Surface modification of implant materials and its effect on attachment and proliferation of bone cells

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Osteoblast-like cell response in variation with the air plasma sprayed (APS) TiO_2 coating process parameters correlated with coating properties were investigated to evaluate the durability and biocompatibility of the surface-modified implant. The Taguchi technique was used to determine the coating properties affected by plasma spraying parameters on Ti–6Al–4V alloy substrate. The coating properties were characterized by porosity and surface roughness using an image analyzer and surf analyzer, respectively. The MG-63 osteoblast like cell morphology and proliferation data on TiO_2 coated substrate were measured by SEM observation and direct cell counting. It was demonstrated that surface roughness increased as spray distance decreased but gas flow rates and spray distance were major factors in the case of porosity. The osteoblast adhesion morphology and proliferation data indicated that osteoblast-like cell morphology was not influenced by process parameters, but cell proliferation was affected to some extent by surface roughness and porosity among TiO_2 coated specimens. Specifically, the difference between those of substrate and coating layer was relatively more visible.

1. Introduction

Plasma spraying fundamentally consists of the injection of powders into a direct current plasma jet, where they are melted and accelerated, and directing the stream of molten particles onto a substrate where they form a coating as they spread and solidify [1]. In order to study coating properties and apply to a new environment, the coating microstructure and formation mechanism must be understood by investigating the temperature, velocity and size distribution of the incident particles and interrelations. Recently, there has been much research concerning the process modification and much has focused on the control of the energy combination of in-flight particles and this energy combination of in-flight particles mainly depends on gas flow rate, spray distance and powder feed rate [2, 3]. The main purpose of applying hydroxyapatite (HA) coatings on Ti alloys has been to keep the mechanical properties of the metallic substrate and take advantage of the coating biocompatibility and chemical similarity with bone simultaneously. However, in evaluating the performance and stability of HA coating in a load bearing situation after long term follow-up, special consideration should be given to the HA coating/Ti alloy substrate [4] and research suggest that the presence of a potentially weak HA coating/Ti alloy interface results in the detached fragment and this will have an adverse effect on the implant or tissues surrounding it [4, 5]. HA particulate debris has been found in periprosthetic tissue after revision of HA-coated implants. Macrophages with ingested particles of HA have also been found at areas of bone resorption around the prosthesis [6]. Moreover, the amorphous HA phase is unavoidable during the preparation of HA coatings by plasma spraying deposition, which is generally accepted to be more soluble *in vitro* and more degradable *in vivo* than the crystalline HA phase [7, 8]. If the coating layer is dissolved too fast before the growth of bones or the stabilization of the implants, the coating is simply meaningless. Considering all of these defects in HA coatings, plasma sprayed TiO_2 coatings were used. The success or failure of an implant has been shown to be related to the morphology of the surface, including microgeometry and roughness [9–11]. Thomas and Cook [12] examined variables affecting osseous contact by implants and concluded that surface texture was a very significant parameter in implant fixation. In several *in vivo* studies, rough surfaces were found to produce better bone fixation than smooth surfaces [10, 12, 13]. Contrary to this, too rough a surface has also been observed to promote macrophage attraction rather than wound healing. *In vitro* studies have also been demonstrated that surface roughness affects cell response [11, 14] and this appears to be the case for osteoblasts as well, since osteoblast-like cells exhibit greater initial attachment to

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rough Ti surfaces [15, 16]. However, little is known about the effect of surface property of plasma sprayed TiO_2 coating on osteoblast attachment and proliferation. Therefore, the overall aim of this work was to characterize the surface properties of plasma sprayed TiO_2 coating layer on Ti–6Al–4V alloy and to investigate the morphology and proliferation of MG-63 osteoblast-like cells in variation with the air plasma sprayed (APS) TiO_2 coating process parameters.

