Enhancement of cell differentiation on a surface potential-controlled nitrogen-doped TiO2 surface

Masami HASHIMOTO1,2, Satoshi KITAOKA1, Maiko FURUYA2, Hiroyasu KANETAKA2, Kazuhiro HOSHIKAYA3, Hayato YAMASHITA3 and Masayuki ABE3

1Japan Fine Ceramics Center, 2–4–1 Mutsuno, Atsuta-ku, Nagoya 456–8587, Japan
2Graduate School of Dentistry, Tohoku University, 4–1 Seiryo-machi, Aoba-ku, Sendai 980–8575, Japan
3Graduate School of Engineering Science, Osaka University, 1–3 Machikaneyama, Toyonaka, Osaka 560–8531, Japan

MC3T3-E1 cell differentiation is more pronounced after 14 days incubation on charged nitrogen-doped TiO2 surfaces compared to on an untreated, neutral Ti surface. The protein fibronectin (Fn) was detected by an immunogold-labeling technique and Ca and P were detected by time-of-flight secondary ion mass spectrometry. Both techniques revealed that an adhesive protein such as Fn adsorbs equally on negatively-charged, positively-charged, and untreated Ti surfaces in culture medium. However, the adsorption of Ca and P was only detected on charged nitrogen-doped TiO2 surfaces. The enhanced adsorption of inorganic ions and Fn is probably responsible for promoting initial stage of osteoblast differentiation. The conformation of adsorbed Fn was observed by high-speed atomic force microscopy and found to be in the side-on orientation on the positively-charged surface. This finding may help elucidate the relation between Fn conformation and cell activity on surface potential-controlled nitrogen-doped TiO2 surfaces in future.

Key-words : TiO2 surface, Surface potential, Cellular response, Fibronectin, Immunogold-labeling technique, High-speed atomic force microscopy

©2019 The Ceramic Society of Japan. All rights reserved.

1. Introduction

Sequential cellular events such as adhesion, migration, proliferation and differentiation on a material surface occurring in vivo and in vitro result in the formation of tissue. Surfaces conducive to cell-adhesion are suitable for tissue-contacting devices and may serve as cell-adhesive matrices for tissue-engineered devices. Protein adsorption on a surface is a likely initial event when implanted surfaces contact fluids in living organisms and is affected by the surface functional groups, surface potential, and the distribution or density of proteins.1–5 Several adhesive plasma proteins, such as fibronectin (Fn) and vitronectin, facilitate cell adhesion in a biologically specific manner through ligand-receptor interactions.6 Positively charged proteins and peptides such as lysozyme and poly-(L-lysine) strongly adsorb on negatively-charged surfaces and promote cell adhesion via electrostatic interactions, and thus controlling the adsorption of these proteins is essential for the design of cell-adhesive matrices.

Bioactive materials in the body bond to living bone through a hydroxyapatite (HAp) layer. The generation of HAp is initiated by calcium or phosphate ion adsorption and complexation with negatively or positively charged surfaces, respectively. Materials that bond to bone typically form a HAp layer in simulated body fluid (SBF) with an ion concentrations similar to that of human blood plasma.7,8 Characterization of the mechanism underlying HAp formation requires understanding the electrostatic interaction between Ca2+ and PO43− ions. TiO2 surfaces are promising candidates because electrostatic interactions are governed by the surface potential of the TiO2 surface.

We previously determined that the surface potential of a rutile-type TiO2 surface can be controlled by introducing nitrogen-based defects.9–14 Cross sectional scanning transmission electron microscope images and N-K edge energy-loss near edge structure spectra of TiO2 surfaces with negative or positive zeta potentials indicate that nitrogen atoms incorporated into TiO2 crystals produce charged surface defects [(NO)x]−1 and [(N2)x]0,2] following 1 h heating at 873 and 973 K, respectively. The charged states induced by the nitrogen-based defects in TiO2 qualitatively coincide with the macroscopic polarity of the surface potential. Furthermore, HAp was efficiently generated on TiO2 surfaces with large negative or positive zeta potentials.

