

## Research Article

# Improving the Osteoblast Cell Adhesion on Electron Beam Controlled TiO<sub>2</sub> Nanotubes

Sung Wook Yoon,<sup>1</sup> Hyun Ju Shim,<sup>2</sup> and In-Bo Shim<sup>1</sup>

<sup>1</sup> Department of Nano and Electronic Physics, Kookmin University, Seoul 136-702, Republic of Korea

<sup>2</sup> Hankuk Academy of Foreign Studies, Gyeonggi-do 449-854, Republic of Korea

Correspondence should be addressed to In-Bo Shim; [ibshim@kookmin.ac.kr](mailto:ibshim@kookmin.ac.kr)

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Here we investigate the osteogenesis and synostosis processes on the surface-modified TiO<sub>2</sub> nanotubes via electron beam irradiation. The TiO<sub>2</sub> nanotubes studied were synthesized by anodization process under different anodizing voltage. For the anodization voltage of 15, 20, and 25 V, TiO<sub>2</sub> nanotubes with diameters of 59, 82, and 105 nm and length of 115, 276, and 310 nm were obtained, respectively. MC3T3-E1 osteoblast cell line was incubated on the TiO<sub>2</sub> nanotubes to monitor the change in the cell adhesion before and after the electron beam irradiation. We observe that the electron beam irradiation affects the number of surviving osteoblast cells as well as the cultivation time. In particular, the high adhesion rate of 155% was obtained when the osteoblast cells were cultivated for 2 hours on the TiO<sub>2</sub> nanotube, anodized under 20 V, and irradiated with 5,000 kGy of electron beam.

## 1. Introduction

Due to its unique physical and chemical characteristics as well as its biocompatibility, titanium dioxide (TiO<sub>2</sub>) has been actively researched in various fields such as photocatalysis, sensor, solar cell, and biomaterial [1–4]. The advantage of TiO<sub>2</sub> is that it can be easily manufactured in various shapes such as bulk, powder, tube, or grid. In particular, the nanostructured TiO<sub>2</sub>, offering the extremely high surface-area-to-volume ratio, can promote cell propagation and reduce rejection symptoms, which is greatly desirable in biomaterial- and implant-related technologies [5]. Among many forms of nanostructured TiO<sub>2</sub>, TiO<sub>2</sub> nanotube has been reported to strongly affect the propagation of osteoblast cell line, the related ossification process, the mobility of blood cells in blood vessels, and spontaneous differentiation of mesenchymal stem cells [6].

There are a number of TiO<sub>2</sub> nanotube synthesis methods such as sol-gel [7] and hydrothermal synthetic process [8], but the quality of the fabricated TiO<sub>2</sub> layer such as ruggedness must be considered. In this respect, it has been shown that nanotubes prepared by anodization method [9] have far better features when compared to those from the other two

processes above. Also, electron beam irradiation technique can offer a way of improving and/or controlling material characteristics, which can lead to new functionalities and applicability in many technological fields. For example, Jeun et al. [10] have employed the electron beam irradiation to increase the photocatalytic activity in sol-gel based TiO<sub>2</sub> and Jun et al. [11] have observed the increase in the conversion rate of CH<sub>4</sub> and CO<sub>2</sub> by 5~10% after electron beam treatment of various catalysts at an absorbed dose of 2 MGy.

In this study, we have investigated the effect of the electron beam irradiation on the osteogenesis and synostosis processes on the TiO<sub>2</sub> nanotube layer prepared by the anodization process. In particular, we studied the interrelation between the change of Ti oxidation state and the adhesion characteristics of osteoblast cells.

The work presented here suggests the possible application of surface-modified TiO<sub>2</sub> nanotube via electron beam treatment as a bio-osteology restoration material.