2. Materials and methods

2.1. Specimen fabrication and tests of coating properties

Specimens (70 mm × 50 mm × 2 mm) used as substrates were cut from Ti–6Al–4V ELI plate. The feedstock material was 99% TiO_2 powder prepared by fusing and crushing methods (Metco 102 powder). The particle shape was angular/blocky and particle size range was $-88 + 7.8\,\mu m$. The substrate was ultrasonically cleaned with acetone and alcohol, Al_2O_3 grit-blasted and then placed into an APS system (Metco 9M system) for TiO_2 coating. The arc current was fixed at the value of 500 Å. This study used the basics of the Taguchi technique to determine and partially quantify which deposition variables had the greatest influence on the properties of APS TiO_2 coating. The Taguchi technique is a statistical method which is used to efficiently determine the influence of various variables on a given process [29]. Increased efficiency was achieved by the use of orthogonal arrays that allows engineers to study a small fraction of the possible combination of factors. Four variables with three levels which are considered to be an important factor in APS coating system were chosen, therefore an $L_9(3^4)$ matrix was determined to be the most suitable. An $L_9(3^4)$ matrix can study four variables and three levels with only nine experimental runs. Table I outlines the Taguchi matrix where the columns represent the plasma spray variables with appropriate three levels chosen for each variable and the rows represent the nine experimental spray runs. Analysis of the interaction affects has been excluded from this study. All specimens were sectioned with a diamond saw and then the specimen was epoxy mounted and fine-polished to minimize the smearing and pull-out of particles. Optical microscopy (Leica Leitz DMR, Germany) was used to characterize the microstructure of the coating layer and coating/substrate interface. Porosity and pore size were measured with an image analyzer (Image pro v4.0, Switzerland). The roughness of grit-blasted substrate as well as the various plasma sprayed surfaces was measured using a surface roughness tester (Mahr surfanalyzer 5000, Germany). The measured length was 10 mm at a probe speed of 0.25 mm/s.

2.2. Cell culture

2.2.1. Cell morphology

MG-63 osteoblast-like cells were used for this experiment. The cell culture was performed in 6-well cell culture plates (Corning, USA). Per each TiO_2 coating specimens (15 mm × 15 mm × 2 mm) 1.0×10^4 cells per ml of medium were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). This low cell-seeding concentration was chosen so that single cell could be observed at the early time point, as could the proliferation of osteoblasts during the experiment. Because it was impossible to determine the extent of confluence of cells grown on the TiO_2 plasma sprayed on Ti–6Al–4V substrate, confluence was determined by cell growth on the culture plate plastic surface. Cultures were maintained in a 5% CO_2 atmosphere at 37 °C and 100% humidity. After three days, the cells were washed with 37 °C prewarmed PBS and fixed with 2.5% glutaraldehyde, 0.1 M phosphate buffer, 1% saccharose, pH 7.3. After fixation, the specimens were rinsed with PBS, sequentially incubated for 10 min in 70, 90 and 100% ethanol, dehydrated through a graded propanol series, and critical point dried from CO_2 (CPD030, Bal-Tec, Germany). A thin layer of Au was sputter-coated onto the specimens prior to examination in a scanning electron microscope (JEOL 6400, Japan). Thirteen-millimeter-diameter Thermanox coverslips (polyethylene terephthalate; Germany) were punched under sterile conditions and used as a control substrate.

TABLE I Taguchi matrix variables and constants

No. of experiment	H_2 gas flow rate (A)	Spray distance (B)	Powder feed rate (C)	Ar gas flow rate (D)
1 (TT1)	5(1)	65(1)	20(1)	75(1)
2 (TT2)	5(1)	80(2)	30(2)	100(2)
3 (TT3)	5(1)	95(3)	40(3)	125(3)
4 (TT4)	10(2)	65(1)	30(2)	125(3)
5 (TT5)	10(2)	80(2)	40(3)	75(1)
6 (TT6)	10(2)	95(3)	20(1)	100(2)
7 (TT7)	15(3)	65(1)	40(3)	100(2)
8 (TT8)	15(3)	80(2)	20(1)	125(3)
9 (TT9)	15(3)	95(3)	30(2)	75(1)

Constants	Variables	Levels		
		1	2	3
Gun: Metco 3MB	Primary gas (Ar) flow rate (SCFH)	75	100	125
Nozzle: Metco 531	Secondary gas (H_2) flow rate (SCFH)	5	10	15
Primary gas pressure: 100 psi	Spray distance (mm)	65	80	95
Secondary gas pressure: 50 psi	Powder feed rate (g/min)	20	30	40

2.2.2. Measurement of cell proliferation

The MG-63 osteoblast-like cells were seeded at 3.0×10^4 cells per specimen in a 12-well plate in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). At harvest, cells were released from the culture surface by the addition of 0.25% trypsin in Hank's balanced salt solution (HBSS) containing 1 mM ethylenediamine tetraacetic acid (EDTA) for 10 min at 37 °C. The reaction was determined by the addition of DMEM containing 10% FBS. A second trypsinization was performed to ensure that any remaining cells had been removed form the surface. SEM examination of disks used in this study showed that following two trypsinizations, all cells were detached from the specimen surfaces. Cell suspensions from both trypsinizations were combined and centrifuged at 1200 rpm for 5 min. The supernatant was decanted and the cell pellet was washed with PBS and resuspended in physiological saline. Cell number was determined by direct counting under phase contrast microscope and all countings were run in triplicate.

