OSTEOBLAST PROLIFERATION ON GRAPHENE OXIDE ELECTRODEPOSITED ON ANODIZED TITANIUM FOR ORTHOPEDIC IMPLANTS

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ABSTRACT Using graphene oxide (GO) coating on biomaterial surfaces is a great potential for long-term use of orthopedic implants due to its biocompatibility and antibacterial property. Anodization is one of many methods that have been used to modify titanium implant surfaces in order to increase osseointegration. In this study, anodized titanium coated with GO (ATiGO) was prepared using anodization and electrodeposition of graphene oxide, respectively. Scanning electron microscopy and atomic force microscopy were used to investigate surface morphologies of the samples. Their physiochemical properties were also evaluated by energy-dispersive X-ray spectroscopy and X-ray diffractrometry. Cell proliferation of mouse osteoblastic cell line (bone-forming cell) was investigated on ATiGO, GO electrodeposited on titanium (TiGO), anodized titanium (ATi), and commercially pure titanium (Ti). The result suggests that the electrodeposited GO on Ti or ATi improves the proliferation of osteoblasts. The results showed a significant higher of cell proliferation on ATiGO than TiGO substrates when compared with the samples at day 1, 3, and 5 of cultivation (p<0.05). The result suggests that the electrodeposited GO on Ti or ATi improve the proliferation of osteoblasts.

1. INTRODUCTION

Two major problems after inserted implant into patient body include the lack of bone tissue integration, and the infection caused by bacteria. However, implant surfaces should increase osteoblast functions and possess antibacterial property (Temenoff & Mikos, 2008). Surface properties of implants, such as wettability, roughness and surface energy are determinants of osteoblast behaviors (Bacakova, et al., 2011). Titanium (Ti) is metals that widely used for orthopedic implants because of its physical, mechanical properties, and excellent biocompatibility (Park, et al., 2003). Ti can be resistant to corrosion and high biocompatible due to its titanium dioxide (TiO_2) layer on its surface (Eillingsen & Lyngstadass, 2000). However, TiO_2 layer on Ti surface which two to ten nanometers in thickness, is unable to prevent corrosion for long-term implants. A modification of Ti surface is needed to protect an ion release from implants (Joon & Young, 2003). Anodization is known as an electrochemical method, used for fabrication of TiO_2 nanotubes on Ti suface (Minagar, et al., 2012). A TiO_2 nanotubes with 50-60 nm in diameter and 200 nm in length after anodization in hydrofluoric acid solution with 20 V for 15 minutes were studied previously (Sirivisoot & Webster, 2008). Anodized titanium (ATi) increased osteoblast proliferation, because its nano-roughness of TiO_2 given larger surface area which leads to higher protein adsorption and cell adhesion (Oh, et al., 2010). Graphene oxide (GO) is produced by chemical oxidation of graphene. GO has a single atom of carbon atoms, and being a two dimentional (2D) structure with a honeycomb lattice structure. GO has hydroxyl groups and epoxides on its basal. Carboxyl and carbonyl functional groups are present on its edge (Zhu, et al., 2010). GO is biocompatible due to their physical and chemical properties (Pinto, et al, 2013). Moreover, the previous study showed that GO had antibacterial properties (Wang, et al., 2011). This property of GO can prevent infection of implants. Osteoblast behaviors and antibacterial properties of the composites of GO and hydroxyapatite coating on Ti and ATi samples were studied previously (Parcharoen, et al., 2014a), (Parcharoen, et al., 2014b). The aim of this study is to study osteoblast responses on anodized Ti and electrodeposited-GO on Ti and ATi samples without osteoinductive hydroxyapatite.

2. EXPERIMENT

2.1 Anodization

The commercially titanium (Ti) (99.2%; Alfa Aesar, USA) were cut into small pieces (1 cm x 1 cm) and twice cleaned by sonicating (TT230D Powersonic, Crest

Ultrasonic, USA) with acetone **(**Sigma, Thailand) for 15 minutes. The samples were etched in acid solution (1.5 wt% nitric acid (Sigma, Thailand): 1.5 wt% hydrofluoric acid (HF) (Gammaco, Thailand)) in ratio 1:1 for 10 seconds. All solutions were prepared from reagent grad chemicals and deionized (DI) water. Ti were used as anode and platinum wire used as cathode, while the distance was 1 cm. About 1.5 wt% HF (Gammaco, Thailand) was used for electrolyte and contained in polyethylene beaker (Gammaco, Thailand). Electrolyte was stirred using magnetic stirrer during anodization. Anodized was carried out at 10 V using a DC power supply (APS-3005D ATTEN DC power supply digital, China) for 15 minutes. After anodization, anodized titanium (ATi) were rinsed with DI water and dried in room temperature (Sirivisoot & Webster, 2008)..

