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Preparation of self-organized nano-network oxide structure on titanium and the *in situ* biocompatibility monitoring using electrochemical techniques

Abstract

This study was to investigate the preparation of self-organized nano-network oxide structure on titanium (Ti) specimen using the electrochemical anodization treatment. The initial biocompatibility of the nano-network oxide layer was *in situ* monitored using the electrochemical impedance spectroscopy (EIS) measurement. Osteoblast-like U-2 OS cells were used in this study. Higher impedance and better cell spreading indicted better biocompatibility. The results showed that the TiO_2 layer with a self-organized nano-network was developed on Ti specimen using the current-controlled anodization treatment in an alkaline solution. The network size increased with an increase in the applied anodic current. During the initial (5 days) cell culture on the test Ti specimens, the EIS data showed that the anodized Ti specimen with a self-organized nano-network TiO_2 surface layer had higher (1.8-3.8 times) *in situ* impedance (2-4.7 x 10^6 Ωcm^2) and better cell spreading, namely better biocompatibility, than the untreated Ti specimen.

Keywords: Nano-network; Titanium; Cell; Biocompatibility; Impedance

Electrochemical Impedance Spectroscopy, EIS

U-2 OS

TiO_2

EIS TiO_2

5   )   1.8-3.8

2-4.7$\times10^6\Omega cm^2$

;   ;   ;   ;

1. Introduction

Various methodologies are being used in an effort to improve the interfacial properties between the biological tissues and the existing implants, e.g. Ti and Ti-based alloy. Among the methods of surface modifications for Ti-based implants, revision in surface topography using the soaking, chemical and/or thermal treatment has been used to affect the cell responses to implants [1-4]. However, the above mentioned method for producing a bioactive porous Ti surface is still relatively time-consuming. Lately, the electrochemical technique, a more simple and fast method, has been taken as a potential alternative for producing the porous Ti-based metals as medical implants [6,7]. Nevertheless, the information concerning the biocompatibility of the porous Ti-based implants produced by electrochemical technique is not available in the above reports.

Poor implant material biocompatibility will cause harm to both the tissue cells and implant material [8]. The interaction between cells and material surface can be used to investigate the biocompatibility of implant materials in the human body. However, the *in situ* studies on the interfacial behaviors of cell/implant material are still very restricted. The electrochemical impedance spectroscopy (EIS) measurement is an electrochemical method by which the material surface character can be really achieved without interfering with the surface electrochemical behavior [9]. Numerous literatures concerning the EIS measurement of titanium (Ti), which is one of the most popular implant materials, in simulated human body environments have been reported [10-14]. However, the electrochemical character of Ti metal cultured with living cells is rarely reported.

In this study, a rapid electrochemical technique, namely galvanodynamic anodization treatment, was used to create a nano-network-structured oxide layer onto a Ti substrate. The initial *in situ* adhesion of osteoblast-like cells onto the modified Ti surface was evaluated using the EIS measurements.

2. Materials and methods

2.1 Materials preparations

Commercially pure Ti disk was used as the test substrate. The substrate surface was ground with SiC paper to #1500. A galvanostat (Model 5000, Jiehan Co., Taichung, Taiwan) was used to apply the anodic current to the ground Ti substrates from zero up to I_1 and I_2 A ($I_1 < I_2$) in concentrated NaOH solution at a constant current scan rate. The corresponding specimens were designated as I_1 and I_2, respectively. The untreated Ti substrate was designated as I_0. The tested Ti substrates were sterilized with UV light for 1 hour before the cell culture was applied to the specimen surface.

2.2 Surface analyses

Surface characterizations of the anodized layer on Ti substrates were performed using a thin-film x-ray diffractometer (TF-XRD), field emission scanning electron microscope (FE-SEM), and x-ray photoelectron spectroscopy (XPS). Morphological dimensions of the anodized structure were evaluated using the FE-SEM micrographs via an

Image-Pro$^®$ Plus image analysis software (version 4.5.1). To evaluate the proteins adsorbed onto the test specimens, XPS was used to qualitatively analyze the nitrogen (in terms of N_{1s}) spectra on the specimen surfaces after 2 hours immersion in the cell culture medium.