3. Results and discussion

3.1. Relationship between coating properties and process parameters

The mean values of surface roughness and porosity of the coating from Taguchi parameters are shown in Table II and ANOVA (Analysis of Variance) on the mean values of the properties were performed in order to evaluate the effects of the process parameters more quantitatively, these are shown schematically in Fig. 1. Spray distance and H_2 gas flow rate were the important parameters that affected the surface roughness, certainly the effect of the spray distance was more notable than that of the other spray parameters. As the spray distance decreased, the surface roughness value (R_a) increased. This may be due to the increase in the degree of the unmelted particles, irregular impacting particles and accumulation layers by transferring insufficient thermal energy within a very short time interval. The surface roughness of the grit blasted substrate was relatively uniform, so the variance of surface roughness only by process parameters is reliable in this study. From the biological point of view, an enlarged surface area by increasing surface roughness would have given the beneficial effect on the implant materials between bone and biomaterial interface.

H_2 gas flow rate, Ar gas flow rate and spray distance were major factors that affected the porosity. But the contribution degree difference of process parameters was

TABLE II Coating properties from Taguchi parameters

No.	Surface roughness (R_a)	Porosity (%)
1	6.24 ± 0.46	3.41 ± 0.384
2	5.00 ± 0.10	4.99 ± 0.256
3	4.23 ± 0.06	5.06 ± 0.083
4	6.46 ± 0.55	4.26 ± 0.257
5	6.16 ± 0.56	5.15 ± 0.017
6	4.90 ± 0.37	5.15 ± 0.497
7	8.08 ± 0.85	2.09 ± 0.160
8	5.82 ± 0.36	5.12 ± 0.611
9	5.26 ± 0.40	1.34 ± 0.053

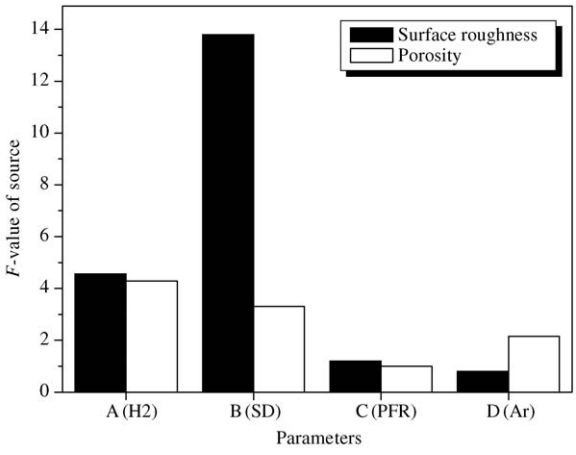

Figure 1 F value of process parameters on the plasma spray coating properties.

relatively smaller than that of the others. This means that the mechanism of formation of pore is relatively complicated and the pore morphology must be also considered. Within the scope of this study, only numerical values of porosity fraction are presented. However, the porosity has a closer correlation with the H_2 (thermal energy term) and spray distance (thermal energy transfer term) than the Ar (kinetic energy term), which has been confirmed by other researchers [17]. Also when the pore morphology is considered, it has been found that small-sized pores mainly resulted from the entrapped gas during plasma spraying while large-sized pores might have resulted from the presence of unmelted particles or the splashing of impacting particles. Therefore, process parameters such as plasma gas composition and spray distance must be considered mainly to control the porosity within the coating layer.

3.2. Cellular morphology

For each specimen, several individual cells were examined by SEM. Because each osteoblast-like cell does not spread at the same rate, the micrographs shown here represent the most typical cell morphology at that time interval. On the Thermanox surface as control, the MG-63 osteoblast-like cells had spread extensively and totally flattened on the polyethylene terephthalate surface. They were of polygonal shape with filopodial extensions indicative of very thin extensions of cell spreading. The cells demonstrated the most significant spreading of all the specimens in this study. They did not have regular orientation and looked scattered in all directions. Some of them appeared thicker in the central area of the nucleus and flattened in the peripheral regions. The nucleus of cells on the Thermanox was visible more obviously than that of cells on Ti–6Al–4V substrate and TiO_2 coated substrate. Sometimes the cells exhibited such close contact with each other that detection of the complete cell perimeter was difficult.

