The effect of UV-photofunctionalization on the time-related bioactivity of titanium and chromium–cobalt alloys

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ABSTRACT
This study examined the possible changes in the bioactivity of titanium surfaces during their aging and investigated the effect of ultraviolet (UV) light treatment during the age-related change of titanium bioactivity. Rat bone marrow-derived osteoblastic cells were cultured on new titanium disks (immediately after either acid-etching, machining, or sandblasting), 4-week-old disks (stored after processing for 4 weeks in dark ambient conditions), and 4-week-old disks treated with UVA (peak wavelength of 365 nm) or UVC (peak wavelength of 250 nm). During incubation for 24 h, only 50% of the cells were attached to the 4-week-old surfaces as compared to the new surface. UVC treatment of the aged surface increased its cell attachment capacity to a level 50% higher than the new surfaces, whereas UVA treatment had no effect. Proliferation, alkaline phosphatase activity, and mineralization of cells were substantially lower on the 4-week-old surfaces than on the new surfaces, while they were higher on the UVC-treated 4-week-old surfaces as compared to the new surfaces. The age-related impaired bioactivity was found on all titanium topographies as well as on a chromium–cobalt alloy, and was associated with an increased percentage of surface carbon. Although both UVA and UVC treatment converted the 4-week-old titanium surfaces from hydrophobic to superhydrophilic, only UVC treatment effectively reduced the surface carbon to a level equivalent to the new surface. Thus, this study uncovered a time-dependent biological degradation of titanium and chromium–cobalt alloy, and its restoration enabled by UVC phototreatment, which surmounts the innate bioactivity of new surfaces, which is more closely linked to hydrocarbon removal than the induced superhydrophilicity.

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1. Introduction

There is a continuing desire to improve osteoconductivity of titanium implants. Recently, our laboratory reported the discovery of ultraviolet (UV) light-mediated photofunctionalization of titanium surfaces enabling an enhancement of their osseointegration capability [1]. When UV treatment is applied to an acid-etched titanium surface, the strength of osseointegration increases more than 3-fold during the early stage of healing in a rat model, along with a virtually complete accomplishment of peri-implant bone formation, i.e., a nearly 100% bone–implant contact. This in vivo improvement is supported by the substantially increased protein adsorption, osteoblast attachment, settlement, and proliferation [1].

The mechanism underlying this bioactivity enhancement remains elusive. Although UV treatment converted the titanium surface from hydrophobic to superhydrophilic, as repeatedly reported in the field of chemical engineering [2–4], the hydrophilic status was unlikely to determine the osteoconductive capacity [1]. For instance, the contact angle of water did not correlate with protein adsorption or cell attachment capacities on titanium treated with UV for various time periods [1]. Instead, the amount of hydrocarbon on the titanium surface showed a very high coefficient of correlation [1]. The surface of bulk titanium is made of a semiconductor TiO₂. Therefore, UV treatment removes hydrocarbons through two possible mechanisms: the induced photocatalytic activity of TiO₂ and a direct decomposition by UV per se [1,3,4]. UV light energy of 3.2 eV is needed to induce TiO₂ photocatalytic
activity to excite an electron from the valence band to the conduction band, which corresponds to approximately 365 nm wavelength, referred to as UVA [2,5]. In contrast, direct hydrocarbon decomposition is enforced by UVC at its peak wavelength of 250 nm.

Titanium constantly absorbs organic impurities such as poly-carbonyls and hydrocarbons, from the atmosphere, water and cleaning solutions [6–8]. Progressive accumulation of organic molecules, particularly those with a carbonyl moiety, onto titanium surfaces is considered unavoidable under ambient conditions. In fact, surfaces of currently used titanium implants, for clinical and experimental use, are found to be contaminated with hydrocarbons [1,4,9–12]. Here, an extremely important question needs to be addressed as to whether the bioactivity of titanium is stable over time as hydrocarbons progressively deposit on its surface. Moreover, if hydrocarbons deposited on titanium surfaces is responsible, at least in part, for the level of their bioactivity, several concomitant questions can be raised: (1) to what level the UV treatment of titanium surfaces, known to remove the deposited hydrocarbons, improves the possibly degrading bioactivity of titanium over time? (2) does UVA or UVC effectively improve the bioactivity of titanium? and (3) do metallic implant materials other than titanium manifest a similar time-related change and UV-mediated recovery in their bioactivity? This study examined osteoblast behavior and responses on titanium surfaces of different ages: new surfaces (immediately after processing), 4-week-old surfaces, and 4-week-old surfaces treated with UVA or UVC. Titanium surfaces with various surface topographies as well as a chromium–cobalt alloy surface were tested.