## 2. Experiments

The TiO<sub>2</sub> nanotubes studied here were fabricated by anodization process. Before anodization, Ti foil (Nilaco, 99.50 purity,

0.1 × 50 × 50 mm, USA) and platinum (Pt) foil (Daehan, 99.90% purity, 0.25 × 50 × 50 mm) were cleaned by ultrasonication in ethanol (Aldrigh, 99% purity, USA) for 3 minutes and dried with nitrogen (N<sub>2</sub>, Daehan Scien. Co, 99.9% purity, KOR.) gas. The 1,000 mL of 0.5 mol% hydrofluoric acid (HF, Duksan, 48% purity, KOR.) solution, prepared by mixing 10.4 mL of HF solution and 989.6 mL of distilled (DI) water, was used for the anodization electrolyte. The anodization was performed with Pt foil as a cathode and Ti foil as an anode in 0.5% HF aqueous solution for 30 minutes with anodization voltage of 15, 20, and 25 V between them. After anodization, the Ti foil was cleaned with distilled water and dried with N<sub>2</sub> gas. Later, the Ti foil was further dried in an electric oven for more than 3 hours at 80°C. Finally, the dried Ti foil was heat-treated in air at 500°C for 1 hour.

For the electron beam treatment, the electron beam accelerator (EB-Tech, Model ELV-8, EB tech. KOR.) was used with the beam energy of 1.0 MeV, acceleration current of 4 mA, beam dimension of 75 mm (length) × 980 mm (width), and dose rate of velocity 20 m/s, resulting in the absorption dose of 0, 50, 500, and 5000 kGy.

To monitor the change in the crystal structure of the TiO<sub>2</sub> nanotube, X-ray diffractometer (Philips, X'Pert PW1830) was used and field emission scanning electron microscope (FE-SEM; JEOL, JSM 7401F, Japan) was used to observe the change in the surface morphology before and after electron beam irradiation. The surface oxidation of the nanotube before and after the electron beam treatment was measured by X-ray photoelectron spectrometer (XPS; VG microtech, ESCA 2000, England) and analyzed with the binding energy of O 1s.

To understand the cell adhesion on the TiO<sub>2</sub> nanotube, MC3T3-E1 mouse osteoblast (CRL-2593, subclone 4, ATCC, Sigma, USA) cell line was incubated. Cultivation was performed by using DMEM culture medium, added with 10% fetal bovine serum in MC3T3-E1, at 37°C for 2, 12, and 24 hours with TiO<sub>2</sub> nanotube. With XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, USA) solution added to the incubated medium, ELISA reader was used to verify the change in the reactant. Also, an image analysis program (Image pro plus, Media Cybernetics) was used to count the number of cells incubated on TiO<sub>2</sub> nanotube. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to determine the number of surviving osteoblast cells and toxicity during the adhesion of the osteoblast cells on the TiO<sub>2</sub> nanotube surface with MTT 5 mg/mL phosphate buffer solution (PBS, Sigma, USA) as reagents. More specifically, MC3T3-E1 cells were dispensed on TiO<sub>2</sub> nanotube surface by 5 × 10<sup>4</sup> cell/well. Then, cultivation was conducted for 2 and 24 hours for media suction and cleaned with 1 × PBS. Here, 100 µg/mL of MTT solution was added to the media and maintained for 4 hours at 37°C in the CO<sub>2</sub> incubator. Later, the media were removed and TiO<sub>2</sub> nanotube was placed to a new 12-well plate. Dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) was added and well mixed with a pipette to be incubated for 5 minutes. Finally, 200 µL of the sample was dispensed into the 96-well and ELISA reader was used to measure the absorbance at 540 nm. The graphs show the average ±

TABLE I: Dimensions of TiO<sub>2</sub> nanotubes fabricated under different anodization voltage.

Anodizing voltage (V)	Inner diameter (nm)	Out diameter (nm)	Wall thickness (nm)	Length (nm)
15	59	102	42	115
20	82	124	41	276
25	105	162	57	310

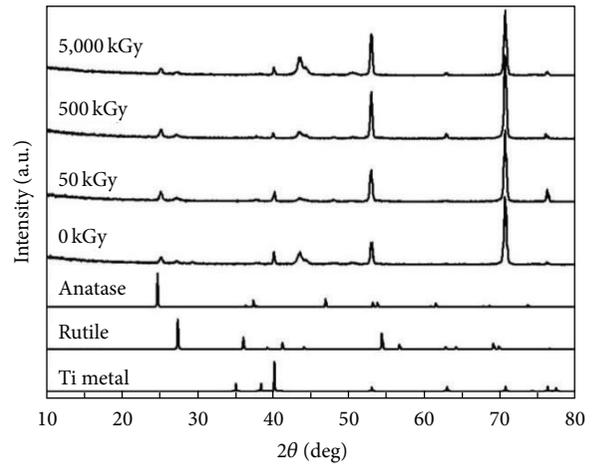


FIGURE 1: XRD patterns of the TiO<sub>2</sub> nanotubes with or without the electron beam treatment.

standard error bars associated with the sample size (or N values) shown in a box in the upper portion of each graph.