On Ti–6Al–4V alloy substrate surface, the MG-63 osteoblast-like cells had spread polygonally. The central area of the nucleus appeared thicker and the peripheral regions less flattened than that of the Thermanox as can be shown in Fig. 2(a). The cell boundaries of adjoining cells fused with each other frequently. Unlike the

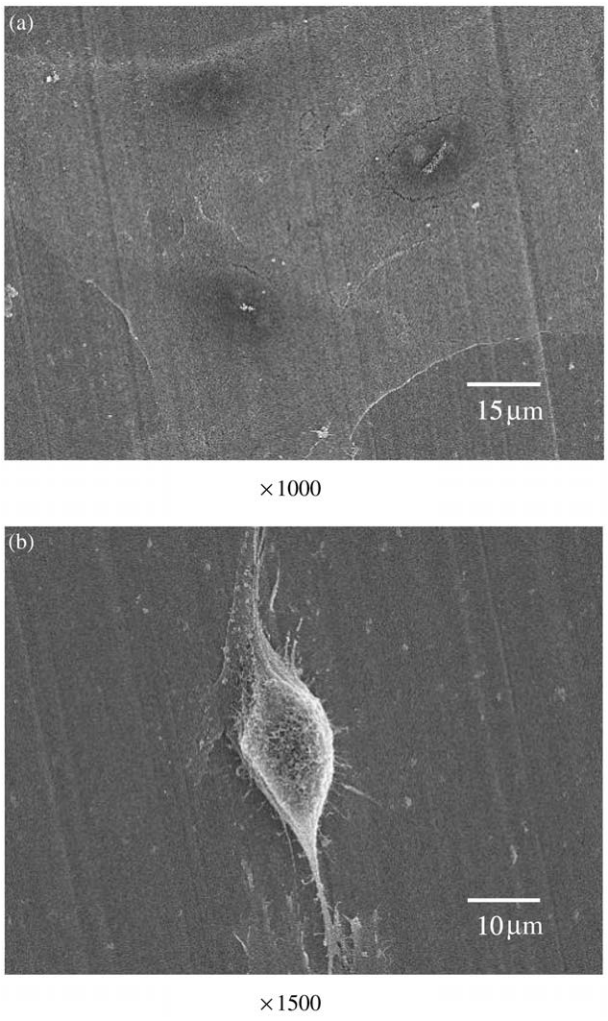

Figure 2 MG-63 osteoblast-like cells on the Ti–6Al–4V alloy substrate after three days.

Thermanox, the cells had a more elongated appearance than polygonal. This may be due to the regular scratch formed by 1200 grit silicon carbide grinding. However, the cells spherical in shape were detected occasionally as shown in Fig. 2(b). This means that the early signs of filopodial extensions extending to adjacent areas of the prepared surfaces and that may be due to the leaching of cell toxic ions such as aluminum or vanadium, which can be generated in this culture system although possible adverse substances are removed by careful experiment.

It is known that topographical features in the micrometer range can affect activation, adhesion, orientation, morphology and movement of cells and the expression of genes [18, 19]. As can be seen in Fig. 3(a), the MG-63 osteoblast-like cells were found to be spread out on the surface of the TiO_2 plasma sprayed specimens and especially more spread around the pore that was formed during the air plasma spray coating process or in the groove. These pores could be divided into two groups with variation of pore morphology and size; small-sized pores chiefly due to the entrapped gas during plasma spraying while large-sized pores due to the presence of unmelted particles or the splashing of impacting particles. The cells appeared to be thicker than on the smooth control Thermanox and Ti–6Al–4V substrate. Moreover, as can be shown in Fig. 3(b), the overlapping of spreading cells was detected more frequently around