2. Materials and methods

2.1. Titanium samples and surface characterization

Commercially pure grade-2 titanium disks (diameter, 20 mm) were prepared either by acid-etching with 67% (w/w) sulfuric acid (H2SO4) at 20 °C for 75 s, machine turning, or sandblasting with 50 μm Al2O3 particles for 1 min at a pressure of 3 kg/m2. In addition, chromium–cobalt alloy disks (Co 63%, Cr 28%, Mo 5%) were prepared by sandblasting. The surface morphology was examined by scanning electron microscopy (SEM) (XL30, Philips, Eindhoven, Netherlands). The acid-etched and sandblasted surfaces exhibited typical micro-roughened surfaces, and the machined surfaces had relatively smooth morphology with turned traces (Fig. 1), as previously reported [11,13,14].

Prepared surfaces were used for the experiments either immediately after their processing (new surface) or after storing them under dark ambient conditions for 4 weeks (4-week-old surface). Half of the 4-week-old disks were treated with UV light for 48 h under ambient conditions. We used two different light sources UVA and UVC. UVA was generated by using a 6 W mercury lamp (UVP, Cambridge, UK); intensity; ca. 2 mW/cm2 (λ = 360 ± 20 nm) and 0.0 mW/cm2 (λ = 250 ± 20 nm), and UVC was generated by using a 15 W bactericidal lamp (Toshiba, Tokyo, Japan); intensity; ca. 0.05 mW/cm2 (λ = 360 ± 20 nm) and 2 mW/cm2 (λ = 250 ± 20 nm). The hydrophilicity of the titanium surfaces was measured using an automated contact angle measuring device (DCA-VZ, Kyowa Interface Science, Saitama, Japan) as a contact angle of 1 μl H2O. Additional image analyses were undertaken to evaluate the spread area of 10 μl H2O on the titanium disks. The chemical composition on the titanium surfaces was evaluated by electron spectroscopy for chemical analysis (ESCA). ESCA was performed using an X-ray photoelectron spectroscopy (XPS) (Multiprobe Surface Science System, Omicron Nanotechnology, Eden Prairie, MN, USA) using a standard Al Kα x-ray (1486.6 eV) as the excitation source.

2.2. Osteoblast cell culture

Following the previously established protocol [14,15], bone marrow cells isolated from the femur of 8-week-old male Sprague–Dawley rats were placed into alpha-modified Eagle’s medium supplemented with 15% fetal bovine serum, 50 μg/ml ascorbic acid, 10 μM Na-glycerophosphate, 10 −5 M dexamethasone, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air; and 5% CO2 at 37 °C. At 80% confluency, the cells were detached using 0.25% trypsin–1 mM EDTA–4Na and seeded onto titanium disks at a density of 3 × 104 cells/cm2. The culture medium was renewed every three days.

2.3. Cell attachment, density and proliferation assays

The initial attachment of cells was evaluated by measuring the number of the cells attached to titanium substrates after 2 h and 24 h incubation. In addition, the propagated cells were quantified as cell density at culture days 3 and 5. The cells were gently rinsed twice with PBS and treated with 0.1% collagenase in 300 μl of 0.25% trypsin–1 mM EDTA–4Na for 15 min at 37 °C. A hemocytometer was used to count the number of detached cells. SEM analysis was performed for the selected culture to confirm the absence of cell remnants on the substrate. The proliferative activity of these cells was measured by BrdU incorporation during DNA synthesis. At day 3 of culture, 100 μl of 10 μM BrdU solution (Roche Applied Science, Mannheim, Germany) was added to the culture wells that were then incubated for 10 h. After trypsinizing the cells and denaturing their DNAs, the cultures were incubated with anti-BrdU conjugated with peroxidase for 90 min, and reacted with tetramethylbenzidine for color development. Absorbance at 370 nm was measured using an ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

Fig. 1. Scanning electron microscopy images of titanium and chromium–cobalt alloy disk used in this study. Bars are 10 μm for 5000× images and 1 μm for 30000× images.
2.4. Morphology and morphometry of cells

The spreading and settling behaviors of osteoblasts were evaluated by examining their morphology and cytoskeletal arrangement shortly after seeding onto the titanium substrates. After 0.5 h and 3 h of culturing, osteoblasts were fixed in 1% formalin, and stained using fluorescent dyes, DAPI (nuclei green color, Vector, CA) and rhodamine phalloidin (actin filament red color, Molecular Probes, OR). Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement. Cell area, perimeter and Feret’s diameter were quantitatively assessed using an image analyzer (ImageJ, NIH, Bethesda, MD).