### 3. Results and Discussion

Figure 1 shows the X-ray diffraction patterns of the TiO<sub>2</sub> nanotubes with or without the electron beam treatment. It could be seen that all the samples have typical anatase structures and the anatase structure has been reported to play more important role in cell propagation and formation when compared to other structures [12–14]. The X-ray diffraction patterns observed before and after electron beam irradiation confirm that the amount of the electron beam dose does not affect the crystal structure of TiO<sub>2</sub> nanotubes.

Figure 2 shows the FE-SEM images of TiO<sub>2</sub> nanotubes fabricated under different anodization voltage between 15 and 25 V, and the resulting dimensions of the nanotubes are listed in Table 1. The scanning electron micrograph shows that the nanotubes are well-arrayed in vertical direction, but the tube diameter varies between 59 and 105 nm depending on the anodization voltage. Also, one can note that both the diameter and length of the nanotube are proportional to the applied voltage during the anodization process. Comparing between the SEM images before and after the electron beam irradiation, we cannot find any morphological difference. From these results, we found that the osteoblast cell adhesion

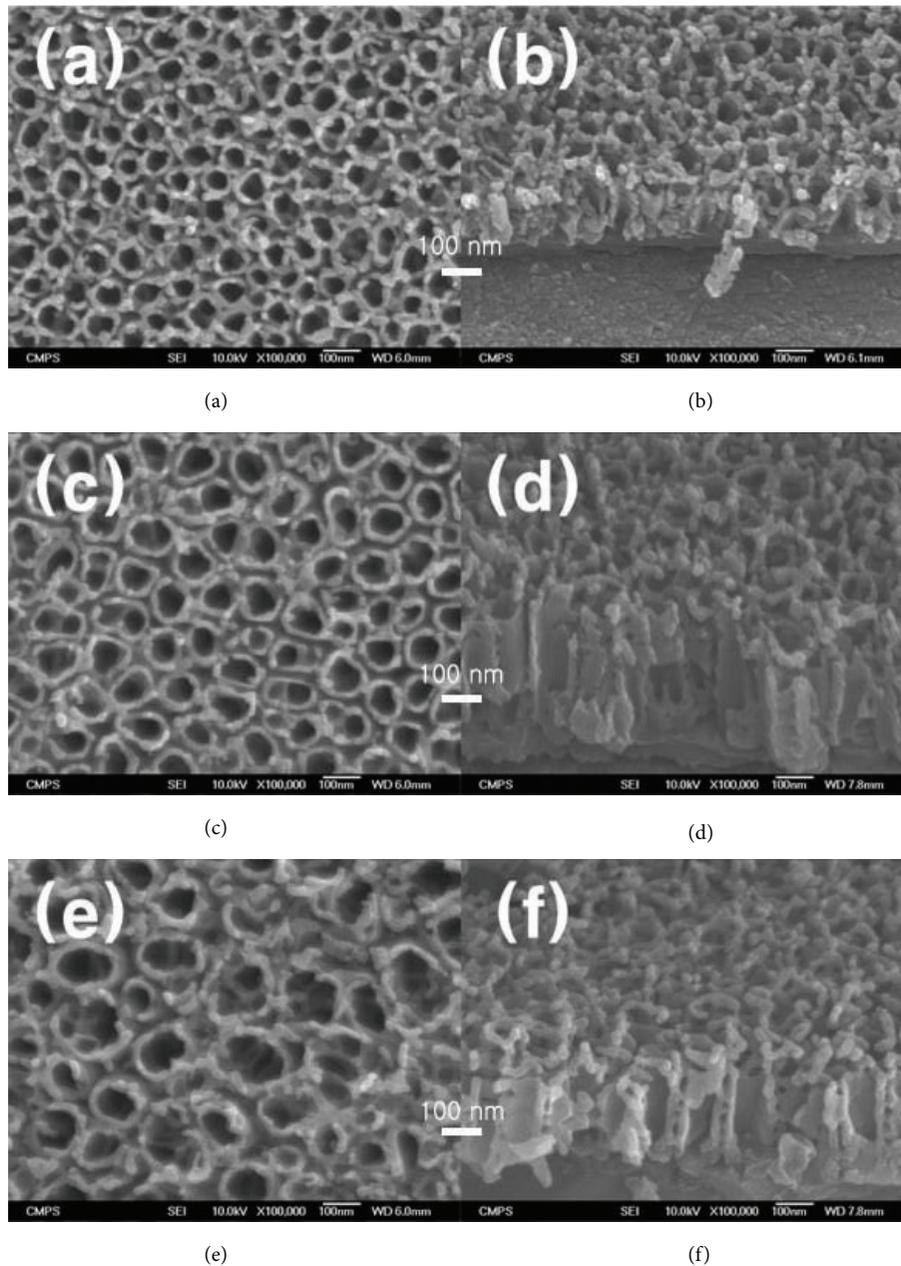


FIGURE 2: FE-SEM images of synthesized  $\text{TiO}_2$  nanotube under different anodization voltage. (a), (c), and (e) show surfaces of the nanotubes, and (b), (d), and (f) display the cross-sectional view of nanotubes fabricated at 15 V, 20 V, and 25 V, respectively.

