Surface Engineering of Titania for Excellent Fibroblast 3T3 Cell-Metal Interaction

Roshasnorlyza Hazan[1*], Srimala Sreekantan[1], Adilah Abdul Khalil[2], Ira Maya Sophia Nordin[2] and Ishak Mat[2]

[1]School of Materials and Mineral Resources Engineering, Engineering Campus, 14300 Nibong Tebal, Pulau Pinang, Malaysia
[2]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Suite 121 & 141, Kompleks EUREKA, 11800 USM, Pulau Pinang, Malaysia

*Corresponding author: roshasnorlyza@gmail.com

Abstract: *The present study is focussed on clarifying the influence of different surface structures (nanotubes, thin film and foam) of titania (TiO_2) on the cell interactions of fibroblast (3T3) cells. The nanotubes were prepared by an anodisation process; thin film by a sol-gel method; and foam by the sacrificed polymeric sponge method. Their* in vitro *bioactivity was investigated by soaking the sample in complete growth medium (RPMI-1640/DMEM) with 3T3 cells. Field Emission Scanning Electron Microscope (FESEM) micrograph and optical density results showed that self-arrayed TiO_2 nanotubes strongly enhanced cellular activities, followed by the foam structure and the thin film. Atomic Force Microscope (AFM) results provided evidence that the enhanced cell interaction in nanotubes is associated with the roughness of the surface.*

Keywords: surface engineering, TiO_2 nanotubes, TiO_2 thin film, TiO_2 foam, cell-metal interaction

Abstrak: *Kajian ini difokuskan untuk menjelaskan kesan struktur permukaan berbeza (tiub-nano, filem nipis dan busa) bagi titania (TiO_2) ke atas tindak balas sel fibroblas (3T3). Tiub-nano disediakan melalui proses penganodan; filem nipis melalui kaedah sol-gel; manakala busa dengan kaedah pengorbanan busa polimer. Bioaktiviti* in vitro *dikaji dengan merendam sampel di dalam medium tumbesaran lengkap (RPMI-1640/DMEM) dengan sel 3T3. Keputusan mikrograf* Field Emission Scanning Elektron Microscope *(FESEM) dan ketumpatan optik menunjukkan tiub-nano tersusun sendiri telah meningkatkan aktiviti sel dan diikuti oleh struktur busa dan filem nipis. Keputusan* Atomic Force Microscope *(AFM) membuktikan bahawa interaksi sel pada tiub-nano disebabkan oleh kekasaran permukaannya.*

Kata kunci: kejuruteraan permukaan, tiub-nano TiO_2, filem nipis TiO_2, busa TiO_2, tindak balas sel-logam

1. INTRODUCTION

Titanium has been studied and used extensively as an implant material in the human body. However, there are unsolved technical problems associated with the surface of titanium as an implant material. The bio-inert character of the naturally forming surface oxide does not readily form a strong interface with surrounding tissue. To address this issue, current attempts at implant materials have been shifted from discovering new materials to developing or employing titanium with a passive interface that enhances osseointegration.

In the case of titanium implants, rough surfaces result in good osseointegration as compared to smooth surfaces. For instance, Lee and co-workers demonstrated that a porous structure produced by alkali heat treatment can improve and accelerate the healing response, thereby improving the potential for implant osseointegration.[1] Similar results were also obtained by Cachinho and Correia, whereby a porous titanium scaffold prepared by sponge reactive sintering method improved the *in vitro* bioactivity.[2] In addition, Carbone et al. [3] and several other researchers.[4,5] have reported cell interaction on sol-gel coated titanium surfaces. Cells showed good attachment, spreading and proliferation on such surfaces.