2.5. Alkaline phosphatase (ALP) activity

ALP activity of the cultured osteoblasts was examined by culture area- and colorimetry-based assays. For the culture area-based quantification, osteoblastic cells cultured for 10 days were washed twice with Hank’s solution, and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM fast red TR for 30 min at 37 °C. The ALP-positive area on the stained images was calculated as [(stained area/total disk area) x 100] (%) using image analyzing software (Image Pro-plus, Media Cybernetics, Silver Spring, MD, USA). For colorimetry, the culture was rinsed with ddH2O and 250 µl p-nitrophenylphosphate (LabAssay ATP, Wako Pure Chemicals, Richmond, VA) was added before incubation at 37 °C for 15 min. The ALP activity was evaluated as the amount of nitrophenol released through the enzymatic reaction and measured at 405 nm wavelength using a colorimetry reader (Synergy HT, BioTek Instruments, Winooski, VT), and further standardized relative to cell number measured using calcein-AM staining (Molecular Probes, Inc, Eugene, OR).

2.6. Mineralization assay

The mineralization capability of cultured osteoblasts was examined by mineralized nodule area- and calcium colorimetry-based assays. Alizarin red staining was performed in the former assay. At day 20 of culture, the specimens were washed twice with 1% PBS at 37 °C and stained for 5 min using 1% alizarin red (pH ~6.3–6.4), after which the specimens were rinsed twice with distilled water and dried in air. The stained specimens were photographed and the images were processed using the same software employed for the ALP activity assay. The alizarin red positive area was calculated using the following equation: [(stained area/total disk area) x 100] (%) using image analyzing software (ImageJ, NIH, Bethesda, MD, USA). The stained specimens were photographed and the images were processed using the same software employed for the ALP activity assay. The alizarin red positive area was calculated using the following equation: [(stained area/total disk area) x 100] (%) using image analyzing software (ImageJ, NIH, Bethesda, MD, USA).

2.7. Gene expression analysis

Gene expression was analyzed using a reverse transcriptase-polymerase chain reaction (RT-PCR) on culture days 10 and 20. Total RNA in these cultures was extracted using TRIzol (Invitrogen, Carlsbad, CA) and a purification column (RNeasy, Qiagen, Valencia, CA). Following DNAse I treatment, reverse transcription of 0.5 µg total RNA was performed using MMLV reverse transcriptase (Clontech, Carlsbad, CA). PCR was performed using Taq DNA polymerase (EX Taq; Takara Bio, Madison, WI) to detect osteopontin and osteocalcin mRNA using the primer designs and PCR condition established previously [14,15]. PCR products were visualized on 1.5% agarose gel by ethidium bromide staining. Band intensity was detected and quantified under UV light and normalized with reference to GAPDH mRNA.

2.8. Statistical analyses

Three samples were used for the assays described above, except for cytometry, which required 15 cell samples. Two-way ANOVA was performed to examine the effects of culture time and titanium surfaces of different ages with or without UV treatment. If necessary, a post-hoc Bonferroni test was conducted to perform multiple comparison tests; p < 0.05 was considered statistically significant. If data were available at only one time point, one-way ANOVA was used to determine the differences among the experimental groups.

3. Results

3.1. Osteoblast attachment to titanium with different age and UV treatment

We first focused on acid-etched titanium surfaces to examine the effect of titanium aging and UV treatment. The number of osteoblasts attached to titanium surfaces varied significantly among differently conditioned titanium disks (p < 0.01, 2-way ANOVA; Fig. 2A). The number of cells attached to the 4-week-old surface during 2 h of incubation was only one fourth of the number observed for the new surface (Bonferroni, p < 0.01). UVC treatment of the 4-week-old surfaces increased the cell attachment 8-fold, which was equivalent to twice the number observed for the new surface. In contrast, the UVA treatment of the 4-week-old surface showed no significant change. Even at 24 h, there was a significant difference among the cultures (1-way ANOVA, p < 0.01). The number of cells attached to the new surface was double of that attached to the 4-week-old surface, while the UVC-treated 4-week-old surface showed even higher cell attachment than the new surface. The UVA-treated 4-week-old surface showed a similar number to the untreated 4-week-old surface.