characteristic has not affected the microstructure change by electron beam irradiation. Oh and Jin [15] studied the comparative SEM micrographs of MC3T3-E1 cells cultured on pure Ti versus vertically aligned anatase  $\text{TiO}_2$  nanotube. The adhesion/growth of osteoblast cells is also significantly accelerated by the topography of the  $\text{TiO}_2$  nanotubes with the filopodia of the growing cells actually going into the nanotube pores, producing a locked-in cell structure. Figure 3 shows the detailed FE-SEM micrographs of the osteoblast cells cultivated on vertically grown  $\text{TiO}_2$  nanotubes after electron beam irradiation. Adhesion of osteoblast cells could

be observed in all surfaces of the cultivated osteoblast cells on  $\text{TiO}_2$  nanotubes that had been electron beam irradiated.

The scanning electron micrograph was taken after the cultivation of MC3T3-E1 osteoblast cell line and based on this, the number of cells adhesion on a fixed area of  $\text{TiO}_2$  nanotube was analyzed (Figure 4). When cultivated on  $\text{TiO}_2$  nanotubes, high adhesion rate of osteoblast cells was obtained with low applied voltage, since the diameter of the  $\text{TiO}_2$  nanotube was small with low anodization voltage. Thus, average surface roughness decreases as the nanotube shrinks, which in turn increases the adhesion rate of osteoblast cells.

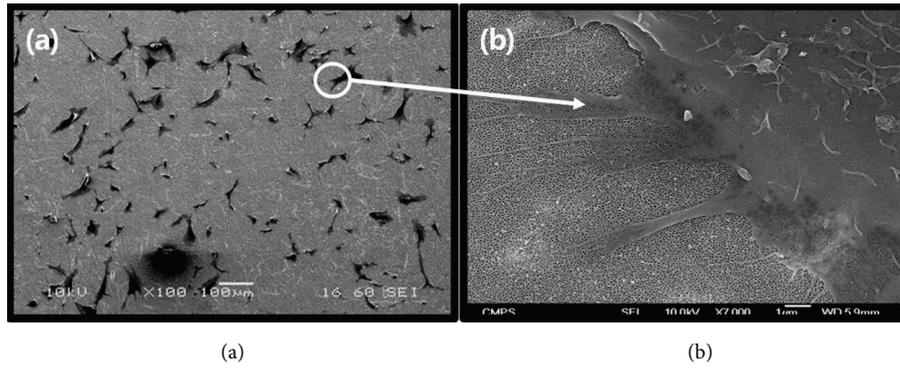


FIGURE 3: (a) Scanning electron micrograph of the osteoblast cell adhered on TiO<sub>2</sub> nanotube after electron beam irradiation. (b) The micrograph taken at high magnification.