Biomaterials implanted in the body are immediately coated with proteins from blood and tissue fluids and these proteins subsequently adsorb onto the biomaterial in
layers. Importantly, all subsequent cellular responses at the implant surface are dependent on the shape of the implant, and its ability to adsorb protein and inorganic ions shortly after implantation. However, the detailed mechanism underlying the cellular response remains unclear. Our aim is to understand the osteoblast response occurring on surface-potential controlled nitrogen-doped TiO₂ (N-TiO₂) surfaces. To this end, we have focused on understanding the adsorption of the adhesive protein Fn by studying its conformation and interaction with inorganic ions such as calcium and phosphate. It was previously reported that Fn has two conformations (an end-on orientation and a side-on orientation)\(^{15,16}\) and that the conformation is influenced by the chemical and physical properties, water wettability, surface roughness and electrostatic force of the substrate.\(^ {17-22}\)

Here, we investigated the correlation between surface potential and MC3T3-E1 cell response to the surface. In addition, we discuss differences in cell responses on the basis of adsorbed species such as Fn, Ca and P ions on surface potential-controlled TiO₂ and untreated Ti surfaces. Finally, the conformation of Fn on surface potential-controlled surface was tried to observe by high-speed atomic force microscopy (AFM).

2. Experimental procedures

2.1 Preparation and characterization of N–TiO₂ surfaces

A sample of commercial, pure Ti (Ti > 99.9%, Nilaco Co., Tokyo, Japan) was placed in a furnace containing N₂ gas at atmospheric pressure with an oxygen partial pressure (P\(O_2\)) of \(10^{-14}\) Pa precision-controlled using a solid-state electrochemical oxygen pump. The gas flow rate was \(3 \times 10^{-4}\) m³/min. The N–TiO₂ surface was prepared by heating Ti from room temperature to 873 or 973 K at a rate of 5 K/min, holding at this temperature for 1 h, then cooling the Ti to room temperature at a rate of 5 K/min. Our previous study confirmed that the zeta potential of the N–TiO₂ surface formed at 873 and 973 K after 1 h was highly negative (−27 mV) and positive (+20 mV), respectively.\(^ {13,14}\) The zeta potential of Ti cannot be measured due to its conductivity and thus the surface potential of untreated Ti measured by kelvin probe force microscopy was zero.\(^ {14}\)

The surfaces of negatively- and positively-charged N–TiO₂ and untreated Ti surfaces were observed using a scanning electron microscope (SEM) (SU-8000, Hitachi, Co. Ltd., Tokyo, Japan) and their surface roughness and contact angle were measured using a laser microscope (OPTELCIS HYBRID mc200, Lasertec, Yokohama, Japan) and contact angle meter (DM-701, Kyowa Interface Science Co., Ltd., Saitama, Japan), respectively.

2.2 Cell culture and differentiation

Cell responses such as proliferation and differentiation on the N–TiO₂ and untreated Ti surfaces were assessed using osteoblast-like MC3T3-E1 cells. The MC3T3-E1 cell line was grown at 310 K in an atmosphere of 5% CO₂/95% air in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA, USA), and penicillin and streptomycin (PS) (Meiji-Seika Kaisha Ltd., Tokyo, Japan). Cells suspended in DMEM with 20% FBS and PS were seeded at a density of \(4 \times 10^5\) cells/well in a 24 well culture plate and incubated for 3 or 7 days at 310 K with 5% CO₂. The viable cell number was evaluated by DNA quantification using an Allprep DNA/RNA mini kit (Qiagen Inc., Valencia, CA, USA). The cells were differentiated by replacing the medium with DMEM containing 20% FBS (serum-containing medium) and osteoblast-inducer reagent (Takara Bio Inc., Otsu, Japan) containing L-ascorbic acid, hydrocortisone, and \(\beta\)-glycerophosphate. After 14 or 28 days differentiation, the cells were rinsed three times with phosphate buffered saline (PBS) (Wako Pure Chemical Industries Ltd., Osaka, Japan), 200 µl of alkaline phosphate yellow liquid substrate and 2 µl of 10% Triton-X/ PBS was added to each well, then the plate incubated at 310 K for 15 min. After a yellow color developed, the reaction was terminated with 75 µl of 2 M NaOH and the absorbance at 450 nm was read using a microplate spectrometer (GloMax-Multi Detection System, Promega Corp., Tokyo, Japan).