3.2. Effects of titanium age and UV treatment on osteoblast proliferation

The number of osteoblasts that propagated at days 3 and 5 varied significantly among the differently conditioned acid-etched titanium surfaces (p < 0.01; 2-way ANOVA; Fig. 2B). At day 3, the new surface exhibited 90% more cells than the 4-week-old surface (p < 0.01), while the UVC-treated 4-week-old surface exhibited...
120% more cells than the 4-week-old surface \((p < 0.01)\). Such contrasting cell density remained significant at day 5 \((p < 0.01)\). In contrast, the effect of UVA treatment was insignificant. BrdU incorporation at day 3 was also significantly high in the following order: UVC-treated 4-week-old surfaces, new surfaces, and 4-week-old surfaces with or without UVA treatment \((p < 0.01; \text{Fig. 2C})\), confirming the results of cell density.

### 3.3. Osteoblast spreading behavior on titanium with different age and UV treatment

Based on the results showing that initial cell attachment and proliferation were enhanced on the UVC-treated surface but not on the UVA-treated surface, we studied subsequent cellular functions focusing on the effect of UVC treatment. Representative low
magnification images captured from osteoblasts 3 h after seeding clearly showed that the number of cells was greatest on the UV-treated 4-week-old surface and lowest on the 4-week-old surface, confirming the results from the cell attachment assay (top 1000× images in Fig. 3). High magnification images obtained at 3 h of incubation vividly revealed that cells were clearly larger with their processes spread on the new and UV-treated 4-week-old surfaces, whereas cells remained small and round with little cytoskeletal development on the 4-week-old surface (bottom 4000× images). Even at the earlier time point of 0.5 h, the cells appeared settled with cytoskeletal development initiated on the new and UVC-treated surfaces (middle 4000× images). The cells were noticeably larger at 3 h as compared to those at 0.5 h on the new and UVC-treated surfaces, indicating an expedited process of cell spread on these surfaces.

Cytomorphometric evaluations demonstrated that the area, perimeter, and Feret’s diameter of the cells were significantly greater for the new and UVC-treated surfaces than for the 4-week-old surface both at 0.5 and 3 h ($p < 0.01$; histograms in Fig. 3). There were no differences between the new and UVC-treated surfaces. These parameters increased by 2.5–3.5 times from 0.5 to 3 h on the new and UVC-treated surfaces, but only approximately 1.5 times increase on the 4-week-old surface, confirming the observation of an expedited cell spread on the new and UVC-treated surfaces, or in other words, a delayed spread on the 4-week-old surface.

3.4. Osteoblast functional phenotypes on variously conditioned titanium

The ALP-positive area of osteoblast culture measured at day 10 was significantly lower on 4-week-old surfaces than on the new surface ($p < 0.01$; Fig. 4A). The ALP-positive area on the UVC-treated 4-week-old surfaces was even higher than on the new surface, which was 2.5 times greater in number than the area on the 4-week-old surface. Similarly, the area of mineralized nodule detected by alizarin red stain was greatest on UV-treated 4-week-old surfaces and lowest on 4-week-old surfaces with considerable differences among the groups ($p < 0.01$; Fig. 4B).

In contrast, ALP activity and calcium deposition evaluated relative to cell number were similar among the three experimental groups (Fig. 5A, B). RT-PCR analyses at days 10 and 20 also showed that there were no differences in the expression level of bone-related genes tested among the three titanium surfaces (Fig. 5C).

3.5. Physicochemical characterization of titanium with different age and UV treatment

Newly prepared acid-etched surfaces showed a water contact angle of $0^\circ$, indicative of their superhydrophilic nature (contact angle $<5^\circ$) (Fig. 6A). The surfaces of the 4-week-old acid-etched titanium disks showed a contact angle of approximately $50^\circ$, indicative of their hydrophobic nature. The contact angle of the 4-week-old surface decreased to $0^\circ$ after either UVA or UVC treatment, indicating the restoration of superhydrophilicity. Accordingly, the area of 10 $\mu$l of water spread on the new, UVA-treated and UVC-treated 4-week-old surfaces was substantially greater than that on the 4-week-old surface ($p < 0.01$). There was no significant difference in the area of water spread between the UVA-treated and UVC-treated surfaces.