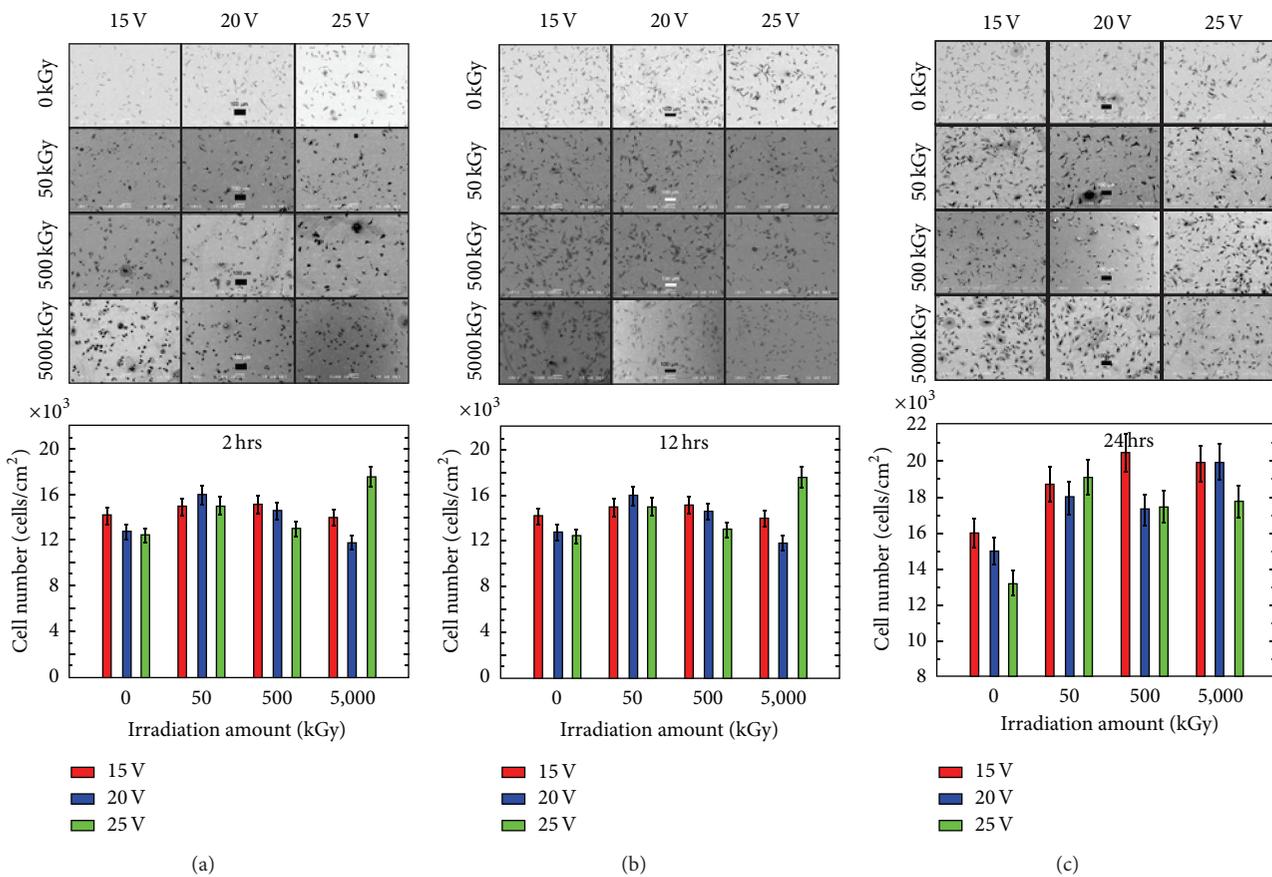


FIGURE 4: Number of cells adhesion on the fixed area of TiO<sub>2</sub> nanotube based on the scanning electron micrograph after cultivation of MC3T3-E1 osteoblast cell line for (a) 2 hr, (b) 12 hr, and (c) 24 hr.

