99–108.