2.2 Electropheretic of graphene oxide

Graphene oxide (GO) (Graphene Square, South Korea) were used at a concentrations of 150 µg/ml or 200 µg/ml. The GO solution was mixed in isopropanol alcohol (Sigma, Thailand) and used as electrolyte. The samples (Ti or ATi) were used as anode and platinum wire were used as cathode. The electrophoretic deposition of GO onto samples was carried out at constant potential of 10 V (APS-3005D ATTEN DC power supply digital, China) for 10 minutes. The electrolyte was stirred using a magnetic stirrer during eletropheretic. The GO was electrodeposited at the concentration of 150 µg/ml on Ti (TiGO150) and ATi (ATiGO150), and at the concentration of 200µg/ml on Ti (TiGO200) and ATi (ATiGO200).

2.3 Surface characterizations

The surface morphology was evaluated using 50,000x magnification of scanning electron microscope (SEM-EDX, Bruker, USA). Image analysis software (ImageJ version 1.32, National Insitutes of Health, USA) was used for analyzing diameter of TiO_2 nanotubes. The elemental composition of the samples was analyzed using an energy dispersive X-ray spectrometer (EDX, SEM-EDX, Bruker, USA). The crystal structure of samples was evaluated using X-ray diffraction (Bruker Axs D8 Discover XRD System, Bruker, USA) at scaning rate of 1°/min. About 10µl of distilled water was dropped on samples surface for evaluating surface wettability using a contact angle tester (DSA 10 MK2, KRUSS, Germany), according to the sessile drop technique.

2.4 Cell proliferations

Mouse pre-osteoblast cells (MC3T3-E1,p=13-15, MTEC, Thailand) were cultured in alpha-modified minimum essential medium (Hyclone, USA) with 10 vol% fetal bovine serum (Gibco, USA), and 1 vol% penicillin/streptomycin (Invitrogen, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were detached when 80-85% confluence via trypsin/EDTA (Invitrogen, USA). All of samples were kept in 24-well culture plates and UV sterilized for 4 hours. MC3T3-E1 cells with density of $5x10^4$cells/cm^2 were seeded on each sterile samples and culture plate used as a control. Then, the samples were incubated at 37 °C in humidified atmosphere of 5% CO_2. The culture media were replenished every 48 hours. After cultured for 5 days, the media were removed and the samples were rinsed for three times with 1X phosphate buffer saline. Cell proliferation assay was assessed using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) MTT assay were used as reported in previous study [Tanurat & Sirivisoot, 2015].

3. Results and Discussion

3.1 Surface Characterization

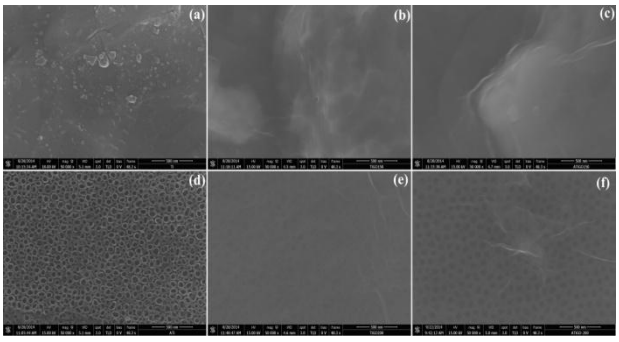


Fig. 1 SEM images of: (a) Ti; (b) TiGO150; (c) TiGO200; (d) ATi; (e) ATiGO150; and (f) ATiGO200. Scale bars are 500 nm.

The commercially pure Ti features a smooth surface, as shown in Fig. 1(a). Anodization was used to fabricate the TiO_2 nanotubes on Ti surfaces with 50 nm in diameters, as shown in Fig. 1 (d). The results suggested that anodization produced a nano-structure surface and increased a roughness of Ti. After GO electropheretic deposition, SEM images show a thin layer of GO covering on the samples. The concentration of GO at 150 µg/ml was used for electrodeposition on Ti (TiGO150) and ATi (ATiGO150), as shown in Figs. 1(b)-(e), respectively. The GO concentration at a 200 µg/ml was also used for electrodeposition on Ti (TiGO200) and ATi (ATiGO200), as Figs. 1(c)-(f).