XPS analyses revealed that the atomic percentage of carbon, which was 19% on the new surface, increased to 58% on the 4-week-old surface (Fig. 6B). UVC treatment of the 4-week-old surface reduced the carbon level to 18.5%, whereas the UVA-treated 4-week-old surface contained 35% carbon.

3.6. Effects of age and UV treatment on other substrates

To address whether the abovementioned findings apply to surface types of titanium other than the acid-etched titanium surface, cell density and ALP activity were examined on machined and sandblasted titanium surfaces as well as a sandblasted chromium–cobalt alloy surface of different ages and with and without UVC treatment.

On these surfaces, the cell density was consistently and remarkably greater on the new surfaces than the 4-week-old surfaces (Figs. 7A, C and 8A). The cell density at day 5 on the new...
surfaces was more than twice than that on the 4-week-old surfaces for all the surface types tested. For the cell density at day 5, UVC treatment of the 4-week-old surfaces increased the number to a level even higher than on the new surface on both the titanium surfaces tested (Fig. 7A and C; \( p < 0.05 \)). The cell density at day 5 was comparable between the new and UVC-treated surfaces on the chromium–cobalt disks (Fig. 8A).

The ALP-positive area of the machined titanium was significantly contrasted in the following order: UVC-treated 4-week-old surface > new surface > 4-week-old surface (Fig. 7B). However, the ALP-positive area of the sandblasted titanium and chromium–cobalt surfaces did not significantly differ between the new and UVC-treated surfaces (Figs. 7D and 8B). Regardless of titanium or chromium–cobalt substrates, the difference between the 4-week-old surface, and the new and UVC-treated surfaces was sizable, ranging from 2 to 3 times.

4. Discussion

This study revealed that the bioactivity of newly processed titanium surfaces substantially degrades within 4 weeks and that UVC treatment, and not UVA treatment, of the degraded surfaces

\[ \text{Relative alkaline phosphatase activity (cell)} \]

\[ \text{Relative calcium deposition (cell)} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Relative alkaline phosphatase activity (cell)} \]

\[ \text{Relative calcium deposition (cell)} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{New 4-W UV} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{GAPDH} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]

\[ \text{New} \]

\[ \text{4-week-old} \]

\[ \text{4-week-old +UVC} \]

\[ \text{Osteopontin} \]

\[ \text{Osteocalcin} \]

\[ \text{Gene expression (arbitrary unit)} \]

\[ \text{Day 10} \]

\[ \text{Day 20} \]
can restore and even increase the bioactivity beyond a level at which the newly processed surfaces innately acquire. The former phenomenon seems like the biological aging of titanium, and the latter, titanium reactivation beyond revitalization.

The time-related degradation of bioactivity occurred within 4 weeks is worthy of attention, as represented by the reduction in initial cell attachment to one quarter to half, cell proliferation reduced by half, and the severely delayed cell spread. More importantly, these impaired initial biological processes seemed to have adversely affected the functional phenotypes in the subsequent osteoblastic stages. The aged titanium surfaces showed less than half of ALP-positive and mineralized nodule areas as compared to the new surfaces. From the opposite perspective, when we simply used new titanium surfaces, these osteoblastic phenotypes doubled. Furthermore, the use of UVC treatment on the aged titanium surfaces robustly increased the degraded osteoblastic phenotypes and even surpassed the level of the new surface, suggesting immediate necessity for an in vivo testing of these titanium surfaces. We carefully examined the possible modulation of osteoblastic differentiation. Our hypothesis was that an increased interaction among the propagated cells on the new and UVC-treated surfaces might promote the rate of differentiation.

However, both ALP activity and calcium deposition, when standardized relative to cell number, did not vary significantly with aging or UV treatment. Furthermore, RT-PCR results did not show the modulated expression of the osteoblast differentiation marker genes tested, thereby indicating that the altered ALP-positive and mineralized nodule areas were likely to be ascribed to the altered number of cells in the culture caused by altered cell attachment and proliferation.