With increasing both electron beam dose and cell cultivation time, one can observe that the cell adhesion rate increases. We have obtained the highest adhesion rate of 155%, when the osteoblast cells were cultivated for 2 hours on top of TiO<sub>2</sub> nanotube, anodized with 20 V, and irradiated with 5000 kGy of electron beam.

Figure 5 shows the experimentally measured optical density (OD) of osteoblast cells and MTT solution with different cultivation time as well as various electron beam

irradiation of 0, 50, 500, and 5000 kGy on TiO<sub>2</sub> nanotube surface. Regardless of the electron beam irradiation dose, it could be seen that the number of surviving osteoblast cells increases with increasing cultivation time. Also, we have observed that there is no significant change due to the anodization voltage and the detailed structure of TiO<sub>2</sub> nanotube.

To understand the observed increase in the adhesion rate, XPS analysis was performed and the condition of

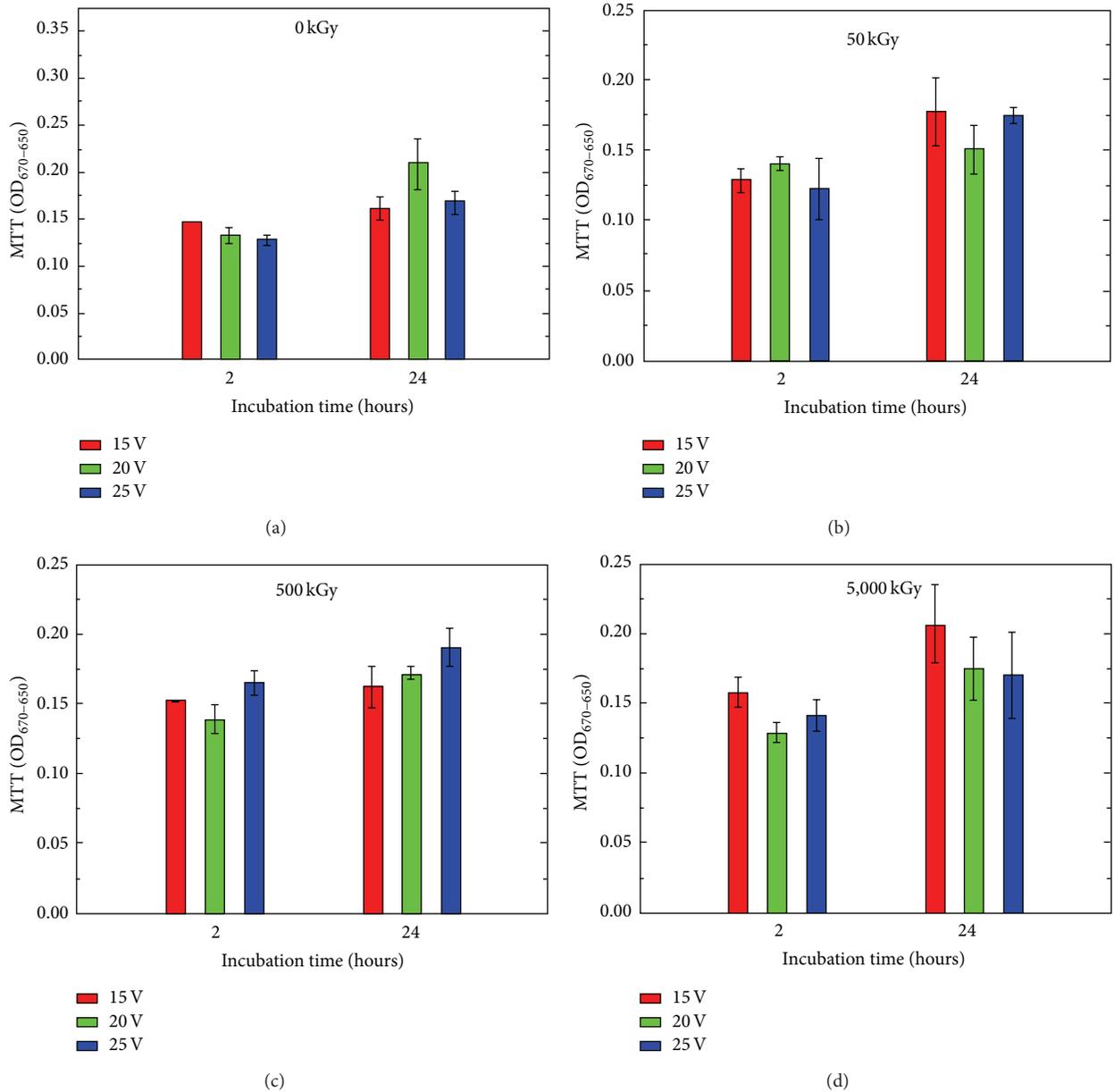


FIGURE 5: Optical density (OD) of osteoblast cells measured with MTT assay for different electron beam irradiation of (a) 0, (b) 50, (c) 500, and (d) 5000 kGy on TiO<sub>2</sub> nanotube surface.

the chemical species on TiO<sub>2</sub> nanotube was monitored before and after the electron beam irradiation. Figure 6 shows the XPS analysis of both untreated TiO<sub>2</sub> nanotube and TiO<sub>2</sub> nanotube irradiated with 5,000 kGy of electron