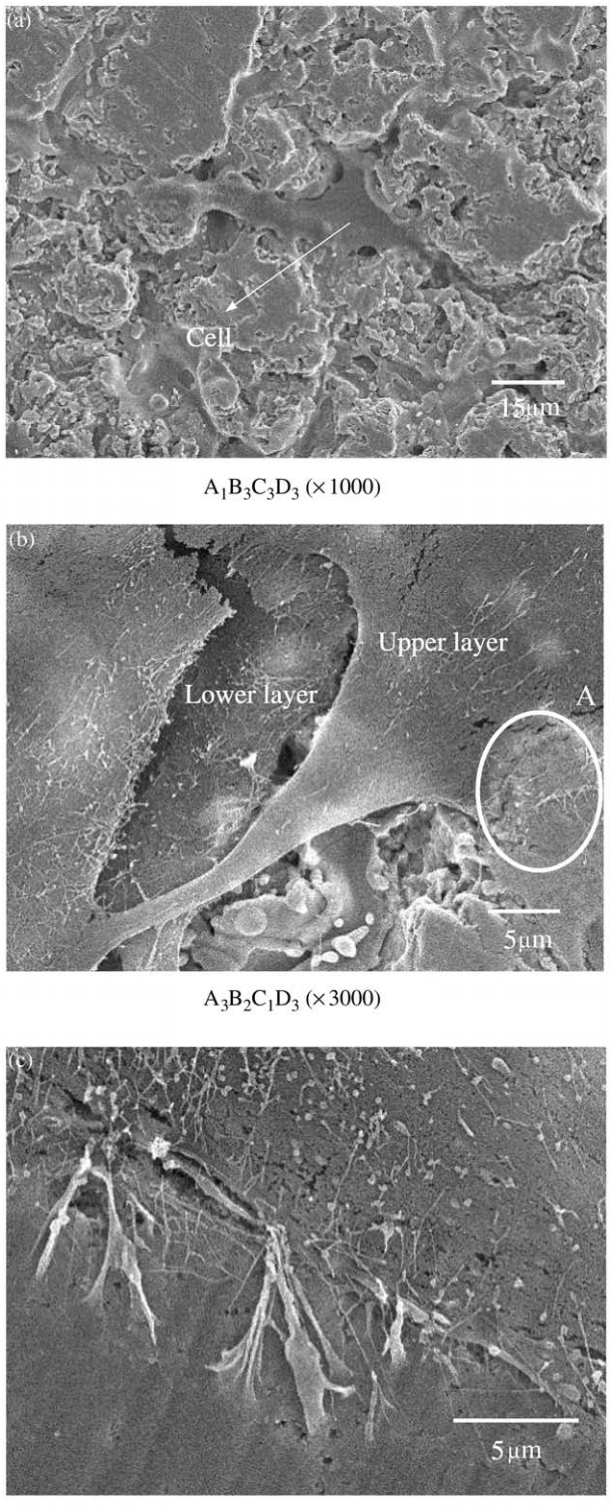

Figure 3 MG-63 osteoblast-like cells on the TiO_2 plasma sprayed specimen after three days: (a) $A_1B_3C_3D_3$ (× 1000); (b) $A_3B_2C_1D_3$ (× 3000); (c) Magnification of region A (× 5000).

the pore and groove, which is formed by the degree of unmelted particles, irregular impacting particles and accumulation layers owing to transferring insufficient thermal energy within some time interval. Notably, the cells attached and spanned from the lowest level of pore or groove. By the plasma spray coating process, a three-dimensional (3-D) network of pores or grooves form and will supply a larger contact area to the cell. Therefore, a crooked and peak and valley area of specimen surface offers an advantage for cells to attach and stretch out.