Lately, several works have been attempted on tube-like structures,[6] discovering that such structures enhance *in vitro* behaviour.[7,8] The adhesion, growth and differentiation of the cells were found to be critically dependent on the size of the tube[9] and surface roughness.[10] However, there is insufficient information regarding comparison of a specific cell on the different types of TiO_2 surface structure. Therefore, in this study, we report the interaction of the 3T3 cell on the three aforementioned types of modified TiO_2 surface: self-array TiO_2 nanotubes, TiO_2 thin film and TiO_2 foam.

2. EXPERIMENTAL

2.1 Formation of TiO_2 Nanotubes

Titanium (Ti) foil (0.27 mm thick, 99.6%, Strem Chemicals) was degreased by sonicating in ethanol (Technical Grade, 95%) for 5 min. The foil was then anodised in a 2-electrode bath with Pt electrode as the counter electrode. Prior to anodisation, the foil was cut into 1 x 3 cm^2 pieces and exposed to the electrolyte, which consisted of 100 ml glycerol (Merck, 87%) with 0.7 g ammonium fluoride, NH_4F (Merck, 98%). All anodisation experiments were performed at 20 V with a DC power supply (Hewlett–Packard 0–60 V/0–50 A, 1000 W) with a sweep rate of 1 V s^{-1} and holding for 30 s every 10 V. The Ti foil

was anodised for one hour. After anodisation, the foil pieces were rinsed with deionised water. The anodised samples were allowed to dry in air.

2.2 Formation of TiO_2 Thin Film

TiO_2 thin film was prepared based on work by Chrysicopoulou et al.[11] with slight modification. The process involves the dissolution of 10.5 ml tetrabutyl orthotitanate (TBOT, Merck, 98%) as the precursor in 111 ml ethanol as a solvent. Nitric acid (HNO_3), 1.5 ml, was added afterward into the transparent solution. Precipitation readily occurred when distilled water, 0.3 ml, was added to the complex. The mixture was sealed with Parafilm and magnetically stirred at room temperature for 2 h. Glass slides were used as the support substrates. Uniform amorphous gel coatings were formed on both sides of 1 mm thick glass microscope slides (Sail Brand) using a dip-coating process. The withdrawal speed of the substrate is 10 cm per minute. The deposited films were aged and dried at 100°C for 30 min in an electric oven and then carefully heat-treated at 500°C in air for one hour.

2.3 Formation of TiO_2 Foam

TiO_2 foam was prepared by the sacrificed polymeric sponge method from a slurry containing 40 wt. % TiO_2 powder in distilled water. The TiO_2 powder was purchased from Merck with 99% purity and had a mean particle size of 0.5 μm. Vigorous mixing was needed to ensure that the slip is homogenous. After vigorous stirring using a magnetic stirrer for one hour, 1 g of polyethylene glycol (PEG, Merck) *600* was added as a binder. The stirring was continued for another 10 min to ensure homogenisation of the suspension. The polymeric sponge was dipped into and infiltrated by the ceramic slurry. After withdrawal, the excess slurry was removed by gentle compression, followed by drying at room temperature for 18 h and in an electric oven at 110°C for another 24 h. Removal of the sponge and sintering of the green body was performed as follows: slow heating to 500°C with 1°C min^{-1}, 2 h holding time at 500°C and heating to 1300°C with 1°C min^{-1}, followed by cooling to room temperature at a rate of 3°C min^{-1}.

2.4 Sample Characterisation

The surfaces of prepared TiO_2 were observed under a FESEM and an X-ray diffraction using the Bruker D8 powder diffractometer operating in the reflection mode with Cu Kα radiation (40 KV, 30 mA) diffracted beam monochromator, using a step scan mode with the step size of 0.1° in the range of 25°–70°, to confirm the formation of TiO_2 nanotubes. The step time was of 3 s, adequate to obtain a good signal-to-noise ratio in the main reflections of the

titania nanotubes, (1 0 1) anatase (2θ = 25.3°) and (1 0 1) rutile (2θ = 36.1°). The roughness of samples was measured by an AFM SPA 300HV. The roughness for the TiO_2 foam could not be measure because the samples were too thick.