2.3 Quantitative analysis of adsorbed Fn

We examined differences in Fn adsorbed on N–TiO₂ and untreated Ti surfaces after incubation in serum-containing medium or Fn-containing Tris-HCl solution by treating with primary antibodies, followed by immunogold-labeling and electron probe X-ray microanalysis. The adsorption of gold atoms (Au) was governed by Fn adsorption on the sample. The serum-containing medium contained inorganic ions and protein (Ca: 2.0 mM, P: 1.0 mM, Fn: 0.03–0.3 mg/ml)\(^ {23}\) whereas the Fn-containing Tris-HCl solution contained only protein (Fn: 0.05 mg/ml, similar to that of serum-containing medium). These solutions did not contain cells. First, N–TiO₂ and untreated Ti surfaces were incubated in the serum-containing medium or Fn-containing Tris-HCl solution (Funakoshi Co., Ltd., Tokyo, Japan) for 1 min at 310 K, then incubated with primary antibody (anti-Fn) (Funakoshi Co., Ltd., Tokyo, Japan) for 4 h at 310 K, washed with PBS, and incubated in aqueous glutaraldehyde (MP Biomedical LLC, Illkirch, France) solution (2.5 wt %) overnight at 277 K. After rinsing with PBS and incubating in PBS containing glycin (Toronto Research Chemicals Inc., North York, Ontario, Canada) (50 mM) for 20 min at room temperature, the samples were washed with PBS and incubated with gold-conjugated anti-rabbit IgG (Funakoshi Co., Ltd., Tokyo, Japan) (1:120 dilution) for 4 h at 310 K. The samples were washed again with PBS and distilled water and dried at room temperature. The relative concentration of Au against all detected elements was determined using an energy dispersive X-ray spectrometry system attached to a SEM. The surface Fn concentration was calculated using the following equation:
\[
[Fn] (\mu g/\mu m^2) = \frac{[\text{counts (at %)}]([Fn]_{sol} (\mu g/mL))}{[A \text{ sol} (at \%)/mL][SA (\mu m^2)]}
\]

where (at %) is the counts measured for Au on the sample, [Fn]sol is the Fn concentration in solution, A sol is the specific activity of the Fn solution, and SA is the surface area containing the Au atoms.

2.4 High-speed atomic force microscopic observation of Fn

The surface potential-controlled N–TiO2 sample was inappropriate for high-speed AFM due to its surface topography and thus we prepared a positively-charged surface by coating aminosilane on mica. The mica is often used as a substrate for AFM observation because it is flat at the nanoscale. An Fn sample solution (10 μg/mL) was placed on this positively-charged surface and incubated. After rinsing the surface with PBS, we investigated the surface morphology of Fn in PBS using a home-built high-speed AFM\(^{24}\) operating at room temperature and a scan speed of 1.5 s/frame. This high-speed AFM can detect surface-adsorbed Fn shortly after soaking the surface in Fn-containing PBS.