Intriguingly, the cells responded differently to the superhydrophilic surfaces created by UVA and UVC treatments. Although both UVA- and UVC-treated surfaces showed superhydrophilicity, only the UVC-treated surface exhibited enhanced bioactivity. The superhydrophilic nature, at least occasionally, seemed to be associated with an increase in bioactivity of materials. Nevertheless, it is premature to conclude the existence of a direct link between the superhydrophilicity and high bioactivity. For instance, acid-etched titanium surfaces, stored in physiological saline solution, maintain their superhydrophilicity and show an increased bone–implant contact [10]. However, the mechanisms underlying enhanced bone–titanium integration is not fully explained. In their reports, the superhydrophilic surface maintained in saline solution reduces osteoblast attachment and proliferation but promote

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Fig. 7. Osteoblastic phenotypes altered by titanium aging and UVC treatment on surface topographies of titanium other than acid-etched surfaces. The cell density at days 3 and 5 (A) and ALP-positive area at day 10 (B) on machined titanium surfaces: new, 4-week-old and UVC-treated 4-week-old surfaces. The cell density at days 3 and 5 (C) and ALP-positive area (D) on sandblasted titanium surfaces. Data are shown as mean ± SD (n = 3) for all panels.
composition promoted the healing of bone defects [22]. As indi-
hydrophobic polymer scaffold materials created by altering the
ing improved hydrophilicity, increased osteoblast proliferation
osteogenic cells, biofilms coated with calcium phosphate, exhibit-
improved hydrophilicity [19]. Regarding the response of stem/
was found in fibroblasts cultured on polyhydroxyalkanoates with
ment and proliferation [18]. In contrast, a lower rate of proliferation
change. For instance, poly(lactide-co-beta-benzyl malolactonate)
ment upon the type of cells, substrates, and the range of wettability
behavior and response has been contentious and may be depen-
dent upon the type of cells, substrates, and the range of wettability
change. For instance, poly[laque-co-beta-benzyl malolactonate]
with improved hydrophilicity promotes NIH3T3 fibroblast attach-
ment and proliferation [18]. In contrast, a lower rate of proliferation
was found in fibroblasts cultured on polyhydroxyalkanoates with
improved hydrophilicity [19]. Regarding the response of stem/
osteogenic cells, biofilms coated with calcium phosphate, exhibit-
ing improved hydrophilicity, increased osteoblast proliferation
[20]. In contrast, superhydrophobic TiO2 nanotubes attract more
esenchymal stem cells than hydrophobic ones [21]. More
hydrophobic polymer scaffold materials created by altering the
composition promoted the healing of bone defects [22]. As indi-
cated in these studies, it has been understandably difficult to obtain
consistent results and make definitive conclusions on the impor-
tance of the hydrophilic status of materials in determining their
biocompatibility because of the following reasons: (1) the surface
topography and composition of the substrates, together with the
change in the hydrophilicity, vary significantly, (2) the ranges of the
previously tested change in hydrophilicity are relatively small, and
(3) the superhydrophobic nature (<5° of contact angle) of the
substrate was rarely tested. As shown in this study, UV treatment of
titanium creates a substantial change of wettability from hydro-
phobic (>50° contact angle) to superhydrophilic (<5° of water
contact angle), while maintaining an identical surface topography
of the substrates. In addition, UVA- and UVC-treated surfaces are
implicated to involve other surface properties that discriminate
each other, although both surfaces are superhydrophilic. These
surfaces may be useful as model surfaces to study the biological
role and significance of surface hydrophilicity in biomaterials.
Further studies implementing gradually changing levels of hydro-
philicity, and greater levels of titanium aging, should be conducted
to explore their biological effects.

The amount of surface carbon, a change in surface chemistry
occurring during aging and UV treatment apart from the hydro-
philic status, may be responsible, at least in part, for the different
bioactivity levels between the aged, new, and UVC-treated surfaces.
The bioactivity not restored on the UVA-treated 4-week-old surface
was associated with the higher percentage of carbon still remaining
on its surface as compared to the new and UVC-treated 4-week-old
surfaces. Our previous study applying different doses of UVC
revealed a strong correlation between the amount of carbon and
osteoblast attachment on titanium surfaces. From this perspective,
UVA treatment should have resulted in an improvement of the
degraded bioactivity, although not as great as the UVC treatment,
along with its partial removal of carbon; however, it turned out to
be negative. In terms of the different abilities of UVA and UVC for
removing hydrocarbons, UVC is generally capable of a direct
photodecomposition of organic components, whereas UVA
removes hydrocarbons via its photocatalysis [23–25]. However, our
recent study showed that light absorbance bands of titanium bulk
peaked around 300 nm [1], implying that bulk titanium may
respond to a slightly lower range of wavelength than UVA for
initiation of photocatalysis. In addition, recent studies in environ-
mental sciences compared various wavelengths of UV and
demonstrated that UVC is more effective than UVA in degrading
hydrocarbons [24–27]. These studies may explain why the UVC-
treated surface contained less carbon.