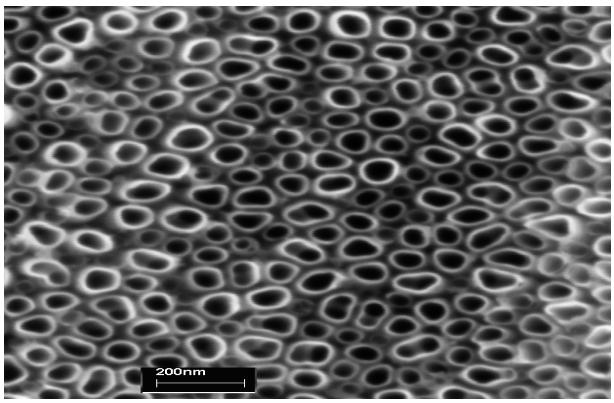
2.5 *In vitro* Testing

The ability of cell integration was evaluated by investigating the ability of 3T3 cells to attach to the TiO_2 surface by soaking in the complete growth medium (RPMI-1640/DMEM) with 3T3 cells. Prior to cell interaction, TiO_2 samples were cut into 5 x 5 mm^2 and autoclaved at 120°C for 20 min. The samples were then immersed in a multi-well plate, which contained 400 µl of complete growth medium (RPMI-1640/DMEM) with 3T3 cells. The cell concentration in each well was approximately 2 x 10^4 cells ml^{-1}. The samples were incubated for 3 days at 37°C in 5% CO_2 + 95% air. The cell interactions with TiO_2 surfaces were analysed by optical microscopy. After 3 days, the remaining solutions in the well were removed, and the TiO_2 was slowly rinsed. The surfaces of TiO_2 were characterised by an optical density test, and the morphology was observed via FESEM.

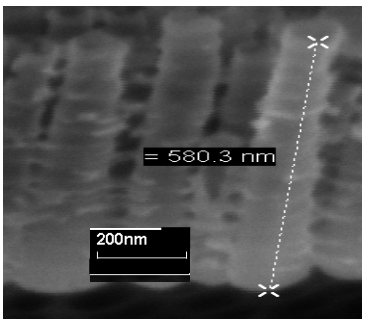
3. RESULTS AND DISCUSSION

3.1 Formation of TiO_2 Nanotubes

Anodising the Ti foil for one hour in glycerol has resulted in self-organised TiO_2 nanotubes. A representative FESEM image of the nanotubes is shown in Figure 1, with the insert displaying the length of the tube, which is ~1.1 µm. The diameter of the tubes was approximately 100 nm, with wall thickness of 15 nm. Figure 2 shows an XRD diffractogram of TiO_2 nanotubes. As anodised, the sample was amorphous with a small peak of anatase at 25°. The detected peak originated from the Ti substrate. The surface roughness of the sample produced in glycerol was characterised using AFM. Figure 3 shows the 3D morphology of TiO_2 nanotubes. The surface roughness measured by AFM is approximately 25.89 nm.



(a)



(b)

Figure 1: FESEM micrograph of TiO_2 nanotubes of (a) top view; and (b) the lengths.

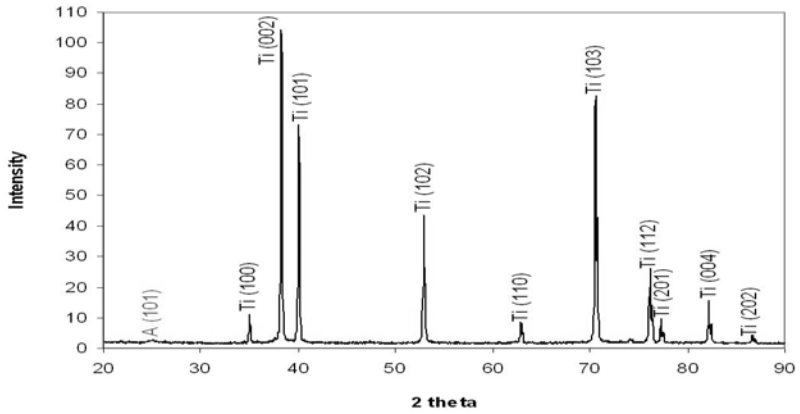


Figure 2: X-ray diffraction of as anodised TiO_2 nanotubes (Ti: Titanium; A: Anatase).

