New Surface Modification of Titanium Implant with Phospho-amino Acid

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The purpose of this study was to investigate a new biochemical surface modification technique for titanium implants using phospho-amino acid. Pure titanium disks were pretreated with 10 N HCl and ultrapure water at room temperature for 30 minutes respectively. Then these disks were modified with either L-threonine (Thr) or O-phospho-L-threonine (P-Thr) at 37°C for 12 hours. X-ray photoelectron spectroscopy (XPS) chemically analyzed the modified surfaces. It was revealed that the N 1s peak which originated from Thr was not detected in the wide-scan spectrum of Thr-modified surface, whereas three peaks of N 1s, P 2s, and P 2p which originated from P-Thr were detected in the wide-scan spectrum of P-Thr-modified surface. Moreover, the P 2p peak of P-Thr which reacted with the surface significantly shifted to a lower binding energy (p < 0.05). Based on the results of this study, it was concluded that P-Thr chemically bonded to the titanium surface treated with HCl.

Key words: Titanium implant, Phospho-amino acid, Surface modification

INTRODUCTION

Biochemical modification of biomaterial surface induces specific cell and tissue responses by means of immobilizing peptides, proteins, or growth factors on the surface1). This procedure offers the advantage of improving the biocompatibility of a surface without adversely affecting the bulk properties of the system, and introduces a new concept of controlling and guiding the cell behavior on an ultra-thin layer of bioactive molecules of a biomimetic surface. Titanium and its alloys are highly favored as implant materials in contact with bone. One strategy to improve osseointegration is to precoat implants with extracellular matrix components such as collagen, fibronectin, laminin, and vitronectin to enable specific cell-extracellular matrix interactions, as well as with growth factors to facilitate differentiation of osteoblasts2). In surface immobilization of poly(ethylene glycol) or peptide3-6, it was found that such modified surfaces involving silanized functionalities were generally unstable and subject to hydrolytic degradation over time when in contact with aqueous fluids3). In studies where a hydrophilic graft copolymer or peptides were spontaneously assembled over a stable PEG-modified surface7), potentially high molecular surface modification was limited either by the stability of the PEG-modified surface, or the availability of peptides because such adsorption was reversible and adsorbed peptides could be easily washed away with fresh buffer or replaced by other molecules in solution. Ferris et al.9) suggested surface modification with the peptide sequence, Arg-Gly-Asp-Cys (RGDC), using gold-thiol chemistry since small peptides can be fabricated synthetically to very high purity and are not dependent on tertiary structure for bioactivity. However, this modification process is complex and the biocompatibility of titanium cannot be applied because its surface is gold-coated. Healy and Ducheyne10) demonstrated that phosphate has a high affinity for titanium oxide surface. Vionnery et al.11) suggested that phosphonic acid molecules covalently attached on a titanium surface might form a scaffold for new bone formation, ultimately leading to interfacial bonding between implant and host tissue.

In the midst of various biochemical surface modification techniques that are being investigated and pursued, there arises an idea to chemically bond amino acid as a low molecule to titanium surface by means of phosphorylation of amino acid. In the body, neutral amino acids as targets of phosphorylation or dephosphorylation are namely L-threonine, L-serine, and L-tyrosine. In particular, the peptide bond in L-threonine is hardly hydrolyzed. The purpose of this study was to investigate, by means of X-ray photoelectron spectroscopy (XPS), a new biochemical surface modification technique for titanium implants using O-phospho-L-threonine acid.
MATERIALS AND METHODS

Specimens preparation
Pure titanium disks (diameter: 5.8 mm, thickness: 2 mm; JIS 2; GC, Tokyo, Japan) were decontaminated by the following means of surface decontamination that Takeuchi et al.12 recommended: ultrasonic treatment with 10 N hydrochloric acid (HCl) for 30 minutes, followed by ultrasonic rinsing with ultrapure water (milli-Q water; > 18 MΩ·cm) for 30 minutes. Then these disks were modified with either 50 mM L-threonine (Lot No. 32K0896, Sigma-Aldrich, St. Louis, Mo, USA) or 50 mM O-phospho-L-threonine (Lot No. 81K4110, Sigma-Aldrich, St. Louis, Mo, USA) (Fig. 1) at 37°C for 12 hours, followed by ultrasonic rinsing with milli-Q water for 10 minutes.

X-ray photoelectron spectroscopy (XPS)
Surfaces of the modified titanium disks, which were mounted on a stub with insulating tape, were chemically analyzed using an XPS instrument (AXIS-HS, Kratos, Manchester, UK). The XPS measurements were performed in a vacuum of less than 10⁻⁷ Pa. Al-Kα monochromatic X-ray with a source power of 150 W was utilized. Charge compensation was achieved with an electron flood gun equipped with the AXIS-HS instrument. Wide- and narrow-scan spectra were acquired at pass energies of 80 and 40 eV respectively. Peak positions were calibrated by referencing a value of 284.6 eV for the peak of C-C, C-H in the C 1s spectrum. Smoothing of narrow scans was done, and a straight line background (for C 1s, N 1s, O 1s, and P 2p) and Shirley-type background (for Ti 2p) were applied in the quantification. The relative sensitivity factors used to calculate the atomic ratios from the peak area ratios were 1.0 for C 1s, 1.68 for N 1s, 2.64 for O 1s, 1.56 for P 2p, and 7.20 for Ti 2p. Reproducibility was guaranteed by taking nine measurements per experimental variable.