2.5 Quantitative analysis of calcium (Ca) and phosphorus (P) after immersion in culture medium

N–TiO\(_2\) and untreated Ti samples were incubated in serum-containing medium or SBF for 1 min at 310 K (pH 7.4). The serum-containing medium contained both inorganic ions (Ca: 2.0 mM, P: 1.0 mM) and protein (Fn: 0.03–0.3 mg/mL) whereas the SBF solution contained only inorganic ions (Na\(^+: 142\) mM, K\(^+: 5.0\) mM, Mg\(^{2+: 1.5}\) mM, Ca\(^{2+: 2.5}\) mM, Cl\(^+: 147.8\) mM, HCO\(_3^-\) 4.2 mM, HPO\(_4^{2-}: 1.0\) mM, SO\(_4^{2-}: 0.5\) mM).\(^{25}\) We used time-of-flight secondary ion mass spectrometry (ToF-SIMS) (ION-TOF GmbH, Munich, Germany) to assess the relative concentrations of Ca and P, which are components of HAp, on N–TiO\(_2\) and untreated Ti. Positive and negative ToF-SIMS spectra of CaPO\(_2\) and PO\(_3\) fragments were obtained using a bismuth primary ion gun, respectively. The peak intensity ratio of P/Ca, Ln(Ip/Ica), was calculated.

2.6 Statistical analysis

The results were expressed as the mean ± standard deviation and p values <0.01 were considered statistically significant. Turkey’s test analysis was performed. Twenty samples for each experiment were analysed to acquire the data.

3. Results and discussion

Figure 1 shows SEM images of the surfaces of untreated Ti, and of negatively- and positively-charged N–TiO\(_2\). These images indicate that each sample had a different surface morphology. The arithmetic mean of the height variation of the roughness profiles, Ra, of untreated Ti, and negatively- and positively-charged N–TiO\(_2\), were 42.0, 41.7 and 40.9 μm, respectively. These results suggest that differences in surface morphology and roughness between these samples can be reduced.

The contact angles of untreated Ti, and negatively- and positively-charged N–TiO\(_2\), were 87.2, 54.3 and 44.3°, respectively. The hydrophilicities of the two N–TiO\(_2\) samples were larger than that of untreated Ti because of their increased H\(^+\) and OH\(^-\) concentrations due to the increased H\(_2\)O affinity of the charged N–TiO\(_2\) surfaces.

Figure 2 shows the degree of cell proliferation on the untreated Ti, and negatively- and positively-charged N–TiO\(_2\) surfaces. All samples supported similar levels of cell proliferation, irrespective of the incubation time (3 and 7 days), suggesting that the surface potential of N–TiO\(_2\) does not affect the proliferation of osteoblast cells.

Figure 3 shows the level of cell differentiation as determined by the alkaline phosphatase activity of MC3T3-E1 cells on untreated Ti, and negatively- and positively-charged N–TiO\(_2\) surfaces. Greater cell differentiation was observed after 14 days incubation on the charged N–TiO\(_2\) surfaces compared to untreated Ti but were similar after 28
days incubation. This finding suggests that the surface potential of N–TiO₂ affects the differentiation of osteoblast cells early during incubation.

We further clarified the mechanism underlying increased cell differentiation on charged N–TiO₂ surfaces by focusing on the adsorption of the model adhesive protein Fn after soaking the surfaces in culture medium containing Fn.

**Figure 4** shows the adsorption of Fn on untreated Ti, and on negatively- and positively-charged N–TiO₂ surfaces after incubating in serum-containing medium or in Fn-containing Tris-HCl for 1 min at 310 K (n = 3).

We next examined the adsorption of Ca and P ions on the three surfaces. No precipitate was observed in SEM images of samples soaked in serum-containing medium for 1 min. ToF-SIMS can detect the initial adsorption of Ca and P ions; using this technique, the peak intensity ratio of P/Ca of samples soaked in serum-containing medium for 1 min showed that Ca and P were detected only on the N–TiO₂ surfaces, with P ions being predominant. The P/Ca peak ratio obtained for samples soaked in serum-containing medium was essentially stoichiometric with that of HAp. Similarly, soaking the three samples in SBF containing Ca and P resulted in Ca and P peaks being detected only for the N–TiO₂ samples, with P ions being predominant (**Fig. 5**). Resolution of ToF-SIMS is ppb level, which is much higher than that of X-ray photoelectron spectroscopy (XPS) (ppm level). So, ToF-SIMS measurement could detect both Ca and P adsorbed on negatively- or positively-charged N–TiO₂ surfaces after incubating in SBF for 1 min. ln(P/Ca) of HAp and α-tricalcium phosphate were 2.48 and 2.84, respectively. After soaking in SBF for 1 min, some kinds of calcium
phosphate was formed on negatively- or positively-charged N–TiO₂ surfaces. This result was not consistent with Ca and P detection by XPS measurement previously reported.¹⁵)