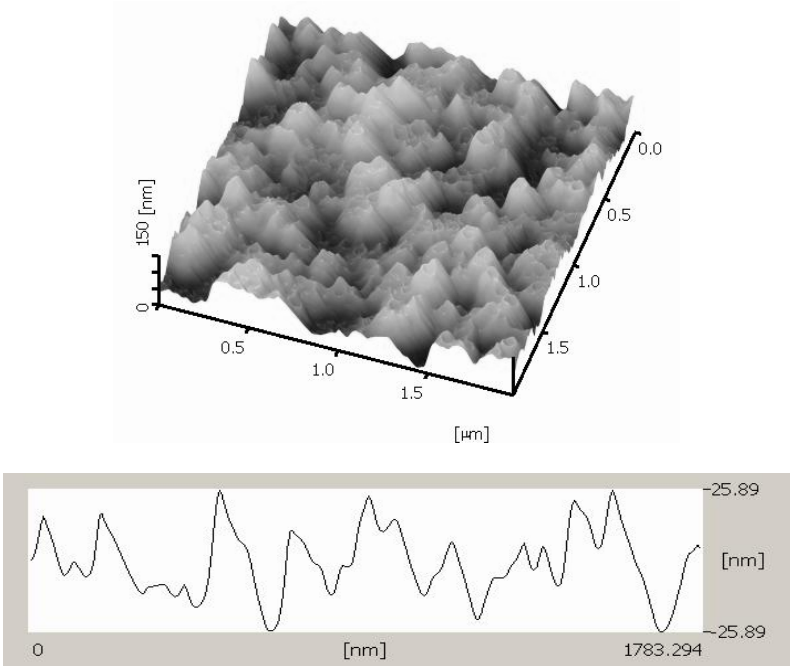


Figure 3: AFM 3D topography and roughness of TiO_2 nanotubes.

3.2 Formation of TiO_2 Thin Film

Figure 4 presents the FESEM micrograph of TiO_2 film prepared by the sol-gel method. Based on the FESEM micrograph, it was found that the surfaces of the thin film were rather smooth as compared to the TiO_2 nanotubes. This was further verified with AFM analysis. The topography of the obtained thin film is shown in Figure 5. The surface roughness was approximately 3.46 nm, with an average surface slope of 5.7°. Thus, this confirms the FESEM observation. Figure 6 shows the XRD spectrum of the TiO_2 heat-treated at 500^{o}C. The thin film cannot be annealed at temperature higher than 500^{o}C because the glass slide tends to bend, causing the film to peel off from the substrate. The spectrum shows the coexistence of both amorphous and crystalline phases. A broad hump in the low 2θ region demonstrates amorphicity originating from the glass substrate, while diffraction peaks were assigned to the anatase peak.

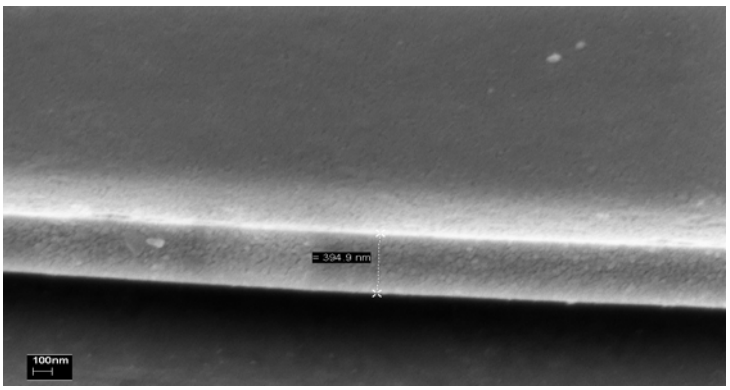


Figure 4: FESEM micrograph of TiO_2 thin film.