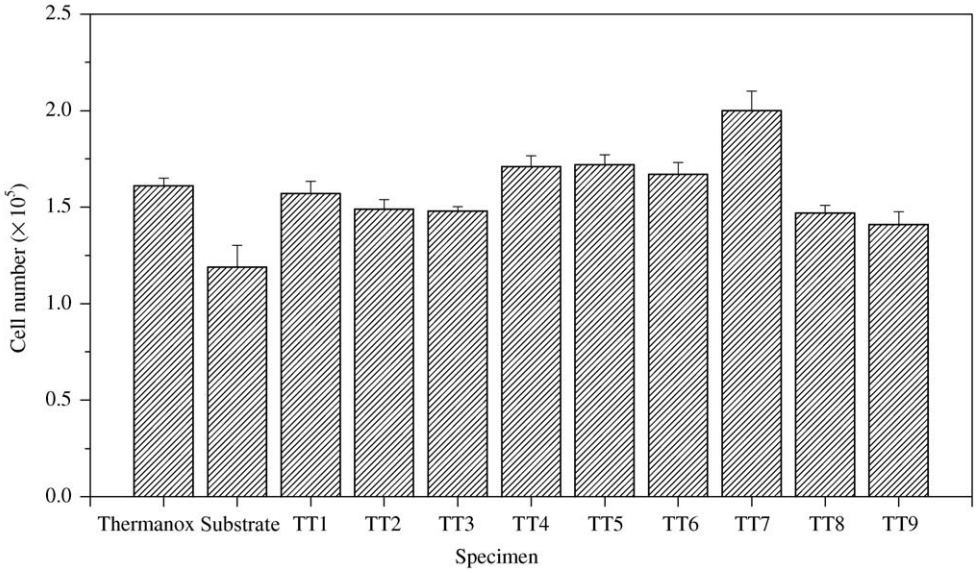

Figure 4 Effects of the coating properties of TiO_2 plasma sprayed specimen and culture substrate on MG-63 osteoblast-like cell proliferation.

The attaching ends of the cells were well spread and formed many filopodia, extending from the base of the cell mass as shown in Fig. 3(c). Sometimes the osteoblasts also possessed many thin, capillary-like extensions.

Despite the differences concerning coating properties in variation with process parameters, the morphologies of MG-63 osteoblast-like cells were similar in this experiment. With few exceptions, the comparison of osteoblast-like cells on the specimens that have different process parameters at the same time revealed only slight differences in cell morphology. The cells on the surface of the TiO_2 plasma sprayed specimen were 3-D oriented and almost filled with pores, resulting in a 3-D network. It is well known that successful bone formation after implantation requires attachment of osteoblast progenitor cells, differentiation into secretary osteoblasts, matrix synthesis and calcification of extracellular matrix [15, 20]. *In vivo* mature osteoblasts exist within a 3-D matrix microstructure. Therefore, it can be proposed that roughened surfaces that provide the possibility to form a 3-D cellular network promote the proliferation of osteoblasts.

3.3. Cell proliferation

The effects of the process parameters of Ti alloy substrate on MG-63 osteoblast-like cell proliferation are illustrated in Fig. 4. The data are mean values of three parallel samples. The results of the cell proliferation data indicated that the number of cells detached from TiO_2 coated specimens was higher than that of smooth surface Ti alloy substrate. Specifically, the cell number of TT4 ~ TT7 specimens was larger than that of Thermanox as control substrate. As indicated in Table II, the surface roughness or porosity of TT4 ~ TT7 specimens is larger than that of the other specimens and this contributes to the more activated proliferation of MG-63 osteoblast-like cell. According to the cell proliferation and morphology data, it was demonstrated that as the surface roughness or porosity increases, the proliferation of osteoblast-like cell also increases. In other words, the biocompatibility of the implant materials mainly depends on the surface topography such as micropores, crevices and pits. However, if the surface roughness value is too high above the critical value, it has been suggested that the wear particle-associated macrophage response happened and this provokes the osteolysis associated with aseptic loosening by releasing factors which activate osteoclastic bone resorption [21, 22] and by providing a population of mononuclear phagocyte precursors from which these multinucleated bone resorbing cells are formed [23–25]. Although, Bauer *et al.* [26] reported that there is little evidence to show that coating particles on their own can cause osteolysis, *in vitro* studies have shown that the debris associated macrophages release mediators that are known to play a role in osteoclast formation and bone resorption [27, 28]. Consequently, surface topography may be a key factor in determining the morphological and functional responses observed during the osteoblast–substrate interactions. However, complete interpretation of these results requires further investigation of detailed morphological responses and functional responses of osteoblasts to biomaterials such as MTT and ALP.

4. Conclusions

Osteoblast-like cell response in variation with the APS TiO_2 coating process parameters correlated with coating properties were investigated to evaluate the durability and biocompatibility of the surface-modified implant. As the spray distance decreased, the surface roughness value (R_a) increased and this may be due to the increase on the degree of the unmelted particles, irregular impacting particles and accumulation layers by transferring insufficient thermal energy within very short time interval. The H_2 gas flow rate, Ar gas flow rate and spray distance were major factors that affected the porosity. But, the differences of contribution degree of

process parameters were smaller than that of the others. From the biological point of view, it could be suggested that roughened surfaces provide the possibility to form a 3-D cellular network promoting the proliferation of osteoblasts. The cell number detached from TiO_2 coated specimens was higher than that of smooth surface Ti alloy substrate. In particular, the cell number of TT4 $\sim$ TT7 specimens was larger than that of Thermanox as control substrate and this contributed to the more activated proliferation of MG-63 osteoblast-like cells. Putting these results together, surface topography may be a key factor in determining the morphological and functional responses observed during the osteoblast–substrate interactions.

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