2.3 Cell culture

Osteoblast-like U-2 OS cells were chosen for this research to imitate the behavior of osteoblasts because this cell line possesses the osteoblast phenotype [15]. Complete McCoy's 5A medium was supplied to culture the U-2 OS cells onto the test specimens (5×10^4 cells/cm^2 in density) in an incubator with 5% CO_2 content at 37 °C. Specimens immersed in complete McCoy's 5A medium without the cell culture were used as the control group.

2.4 In situ impedance measurement

A custom-made impedance measurement system, including the 5% CO_2 incubator for the cell culture, was developed. During the cell growth process, *in situ* electrochemical impedance spectroscopy (EIS) was measured and used to analyze the impedance of the specimens at different cell culture periods (0.5, 1, 2, 3, and 5 days). An AUTOLAB PGSTAT 30 potentiostat combined with an AUTOLAB FRA 2 frequency response analyzer was used to perform the EIS measurements. The EIS measurements were performed at the open circuit potentials (as dc voltage) of the test specimens at different cell culture periods with a sinusoidal potential of 5 mV (as ac voltage). The impedance obtained at 10^{-3} Hz was used to represent the charge transfer resistance from the Ti substrate outwards to the biological adhesion layer. Higher impedance stands for better proteins and/or cells adhesion (or better biocompatibility) because more proteins and/or cells covering the test specimen surface increase the charge transfer resistance. A saturated calomel electrode and platinum sheet were used as the reference and counter electrodes, respectively. McCoy's 5A cell culture medium was used as the electrolyte for EIS measurements.

2.5 Cells morphology observation

The U-2 OS cells were cultured on the tested Ti substrates (5×10^4 cells/cm^2 in density) after 1 day of incubation. The unattached cells and culture medium were then removed, followed by washing with phosphate-buffer saline. The cells attached to the specimens were sequentially fixed and dehydrated. The cell surface morphology was observed using a FE-SEM after coating a thin platinum film onto the specimens.

3. Results and discussion

The outermost surface of the Ti substrate (I_0) before an anodization treatment has been analyzed using XPS and identified as TiO_2 [16]. After the anodization treatment, the surface oxide layer on the Ti substrate was identified using a TF-XRD and described in detail elsewhere [17]. Regardless of the applied anodic current, the surface layer structure on the anodized Ti substrates (I_1 and I_2) contained mainly anatase TiO_2.

Figure 1 shows the FE-SEM micrographs of Ti substrates with and without anodization treatment: (a) untreated I_0; (b) anodized I_1; (c) anodized I_2. A self-organized network structure appeared on the anodized Ti substrates regardless of the anodized current. Using image analysis software, the size of the network increased with an increase in the applied anodic current, from a few decades to around 100 nm. It has been described that biological tissues interact mainly with the outermost atomic layers of the biomaterials when the biomaterials are implanted into the body environment [18]. In this study, the anatase TiO_2 layer with a nano-network structure was expected to play an important role in the biocompatibility of the anodized Ti substrates, which will be discussed later.

Figure 2 shows the electrochemical impedance (at 10^{-3} Hz) of the tested Ti substrates, with and without cell culture, after different periods in the culture medium. Under the same cell incubation period, the impedances for cell-cultured I_1 and I_2 specimens were higher (1.8-3.8 times) than that for cell-cultured I_0 specimen. The I_2 specimen had higher impedance (3.8-4.7 x 10^6 Ωcm^2) than the I_1 specimen (1.9-3.4 x 10^6 Ωcm^2). On the other hand, the impedance for the cell-cultured specimens only slightly increased upon lengthening the culture period, especially after 1 day of cell incubation. It should be noted that the impedances for the tested specimens without cell culture were approximately 4-9 x 10^5 Ωcm^2 during a 5-day period, and were lower (around 2-10 times less) than that for the cell-cultured specimens.