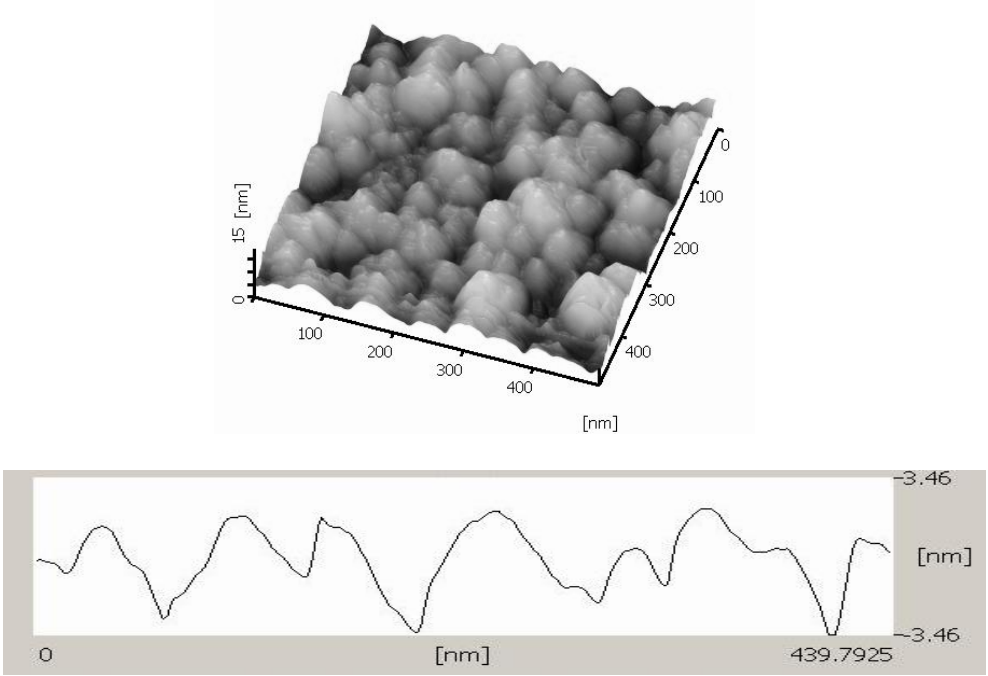


Figure 5: AFM surface topography and roughness for TiO_2 thin films.

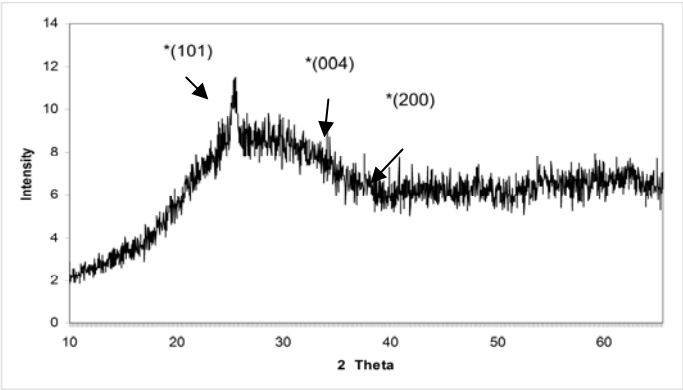


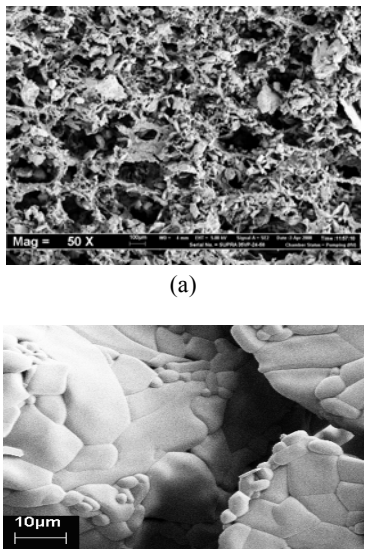
Figure 6: X-ray diffraction of the TiO_2 thin films annealed for one hour (A: anatase).