3.2 Energy dispersive X-ray (EDX) analysis

The chemical characterization was carried out with an EDX analysis to confirm the nanotubular TiO_2 formation and GO electrodeposition. After anodization, the oxygen component of ATi was significantly increased when compared to that on Ti. On the other hand, the ATi substrates had a lower in the titanium component when compared to Ti substrates. Because of the formation of TiO_2 nanotubular involves of two reactions.

$$Ti + 2H_2O \rightarrow TiO_2 + 4H^+ + 4e^- \quad (1)$$

$$TiO_2 + 4H_6F^- \rightarrow {}^1/_2[TiF_6]^{2-} + H_2O \quad (2)$$

When applied an electrical potential, TiO_2 array occurs according to the oxidation of Ti by the following the equation (1). According to the equation (2), the applied potential drives Ti^{4+} ions migrating into fluoride containing electrolyte. The combining between Ti^{4+} with fluoride ion (F^-) was produced hexafluoride complex ($[TiF_6]^{2-}$), which is poorly soluble in water. The competition between the formation of TiO_2 in equation (1) and the dissolution in equation (2), leading to the

formation of TiO_2 nanotubular arrays (Al-Mobarak & Al-swayih, 2014). The carbon components of the samples were significantly increased when GO was used at the concentration of 200 µg/ml. The carbon components were 9.70%, 9.80%, 17.00%, and 22.02% on TiGO150, ATiGO150, TiGO200, and ATiGO200, respectively.

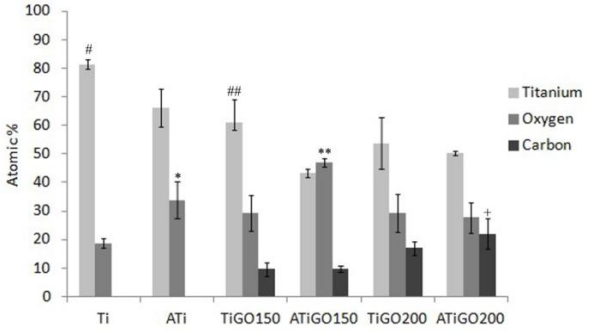


Fig. 2 EDX analysis shows the atomic percentage of titanium, oxygen and carbon on Ti, ATi, TiGO150, ATiGO150, TiGO200, and ATiGO200. Data analysis is mean ± standard deviation (N=3; n=1). Statistic analysis was used one-way ANOVA: #$p < 0.05$ when compared to the titanium component with ATi samples; *$p < 0.05$ when compared to the oxygen component with ATi samples; ##p when compared to the titanium component with other GO-coated samples; **$p < 0.05$ when compared to the oxygen component with other GO-coated samples; and +$p < 0.05$ when compared to the carbon component with other GO-coated samples.

3.3 X-ray diffraction (XRD) analysis

To investigate of the reaction of GO electrodeposition onto Ti and ATi surfaces, the X-ray diffraction (XRD) analysis was used to determine a crystal structure of the electrodeposited GO. The control was the GO solution dropped on ATi surface and dried in room temperature. The sharp peak appears at 2 Theta or 10.16° for the GO control, however, that peak disappears on the other GO-deposited samples (Tanurat & Sirivisoot, 2015). This indicated that a deoxygeneration was occurred during electrodeposition of GO on Ti or ATi. The GO has many oxygen functional groups, such as carboxylate and carbonyl at its edges, and hydroxyl and epoxide on its basal planes. It is possible that the electropheretic deposition of GO involves of the Kolbe reaction (Vijh & Conway, 1967)

$$RCOO^- \rightarrow RCOO\cdot + e^- \quad (3)$$

$$RCOO\cdot \rightarrow R\cdot + \cdot CO_2 \quad (4)$$

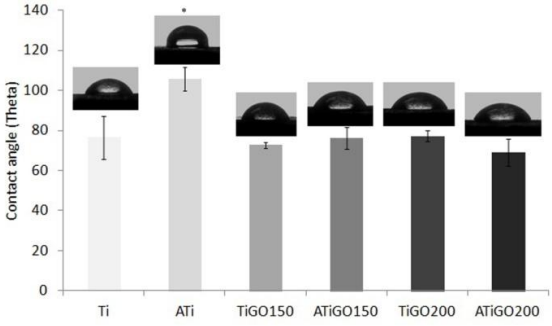
$$R\cdot + R\cdot \rightarrow R\text{-}R \quad (5)$$