As shown in Fig.2, for I_0, I_1, and I_2 specimens without cell culture, the impedance was related to the surface TiO_2 layer and the adsorbed proteins (mainly as soluble albumin) from the culture medium. The corresponding impedance was much lower than that of the cell-cultured specimens. Furthermore, the impedance was nearly constant when increasing the immersion time, indicating the TiO_2 layer and the adsorbed proteins were very stable in the culture medium. On the other hand, for cell-cultured I_0, I_1, and I_2 specimens, the impedance was related to more complex species. In addition to the surface TiO_2 layer and the adsorbed proteins mentioned in the case without cell culture, the attached cells and the proteins [such as extracellular matrix (ECM)] generated during cell growth also played an important role in the impedance variation. At day 0.5, regardless of the surface treatment, the cell-cultured Ti specimens showing higher impedance than the specimens without cell culture was mainly due to the presence of the attached cells and the ECM on the specimen. When increasing the culture time (e.g. at day 1 and 2), the slight increase in impedance was believed to be related to the spreading of the attached cells and the generation of ECM during cell growth. As the culture time increased, the attached cells would shrink before doubling the cell number (i.e. before proceeding the proliferation process), leading to a slight decrease in cell coverage. In this study, the U-2 OS cells doubled their number about every 1.8 days. This might partially explain the fact that a tiny decrease in impedance was observed for the cell-cultured I_1 and I_2 specimens when the culture time increased from day 1 to day 2. However, the impedance of the cell-cultured Ti specimens at this stage was still larger than that of the specimens without cell culture due to the presence of attached cells and adsorbed ECM. As the culture time continued (e.g. at days 3 and 5), the increased cell number (via the proliferation process)

and the generation of ECM (during cell growth) led to a slight increase in impedance, although the shrinkage of cells still occurred every 1.8 days.

Figure 3 shows the FE-SEM micrographs of the attached U-2 OS cells on the Ti substrates after 1 day of cell culture: (a) untreated I_0; (b) anodized I_1. Comparing Figs. 3(a) and 3(b), attached cells exhibiting flatter membranes (indicated by arrows in Fig. 3(b)) were observed on the anodized I_1 specimen compared to the untreated I_0 specimen. Similar results were also observed for the anodized I_2 specimen. Orsini et al. [19] reported that the formation of thin membranes on the material surface is ascribed to the spreading of cell filopodia when the cell adhesion process continues. This is a good index to the quality of cell growth in evaluating the biocompatibility of implant materials. Therefore, the evidence for improved cell/material surface interaction on the anodized Ti specimens included the quantitatively higher impedance (Fig. 2) and the qualitatively better cell spreading (*i.e.* flattened cell membrane)(Fig. 3).

Figure 4 shows the XPS surface analysis results (spectra for N_{1s}) of I_0 and I_1 specimens before and after 2 hours immersion in the culture medium. It showed that higher intensity for N spectra was detected on the I_0 and I_1 specimens after culture medium immersion compared to that before immersion. Furthermore, the anodized I_1 specimen had higher N intensity than the untreated I_0 specimen after immersion in the culture medium. In other words, higher proteins (mainly as albumin) content existed on the anodized Ti substrate with respect to the untreated Ti substrate. It has been reported that the scaffolds with nano-fibrous pore walls adsorb more proteins than the scaffolds with solid pore walls, which mediates the cell interactions with scaffolds [20]. Therefore, the nano-network-structured TiO_2 layer on the anodized Ti substrate was beneficial to the adhesion of nano-scaled proteins ascribed to the components in the culture medium. This increased the electrochemical impedance of the anodized Ti substrates due to the increasing adsorption of proteins onto specimens. At the same time, the following cells spreading process on the anodized Ti substrates would also be enhanced by the adsorbed proteins (Fig. 3(b)), leading to higher electrochemical impedance as well. The above phenomena could explain the results shown in Fig. 2.

4. Conclusions
We developed a self-organized TiO_2 network layer onto a Ti substrate with a network size of under 100 nm through a rapid and simple galvanodynamic anodization treatment. The anodized Ti substrate had higher (1.8-3.8 times) electrochemical impedance of 1.9-4.7 x 10^6 Ωcm^2 and better cell spreading, namely better biocompatibility, compared to the untreated Ti substrate.