3.3 Formation of TiO_2 Foam

A representative FESEM micrograph of the foam produced by the sacrificed polymeric sponge method is shown in Figure 7. The sintered body presents large interconnected macropores. Macropores are sized in the range of 100–300 μm. Micropores can also be observed at pore walls, presumably resulting from volume shrinkage during the reactive sintering process of the TiO_2 powders. The structure of TiO_2 foams with rough surfaces is believed to play an important role in the process of bone formation because it is favourable for cell seeding, cell attachment, proliferation, differentiation and growth of tissue. Figure 8 shows an XRD result of the sintered body of the foam. The result reveals the existence of anatase, rutile and brookite phases. In this case, AFM was not performed due to the features of the foams.

3.4 Cell Interaction Test

Evaluation of bioactivity was conducted by immersing TiO_2 nanotubes, thin film and foam into a complete growth medium (RPMI/DMEM) containing 3T3 cells. The corresponding surface morphology of each TiO_2 sample after soaking in the medium for 3 days is shown in Figure 9. Cytoplasmic spreading was observed over the three different surfaces, indicating good adherence of 3T3 onto TiO_2. However, the adhesion and propagation of the 3T3 cell on TiO_2 nanotubes [Fig. 9(a)] and foam [Fig. 9(c)] was greater than on other surfaces. In the case of TiO_2 nanotubes, it was noticed that the filopodia of 3T3 cells actually propagate and grow into the vertical tubes [insert in Fig. 9(a)]. The rapid adherence and spread of the cells cultured on TiO_2 nanotubes could be caused by the larger surface area and the vertical topology, thus contributing to the locked-in cell configuration. Cells on TiO_2 foam show filamentous network structure



(a)

(b)

Figure 7: Morphology of TiO_2 foam surface.

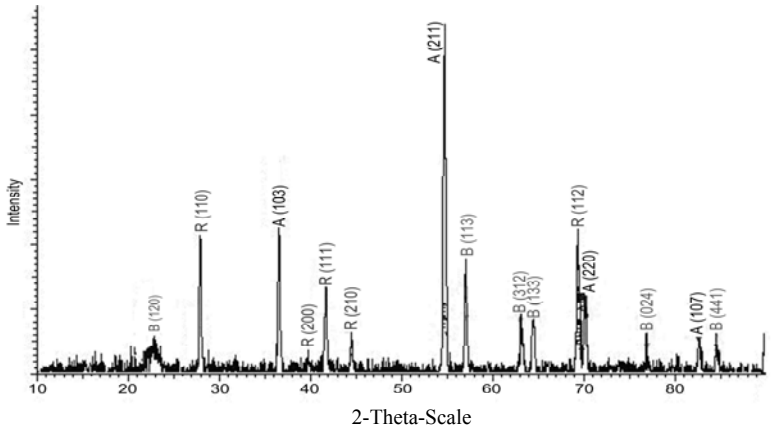
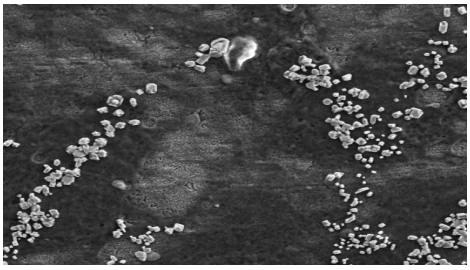