During electrodeposition, the GO nanosheets are negatively charged when electrical potential was applied. Then they migrate to the anodic electrode. Moreover, electrons can be removed from carboxylate groups of GO, according to the oxidation of caboxylate by equation (3). The equation (4) is an oxidative decarboxylation, removing of carbon atom from a carbon chain, producing CO_2. The reaction of equation (5) involves a recombination of two radicals, which bond within in the GO nanosheets. We hypothesized that the Kolbe reaction could be used to explain the anodic oxidation of GO-carboxylate structure during the electrodeposition of GO in this work.

3.4 Water wettability

The averaged water contact angle of Ti samples was 76.5°. However, the averaged contact angle of ATi samples increased to be 105.7°. Nano-roughness formed after anodization of Ti affects to wettability of the surfaces itself. It was shown that a presence of oxygen-containing functional groups (or TiO_2) increased the wettability of ATi samples (Bacakova, et al., 2011). For the GO-coated samples on both Ti and ATi, the water contact angles were similar to Ti samples. Therefore, the GO electrodeposition on ATi samples is lowers its averaged water contact angle.

Fig. 3 A comparison of averaged contact angles of liquid



water at room temperature on Ti, ATi, TiGO150, ATiGO150, TiO200, and ATiGO200. Data analysis is mean ± standard deviation (N=3; n=1) with *$p < 0.05$ when compared to other samples.

3.5 Cell proliferation using MTT assay

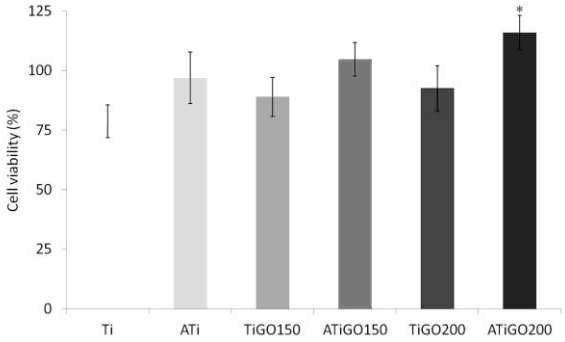


Fig. 4 Cell viability of MC3T3-E1 pre-osteoblast cells evaluated at day 5 of cultivation. Data analysis is mean ± standard error of mean (N=3; n=3) *$p < 0.05$ when compared to other samples.

The MTT assay, which is a colorimetric assay for evaluating cell metabolic activity, was used in this study. The Fig. 4 shows the MC3T3-E1 cell viability on the uncoated-GO samples (Ti and ATi) and the GO-coated samples (TiGO150, ATiGO150, TiGO200, and ATiGO200) after 5 days of cultures, respectively. Osteoblast cells cultured on Ti displayed lowest cell viability among all other samples and showed lower viability than on ATi samples. It is possible that cell proliferation increased on rougher samples since ATi had

rougher surface than Ti, leading to higher protein adsorption, cell adhesion, and cell proliferation (Zhang, et al., 2013). The results indicated that cell proliferation on the GO-coated ATiGO samples was significantly higher when compared to that on the GO-coated Ti samples. Cell proliferation on the ATiGO200 was highest among all other samples, even though the wettibility of ATiGO200 was similar to that on Ti samples (Fig. 3). The presence of carbon element on the GO-electrodeposited samples increased the cell proliferation. The results suggested that surface chemistry had higher influence to osteoblast proliferation on the GO-electrodeposited samples than wettibility of the samples.

CONCLUSION

The EDX results confirmed the presence of carbon element on the GO- electrodeposited samples. The XRD results suggested that the coated-GO had lost of carboxylate group during electrodeposition. The study of wettability indicated no significant difference between the GO-electrodeposited samples and Ti controls. However, cell proliferation was higher on the GO-electrodeposited on ATi than other samples. Surface roughness analysis and study of osteoblast differentiation are still in progress.

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