These results indicate that adhesive-proteins such as Fn adsorb on Ti surfaces irrespective of surface treatment or not, whereas Ca and P ions adsorb only on negatively- or positively-charged surfaces. Therefore, Fn directly adsorbed onto untreated Ti. For the Ti-treated samples, Ca and P adsorbed first, followed by the adsorption of Fn on these CaP adsorbants. Other studies have shown that Ca²⁺,²⁹) and P³⁰) play important roles in elevating ALP activity in MC3T3-E1 osteoblasts. Enhanced adsorption of inorganic species and Fn probably promotes osteoblast differentiation.

**Figure 6** shows a high-speed AFM image of Fn adsorbed on a positively-charged surface. Fn forms fibrils with a maximum height of about 6 nm. Fn is a highly flexible glycoprotein composed of two nearly identical covalently linked subunits, as shown in **Fig. 7** (upper figure) and adopts two adhesion conformations: side-on and end-on orientations (**Fig. 7**, lower figure). The heights of the side-on and end-on orientations are 9.9 ± 1 and 19.6 ± 0.7 nm, respectively.¹¹) Our results suggest that Fn adsorbed in its side-on orientation on the positively-charged surface early during the adsorption process. The change in conformation of Fn with increased soaking time can be measured by high-speed AFM. The relation between the conformational change of Fn with time and its role in cell differentiation may be clarified in future.

4. Conclusions

MC3T3-E1 cell differentiation was enhanced by adsorption on negatively- or positively-charged N–TiO₂ surfaces. Cell adhesive proteins such as Fn and ionic species such as Ca and P adsorbed on these charged N–TiO₂ surfaces in culture media. We confirmed that Fn can be observed by high-speed observation. Fn adsorbed in a side-on orientation on the positively-charged surface early during the adsorption process.

**Acknowledgements** This work was supported in part by a Kibankenkyu C grant (No. 16K06786) from the Japan Society for the Promotion of Science (JSPS).