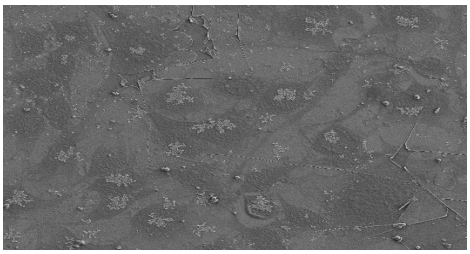
Figure 8: XRD result for TiO_2 foam (A: Anatase; R: Rutile; B: Brookite).

with cell-to-cell attachment, and the cells spread along the grain boundary and edges. The entire TiO_2 foam was covered by 3T3 cells, blocking the view of the grain boundaries [Fig. 9(c)], indicating excellent growth of 3T3 cells on this structure. The presence of cytoplasm can be clearly observed on this sample as well. In contrast, the TiO_2 thin films show insignificant growth of 3T3 cells. This is likely due to the reduced opportunity for the cells to integrate on the smooth surface.

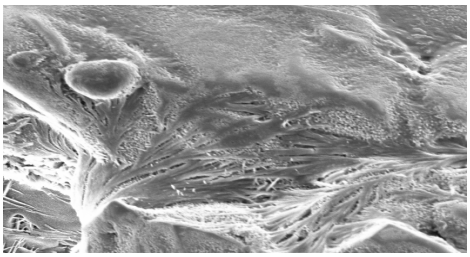
The excellent integration of 3T3 cells, as seen by FESEM observation, was verified with an optical density test. Figure 10 shows the results of optical density after 72 h of culturing 3T3 cells onto TiO_2 surfaces. Cell proliferation rates, as measured by counting cells after 72 h by a CellTiter 96® AQ_{ueous} Assay using a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS), were highest on the TiO_2 nanotubes, exceeding other TiO_2 samples. Cell density for different surfaces decreases in the order of TiO_2 nanotubes > TiO_2 foam > TiO_2 thin film.



(a)



(b)



(c)

Figure 9: Interaction between 3T3 cells with different TiO_2 surfaces; (a) nanotubes; (b) thin film; and (c) foam.

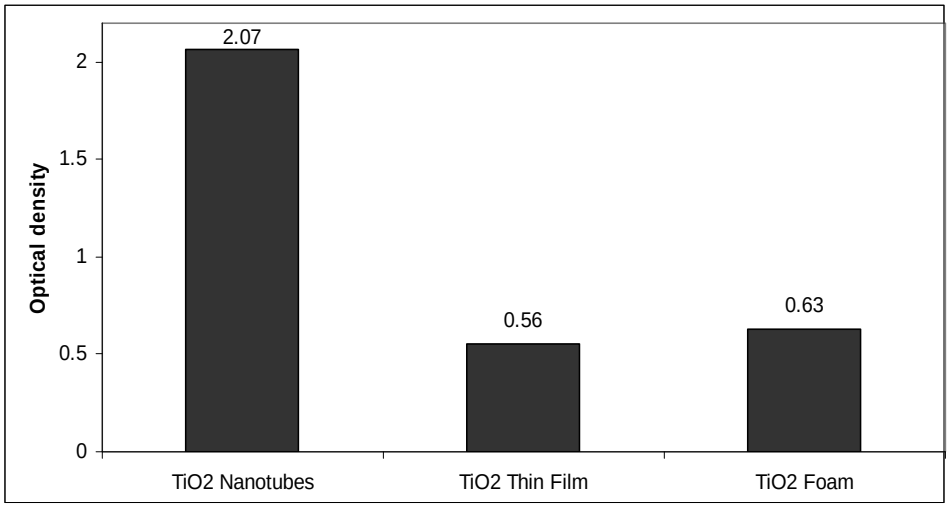


Figure 10: Proliferation of 3T3 cells after culture 72 h.

A possible reason for this is that the rough surfaces in tube-like structure and interconnected pores in foam lead to an increase in focal contact and thus exhibit enhanced osteoblast differentiation as compared to the thin film with a smooth surface. In summary, the results suggest that cell adhesion and cell proliferation were increased at a statistically significant level by modifying the Ti surface into a porous structure with rough surface morphology.