beam. Based on the narrow scan analysis of O 1s peak, we observed the presence of various forms of the oxidized Ti compound such as TiO<sup>+</sup>, TiO<sub>2</sub><sup>+</sup>, Ti<sub>2</sub>O<sup>+</sup>, and Ti<sub>2</sub>O<sub>2</sub><sup>+</sup>. The O 1s binding energy from the XPS database is 532 eV. Therefore, it is impossible to separate the O 1s peak, existing on the TiO<sub>2</sub> nanotube surface, only based on the value of the binding energy. Thus, the peak near 533 eV is caused by different chemical species, which is most likely to be OH hydrates in the atmosphere. Therefore, the contribution of O, having the binding energy

of 533.3 eV, could be considered before and after electron beam irradiation. Here, the area ratio of O/O-H binding was 17085/2580 and there was no significant change before and after electron beam treatment. This suggests that the oxygen existing on TiO<sub>2</sub> nanotube surface is not a decisive factor in determining the adhesion rate of osteoblast cells.

To further determine the Ti surface oxidation condition before and after electron beam irradiation, the experimental Ti 2p narrow scan spectra of TiO<sub>2</sub> nanotube surfaces were fitted. Before the electron beam treatment, the peak in the spectrum can be separated into Ti 2P<sub>3/2</sub>(Ti<sup>4+</sup>) with binding energy of 460.60 eV and Ti 2P<sub>3/2</sub>(Ti<sup>3+</sup>) with 461.50 eV. However, after the electron beam treatment with 5,000 kGy,