5. References
[1]   J.Okumura, H.Ohgushi, P.Ducheyne, *Bioceramics: vol. 6,* eds. P.Ducheyne, D.Christiansen, Pergamon, Oxford, 1993, 305.

[2]   M.Assad, A.Chernyshov, M.A.Leroux, C.H.Rivard, *BioMed Mater Eng.*, 2002,**12**,225.

[3]   Y.Yang, J.Tian, J.Deng, J.L.Ong, *Biomaterials*, 2002,**23**,1383.

[4]   H.M.Kim, T.Kokubo, S.Fujibayashi, S.Nishiguchi, T.Nakamura, *J BioMed Mater Res.,* 2000,**52**,553.

[5]   S.Fujibayashi, M.Neo, H.M.Kim, T.Kokubo, T.Nakamura, *Biomaterials*, 2004,**25**,443.

[6]   X.Zhu, J.L.Ong, S.Kim, K.Kim, *J Biomed Mater Res.*, 2002,**60**,333.

[7]   R.Rosenberg, D.Starosvetsky, I.Gotman, *J Mater Sci Lett.,* 2003,**1**,29.

[8]   L.A.Thomson, F.C.Law, N.Rushton, J.Franks, *Biomaterials,* 1991;**12**:37.

[9]   G.W.Walter, *Corros Sci.,* 1986;**26**:681.

[10] N.Ibriş, JCM Rosca, *J Electroanal Chem.,* 2002;**526**:53.

[11] J.P.Bearinger, C.A.Orme, J.L.Gilbert, *J Biomed Mater Res.,* 2003;**67**:702.

[12] F.Contu, B.Elsener, H.Bohni, *J Biomed Mater Res.,* 2003;**67:**246.

[13] C.Aparicio, F.J.Gil, C.Fonseca, M.Barbosa, J.A.Planell, *Biomaterials,* 2003;**24**:263.

[14] F.Contu, B.Elsener, H.Bohni, *J Biomed Mater Res.,* 2002;**62**:412.

[15] J.M.Nelissen, R.Torensma, M.Pluyter, G.J.Adema, R.A.Raymakers, Y.Van Kooky, C.G.Figdor, *Exp Hematol.,* 2000;**28**:422.

[16] H.H.Huang, *Biochem Biophys Res Commun.*, 2004;**314**:787.

[17] S.J.Pan, *Surface Modification of Biomedical Pure Titanium Using Electrochemical Technique*, M.Sc. thesis, Taiwan: Chung Shan Medical University; 2004.

[18] K.M.Woo, V.J.Chen, P.X.Ma, *J Biomed Mater Res.*, 2003,**67A**,531.

[19] G.Orsini, B.Assenza, A.Scarano, M.Piatelli, A.Piattelli, *Int J Oral Maxillofac Impants.*, 2000,**15**,779.

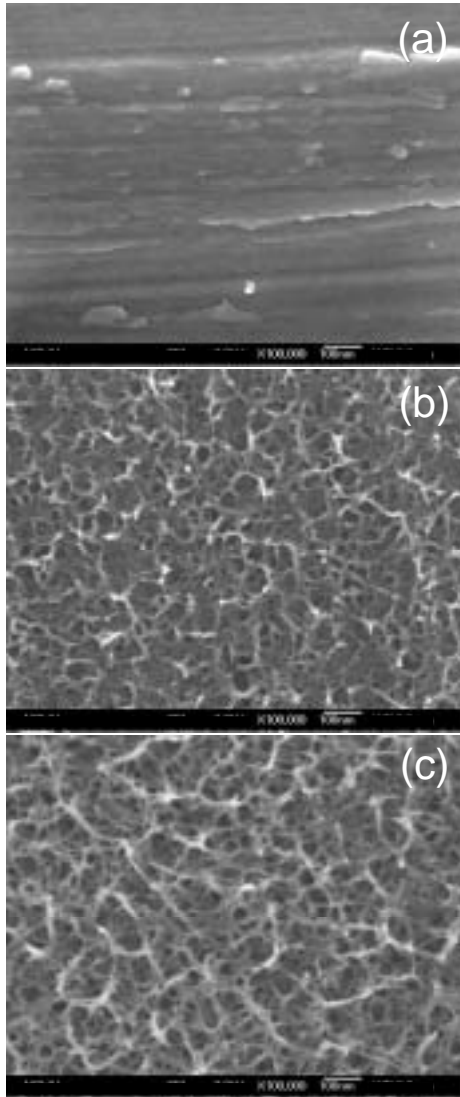[20] B.Kasemo, J.Lausmaa, *CRC Crit Rev Biocomp.*, 1986,**2**,335.