This study revealed the UV-induced enhancement of bioactivity
of a chromium–cobalt alloy. The interpretation of the age-degraded
bioactivity and its UVC-mediated restoration on chromium–cobalt
alloy suggests a hypothesis. Since this alloy has little or no photo-
catalytic potential, age-related accumulation of surface hydrocar-
bons and its direct UVC-mediated photolysis are, at least at this
point, the likely explanation for these phenomena. However, the
role of hydrocarbons in determining the bioactivity of these
materials remains to be explored. One possibility for TiO2 surfaces
is that the removal of hydrocarbon results in the exposure of Ti4
and may facilitate its interaction with biological cells that are
electronnegatively charged [1]. Recent reports suggest that surface
hydrocarbons play a role in determining the hydrophilic nature of
Ti; the more the hydrocarbons absorbed, the higher is the contact
angle of H2O [4,28]. The role of surface hydrocarbons alone as well
as possible synergistic effects of hydrocarbons, hydrophilicity and
other surface properties, is of interest.

The present results regarding the degradation of the bioactivity
of titanium over time may have two potentially major implications.
Clinically, titanium implant products, regardless of dental or

![Fig. 8. Osteoblastic phenotypes altered by aging and UVC treatment on other biological-grade metal substrates. The cell density at days 3 and 5 (A) and ALP-positive area at day 10 (B) on sandblasted chromium–cobalt alloy. Data are shown as mean ± SD (n = 3).](W. Att et al. / Biomaterials 30 (2009) 4268–4276 4275)
orthopedic use, have been considered biologically stable to exert invariable clinical performance. Currently, there is no regulation or expiration of manufacture, distribution, and storage of these products, including dental implants, femur stems, and plates and screws for spine surgery. The time-related deteriorating bioactivity of titanium, as demonstrated in this study, should draw an immediate and strong interest for further investigation as well as for potential clinical implications and public attention. Second, to the best of our knowledge, the age of titanium surfaces has never been standardized in previous literature in the biomaterial field. We have revealed that the effect of time since processing of titanium is critical for determining its bioactivity. For instance, titanium substrates with identical surface topographies could generate a substantial difference in their bioactivities only because of their different ages. All three different surface topographies of titanium we tested as well as the chromium–cobalt alloy, another popular implantable metal, manifested this age-related attenuation of bio-properties, implying that this nature may be applicable for many of implantable metallic materials. Future studies to compare different surfaces for their biological and osteoconductive potentials may require the standardization of the age of experimental samples, or a careful interpretation of the research outcome. In order to confirm these potential impacts, in vivo studies and additional in vitro studies with more titanium samples from different levels of aging are essential. Thus, this study provides a new important insight into further advancing titanium biological research and implant therapeutics.

5. Conclusions

This study examined the possible changes in the bioactivity of titanium surfaces during their aging and investigated the effect of ultraviolet (UV) light treatment during the age-related change of titanium bioactivity. Rat bone marrow-derived osteoblasts were cultured on new titanium disks (immediately after either acid-etching, machining, or sandblasting), 4-week-old disks (stored after processing for 4 weeks in dark ambient conditions), and 4-week-old disks treated with UVA or UVC. Cell attachment, proliferation, alkaline phosphatase activity, and mineralization of osteoblasts were substantially lower on the 4-week-old surfaces than on the new surfaces, while they were higher on the UVC-treated 4-week-old surfaces as compared to the new surfaces. UVA treatment had no such recovering effects. The age-related impairment of bioactivity was also found on a chromium–cobalt alloy, and was associated with an increased percentage of surface carbon. Although both UVA and UVC treatment converted the 4-week-old titanium surfaces from hydrophobic to superhydrophilic, only UVC treatment effectively reduced the surface carbon to a level equivalent to the new surface. These results suggest a time-related degrading bioactivity of titanium and chromium–cobalt alloy, and its restoration enabled by UVC phototreatment, which surpasses the innate bioactivity of new surfaces.

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Appendix

Figures with essential colour discrimination. Parts of Fig. 6 in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.04.048.

References