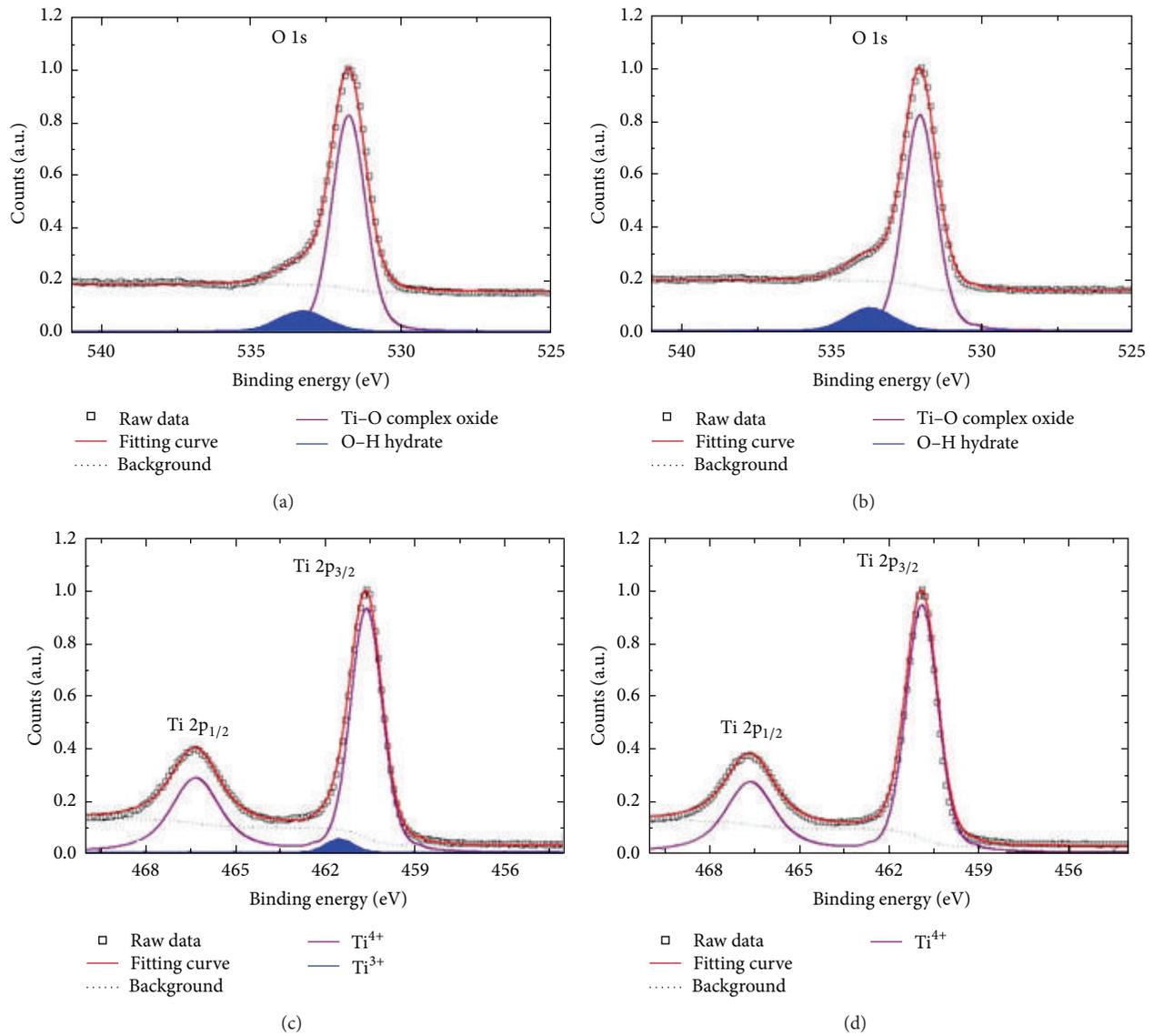


FIGURE 6: XPS analysis of both untreated TiO<sub>2</sub> nanotube and TiO<sub>2</sub> nanotube irradiated with 5,000 kGy of electron beam. (a) O 1s without irradiation, (b) O 1s after irradiating with 5,000 kGy, (c) Ti 2p without irradiation, and (d) Ti 2p after irradiating with 5,000 kGy.

the peak corresponds to Ti 2P<sub>3/2</sub>(Ti<sup>4+</sup>) with 460.90 eV and Ti 2P<sub>3/2</sub>(Ti<sup>3+</sup>) with zero binding energy, suggesting the disappearance of Ti 2P<sub>3/2</sub>(Ti<sup>3+</sup>) after irradiation. These suggest that the electron beam irradiation affects the oxidation of TiO<sub>2</sub> nanotube surface and in return, this modification of the Ti oxidation condition leads to the change in the adhesion characteristics of osteoblast cells on TiO<sub>2</sub> nanotube surface.

#### 4. Conclusion

TiO<sub>2</sub> nanotubes were synthesized via anodization method and the size of nanotube was controlled by the anodization voltage. Also, these nanotubes were electron beam irradiated with dose of 0, 50, 500, and 5000 kGy and the resulting

absorption characteristics of osteoblast cells on TiO<sub>2</sub> nanotube were investigated by cultivating MC3T3-E1 osteoblast cells. We have obtained the high adhesion rate of 155% after cultivating osteoblast cells for 2 hours on the TiO<sub>2</sub> nanotube, anodized under 20 V and irradiated with 5,000 kGy of electron beam. Our experimental XPS measurements suggest that the electron beam irradiation affects the oxidation of TiO<sub>2</sub> nanotube surface and in return, this modification of the Ti oxidation condition leads to the change in the adhesion characteristics of osteoblast cells on TiO<sub>2</sub> nanotube surface.

#### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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