**References**

1) F. Grinnell and M. K. Feld, *J. Biomed. Mater. Res.*, 15, 363–381 (1981).
2) D. L. Coleman, D. E. Gregonis and J. D. Andrade, *J. Biomed. Mater. Res.*, 16, 381–398 (1982).
3) A. S. Hoffman, *J. Biomed. Mater. Res.*, 20, ix–xi (1986).
4) J. M. Schakenraad, J. Arends, H. J. Busscher, F. Dijk, P. B. van Wachem and C. R. H. Wildevuur, *Biomaterials*, 10, 43–50 (1989).
5) S. D. Johnson, J. M. Anderson and R. E. Marchant, *J. Biomed. Mater. Res.*, 26, 915–935 (1992).
6) J. F. Schultz and D. R. Arment, *J. Biol. Chem.*, 270, 11522–11531 (1995).
7) A. Zadpoor, *Mater. Sci. Eng., C*, 35, 134–143 (2014).
8) T. Kokubo and S. Yamaguchi, *Acta Biomater.*, 44, 16–30 (2016).
9) M. Hashimoto, K. Kashiwagi and S. Kitaoka, *J. Mater. Sci.-Mater. M.*, 22, 2013–2018 (2011).
10) M. Hashimoto, K. Hayashi and S. Kitaoka, *Mater. Sci. Eng., C*, 33, 4155–4159 (2013).
11) M. Hashimoto, S. Kitaoka, S. Muto, K. Tatsumi and Y. Obata, *J. Mater. Res.*, 31, 1004–1011 (2016).
12) M. Hashimoto, S. Kitaoka, Y. Obata, S. Muto, T. Ogawa, M. Furuya and H. Kanetaka, *Key Eng. Mat.*, 758, 86–89 (2017).
13) M. Hashimoto, T. Ogawa, S. Kitaoka, S. Muto, M. Furuya, H. Kanetaka, M. Abe and H. Yamashita, *Key Eng. Mat.*, 782, 218–223 (2018).
14) M. Hashimoto, T. Ogawa, S. Kitaoka, S. Muto, M. Furuya, H. Kanetaka, M. Abe and H. Yamashita, *Acta. Mater.*, 155, 379–385 (2018).
15) A. P. Ngankam, G. Mao and P. R. V. Tassel, *Langmuir*, 20, 3362–3370 (2004).
16) S. R. Sousa, M. M. Brás, P. M. Ferreira and M. A.
Barbosa, Langmuir, 23, 7046–7054 (2007).
17) A. G. Hemmersam, K. Rechendorff, M. Foss, D. S. Sutherland and F. Basenbacher, J. Colloid Interf. Sci., 320, 110–116 (2008).
18) B. G. Kaselowsky, D. M. Collard and A. J. Garcia, J. Biomed. Mater. Res., 66A, 247–259 (2003).
19) M. Bergkvist, J. Carlsson and S. Oscarsson, J. Biomed. Mater. Res., 64A, 349–356 (2003).
20) H. M. Kowalczynska, M. Nowak-Wyrzykowska, R. Kolos, J. Dobkowski and J. Kaminski, J. Biomed. Mater. Res., 72A, 228–236 (2005).
21) S. R. Sousa, M. M. Bras, P. Moderas-Ferreira and M. A. Barbosa, Langmuir, 23, 7046–7054 (2007).
22) A. P. Ngankam, G. Mao and P. R. Van Tassel, Langmuir, 20, 3362–3370 (2004).
23) http://www2.kaiyodai.ac.jp/~hasobe/2014-Kit-Level2/Exp%20Data/Set1-TF-Brows.html.
24) H. Yamashita, K. Iinoue, M. Shibata, T. Uchihashi, J. Sasaki, H. Kandori and T. Ando, J. Struct. Biol., 184, 2–11 (2013).
25) T. Kokubo and H. Takadama, Biomaterials, 27, 2907–2915 (2006).
26) M. Hasegawa, T. Kudo, H. Kanetaka, T. Miyazaki, M. Hashimoto and M. Kawashita, Biomed. Mater., 11, 045006 (2016).
27) T. Kokubo and S. Yamaguchi, J. Biomed. Mater. Res. A, 107A, 968–977 (2019).
28) S. Nakamura, T. Matsumoto, J. Sasaki, H. Egusa, K. Y. Lee, T. Nakano, T. Sohmura and A. Nakahira, Tissue Eng. Pt. A, 16, 2467–2473 (2010).
29) S. Maeno, Y. Niki, H. Matsumoto, H. Morioka, T. Yatabe, A. Funayama, Y. Toyama, T. Taguchi and J. Tanaka, Biomaterials, 26, 4847–4855 (2005).
30) G. R. Beck, E. C. Sullivan, E. Moran and B. Zerler, J. Cell. Biochem., 68, 269–280 (1998).
31) G. R. Beck, B. Zerler and E. Moran, P. Natl. Acad. Sci. USA, 97, 8352–8357 (2000).
32) Y.-R. V. Shih, Y. Hwang, A. Phadke, H. Kang, N. S. Hwang, E. J. Caro, S. Nguyen, M. Siu, E. A. Theodorakis and N. C. Gianneschi, P. Natl. Acad. Sci. USA, 111, 990–995 (2014).