RESULTS
Titanium surface modified with L-threonine (Thr)
Fig. 2 shows a XPS wide-scan spectrum of Thr and a narrow-scan spectrum of its C 1s region. In the wide-scan spectrum, O, N, and C were detected. The C 1s peak was attributed to three peaks at 288.4 eV (COO⁻), 286.0 eV (C-O, C-N), and 284.6 eV (C-C, C-H). Fig. 3 shows a XPS wide-scan spectrum of Thr which reacted with titanium treated with HCl and a narrow-scan spectrum of its C 1s region. In the
SURFACE MODIFICATION OF TITANIUM IMPLANT

Wide-scan spectrum

Narrow-scan spectrum of C 1s

binding energy of P 2p of P-Thr

When P-Thr was applied to a titanium surface treated with HCl, the P 2p peak of non-reacted P-Thr – with binding energy at 134.0 eV – significantly shifted to a lower binding energy at 133.7 eV (t-test: p<0.05).
Table 1  P/Ti values of P-Thr at HCl-treated titanium surface and untreated titanium surface (control)

<table>
<thead>
<tr>
<th>Pretreatment of Ti surface</th>
<th>P/Ti</th>
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<tbody>
<tr>
<td>HCl</td>
<td>0.140 ± 0.04</td>
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<tr>
<td>Control</td>
<td>0.046 ± 0.03</td>
</tr>
</tbody>
</table>

Values connected by vertical line are statistically different.

P/Ti value of P-Thr after reaction with titanium treated with HCl

As shown in Table 1, the P/Ti value of P-Thr which reacted with titanium treated with HCl (0.140 ± 0.04) was significantly larger than that of untreated titanium surface (0.046 ± 0.00) (t-test: p<0.01).

DISCUSSION

XPS spectra of the modified titanium surfaces revealed that Thr could not chemically bond to the surface treated with HCl and water, whereas chemical bonding of P-Thr to the titanium surface was detected. Lausmaa et al. reported that glycine monomolecular layers adsorbed on the titanium surface were stable up to above room temperature and did not seem to be significantly affected by the presence of water. MacDonald et al. demonstrated that by modifying the physicochemical properties of titanium surface, it might be possible to alter adsorption of human plasma fibronectin and hence optimize cell attachment. On the other hand, an investigation into the adsorption of albumin and fibrinogen on titanium surface in vivo revealed that 92% of albumin and over 98% of fibrinogen that were adsorbed respectively were desorbed. In this study, Thr might be adsorbed on the titanium surface because the peaks of COO' and C-O, C-N were detected when the duration of ultrasonic rinsing with milli-Q water - after surface modification with Thr - was shortened to less than 10 minutes. However, since ultrasonic rinsing with water for 10 minutes washed Thr away from the titanium surface, Thr would be desorbed from the surface in vivo because Thr was only adsorbed on the surface but not chemically bonded to the surface.

Viorney et al. indicated that a significant increase of collagen type I production was observed on titanium surfaces modified with phosphonic acids compared to unmodified Ti surfaces. Yoshinari et al. reported that titanium surfaces modified with Ca-ion implantation and thin hydroxyapatite coatings caused the immobilization of biphosphonate on the surfaces - which did not occur on the unmodified surfaces. Hence, the idea was floated that phosphonic acid involving one -H₂PO₄ group might bond to the titanium surface more strongly and stably rather than bisphosphonate with two -H₂PO₄ groups. This study indicated that the three peaks of P 1s, P 2s, and P 2p which originated from P-Thr were detected in the XPS wide-scan spectrum of P-Thr that reacted with titanium treated with HCl, and the P 2p peak of P-Thr significantly shifted to a lower binding energy (p<0.05). Moreover, the peaks at 288.4 eV (COO') and 286.0 eV (C-O, C-N) were detected at relatively high intensity in the narrow-scan spectrum of its C 1s region after ultrasonic rinsing with water for 10 minutes. The molecule of P-Thr involves one element of P and N respectively, and in this study each amount of P and N indicated similar ratios by quantitative analysis. Therefore, P-Thr chemically bonded to the titanium surface treated with HCl.

P/Ti value at titanium surface treated with HCl was three times greater than that of untreated titanium surface, and P-Thr covered an approximate 32% area of the Ti surface. After glass surface was treated by amine functionalization, PEG-aldehyde modification, and gold patterning in that sequence, an attempt was made to bond the peptide CGRGDS to the surface. With this procedure, the peptide CGRGDS covered a 21% area of the glass surface. Since the molecular weight of CGRGDS is much larger than that of P-Thr, the 32% surface coverage of P-Thr in this study seemed to be a very large value. Waliavaara et al. demonstrated that oxide thickness and carbon contamination had no influence on protein adsorption or contact activation. In contrast, El-Ghannam et al. indicated that the oxide layer affected the conformation of an adsorbed protein such as C3 and laminin-5, and might therefore alter its biological activity. Krozer et al. reported that the bonding of amino-alcohol applied for cleaning contaminated implant surfaces was stronger to an acid-treated titanium surface than an anodically oxidized titanium surface. Thus the decontamination method using HCl may serve to facilitate a stable chemical modification of the titanium surface and promote a progressive binding of P-Thr to the modified surface.

CONCLUSION

The focus of this study was to investigate the possibility of a new biochemical surface modification technique for titanium implants using a phospho-amino acid versus an amino acid. Consequently P-Thr, a phospho-amino acid by means of phosphorylation of Thr, chemically and stably bonded to the titanium surface treated with HCl - as recommended by Takeuchi et al.

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