4. CONCLUSION

Different surfaces of TiO_2 vary in terms of adhesion, spreading, growth and differentiation of cells. The self-arrayed TiO_2 nanotubes provided accelerated interaction and strongly enhanced cellular activities compared to a smooth TiO_2 thin-film surface. It was clear that rough surface morphology is an important factor for better cell-metal interaction. Surface topography significantly influenced the cell migratory and attachment behaviours at implant surfaces. From the optical density test, the surface interactions of cells were in the order of TiO_2 nanotubes > TiO_2 foam > TiO_2 thin film. From this research, the best surface engineering for cell-metal interaction was a self-array of TiO_2 nanotubes.

5. ACKNOWLEDGEMENTS

The authors would like to thank Universiti Sains Malaysia for the sponsorship through a Short-Term Grant 2007: 6035227 and FRGS: 6070020.

6. REFERENCES

1. Lee, B., Lee, C., Kim, D., Choi, K., Lee, K.H. & Kim, Y.D. (2008). Effect of surface structure on biomechanical properties and osseointegration. *Materials Science and Engineering C*, 28(8), 1448–1461.
2. Cachinho, S.C.P. & Correia, R.N. (2008). Titanium scaffolds for osteointegration: Mechanical, *in vitro* and corrosion behaviour. *Journal of Material Science: Material Medical*, 19(8), 451–457.
3. Carbone, R., Marangi, I., Zanardi, A., Giorgetti, L., Chierici, E., Berlanda, G., Podestà, A., Fiorentini, F., Bongiorno, G., Piseri, P., Pelicci, P.G. & Milani, P. (2006). Biocompatibility of cluster-assembled nanostructured TiO_2 with primary and cancer cells. *Biomaterials*, 27(17), 3221–3229.
4. Harle, J., Kim, H., Mordan, N., Knowles, J.C. & Salih, V. (2006). Initial responses of human osteoblasts to sol–gel modified titanium with hydroxyapatite and titania composition. *Acta Biomaterilia*, 2(5), 547–556.
5. Kommireddya, D.S., Sriramb, S.M., Lvova, Y.M. & Mills, D.V. (2006). Stem cell attachment to layer-by-layer assembled TiO_2 nanoparticle thin films. *Biomaterials*, 27(24), 4296–4303.
6. Das, K., Vasmi Krishna, B., Bandyopadyay, A. & Bose, S. (2008). Surface modification of laser-processed porous titanium for load-bearing implants. *Scripta Materialia*, 59(8), 822–825.
7. Oh, S., Daraio, C., Chen, L., Pisanic, T.R., Fiñones, R.R. & Jin, S. (2006). Significantly accelerated osteoblast cell growth on aligned TiO_2 nanotubes. *Journal of Biomedical Materials Research Part A,* 78A(1) 97–103.
8. Popat, K.C., Leoni, L., Grimes, C.A. & Desai, T.A. (2007). Influence of engineered titania nanotubular surfaces on bone cells. *Biomaterials*, 28(21), 3188–3197.
9. Park, J., Bauer, S., Mark, K. & Schmuki, P. (2007). Nanosize and vitality: TiO_2 nanotube diameter directs cell fate. *Nano Letter*, 7(6), 1686–1691.

10. Kawahara, H., Soeda, Y., Niwa, K., Takahashi, M., Kawahara, D. & Araki, N. (2004). *In vitro* study on bone formation and surface topography from the standpoint of biomechanics. *Journal of Materials Science: Materials in Medicine*, 15(12), 1297–1307.
11. Chrysicopoulou, P., Davazoglou, D., Trapalis, C. & Kordas, G. (1998). Optical properties of very thin (<100 nm) sol-gel TiO_2 films. *Thin Solid Films*, 323(1–2), 188–193.