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Fig. 1 FE-SEM micrographs of Ti substrates with and without anodization treatment: (a) untreated I_0; (b) anodized I_1; (c) anodized I_2.
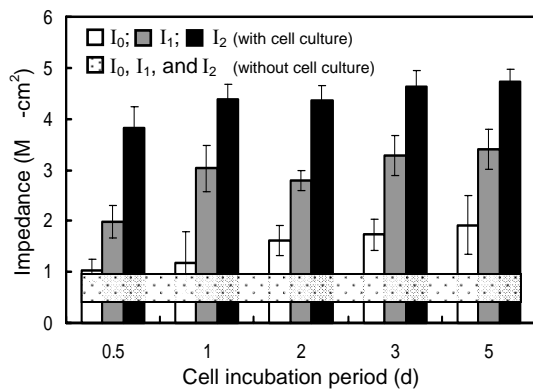

Fig. 2 Electrochemical impedance (obtained at 10^{-3} Hz) of the tested Ti substrates, with and without cell culture, after different periods in the culture medium.
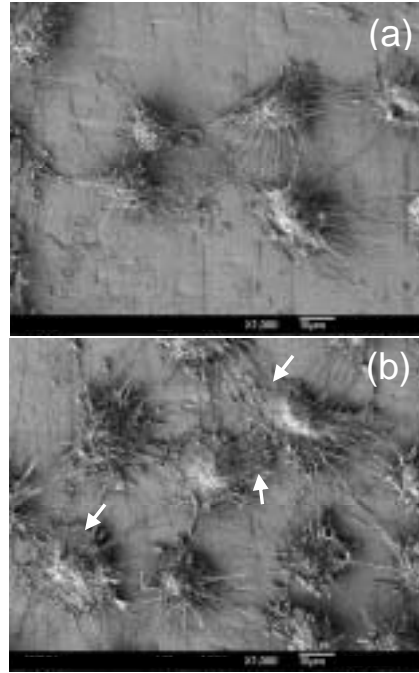

Fig. 3 FE-SEM micrographs of the attached U-2 OS cells on the Ti substrates after 1 day of cell culture: (a) untreated I_0; (b) anodized I_1.
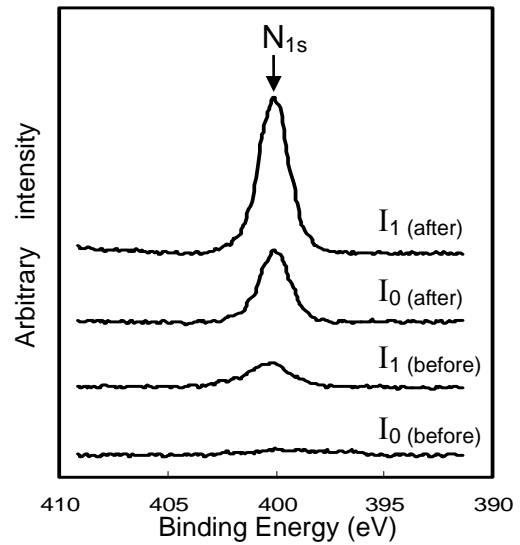

Fig. 4 XPS surface analysis results (spectra for N_{1s}) of I_0 and I_1 specimens before and after 2 hours immersion in culture medium.

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