Biomacromolecules **2007,** *8,* 3965–3972

Functionalized Titanium Oxide Surfaces with Phosphated Carboxymethyl Cellulose: Characterization and Bonelike Cell Behavior

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Received September 17, 2007; Revised Manuscript Received October 10, 2007

The performance of dental or orthopedic implants is closely dependent on surface properties in terms of topography and chemistry. A phosphated carboxymethylcellulose containing one phosphate group for each disaccharide unit was synthesized and used to functionalize titanium oxide surfaces with the aim to improve osseointegration with the host tissue. The modified surfaces were chemically characterized by means of X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy. The investigation of the surface topography was performed by atomic force microscopy measurements before and after the polysaccharide coating. In vitro biological tests using osteoblast-like cells demonstrated that functionalized TiO_2 surfaces modulated cell response, in terms of adhesion, proliferation, and morphology. Phosphated carboxymethylcellulose promoted better cell adhesion and significantly enhanced their proliferation. The morphology of cells was polygonal and more spread on this type of modified surface. These findings suggest that the presence of a phosphate polysaccharide coating promotes osteoblast growth on the surface potentially improving biomaterial osseointegration.

Introduction

Titanium and titanium-based alloys have been used as excellent materials for dental implants, artificial hip joints, and bone screws thanks to their high biocompatibility and their good mechanical properties being similar to bone.[1] Moreover, the presence of a oxide layer formed upon exposure to air guarantees an excellent inertness of its surface, allowing it to readily heal into the bone tissue. Despite all these excellent properties, a true adhesion between the bone and the metal surface has not yet been observed.[2] The osseointegration, especially its speed, is a major determinant of implant success. Surface topography and surface chemistry are crucial for the short- and long-term success of the osseointegration process. A general need for improved healing response and increased speed of osseointegration of the biomaterial with the host tissue is still a relevant topic.[3] For this reason, much effort has been made in the past in order to optimize the properties of titanium oxide surface by manipulating the surface chemical composition and the surface topography.[4]

Concerning the topographical modifications of titanium oxide surfaces, they are all finalized to increase the surface roughness. Different topographies can be achieved with the help of various techniques which span from mechanical methods (like micromachining, particle blasting, polishing, etc.)[5,6] to chemical methods (acid or alkaline, hydrogen peroxide treatments).[7–9] In this way the implant's surface looks like the bone tissue, promoting the osseointegration of the device. The modification of the surface chemistry of titanium oxide includes different possible approaches that allow the tailoring of surface properties. Nonspecific physical adsorption of peptides, proteins, or blood components have been investigated.[10] Layer-by-layer self-assembly of polyanions and polycations have been used to engineer the surface of titanium oxide.[11] Among the different methods which involve the covalent attachment of the target molecule to the surface, silanization and molecular assembly are the most used thanks to the simplicity with which well-defined surface film can be produced. Silanization has often been used to introduce functional groups that can be further functionalized with cross-linker-containing molecules with biological activity such as peptides or proteins.[12–14] Among all the molecules which can be used for molecular assembly, alkane phosphates and alkane phosphonates have received attention because they show a strong chemical affinity toward transition metal surfaces. Several papers deal with the structure and properties of alkane phosphates on metal oxide surfaces.[15] Recently self-assembled monolayers of alkane phosphates or phosphonates have been used on a titanium oxide surface to tailor physical−chemical properties of the surface such as wettability, surface charge, and biofouling properties.[16]

In this paper we describe a method to functionalize titanium oxide surfaces with a phosphated polysaccharide in order to increase the osseointegration to the host bone tissue. Carboxymethyl cellulose (CMC) is a negative charged derivative of cellulose obtained by introducing CH_2COOH groups in the polymer chain. CMC containing one phosphate group for each disaccharide unit (CMCP) has been used to functionalize titanium oxide (TiO_2) surfaces.[17] Thanks to the presence of phosphate groups, which induce osseointegration,[18] the modified TiO_2 surfaces should be able to increase bone cell growth and metabolic activity. Moreover, the presence of a polymer film may allow a further functionalization of the surface exploiting the carboxylate groups and the phosphate groups present in the polysaccharide backbone.

The chemistry of modified TiO_2 surfaces was characterized by X-photoelectron spectroscopy (XPS), and grazing angle equipped Fourier transform infrared (FT-IR) spectroscopy; information about surface topography, roughness, and stiffness

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10.1021/bm701033u CCC: $37.00

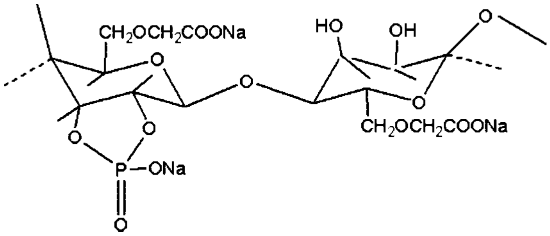

Figure 1. Schematic representation of a CMCP-Na disaccharidic unit containing one phosphate group.

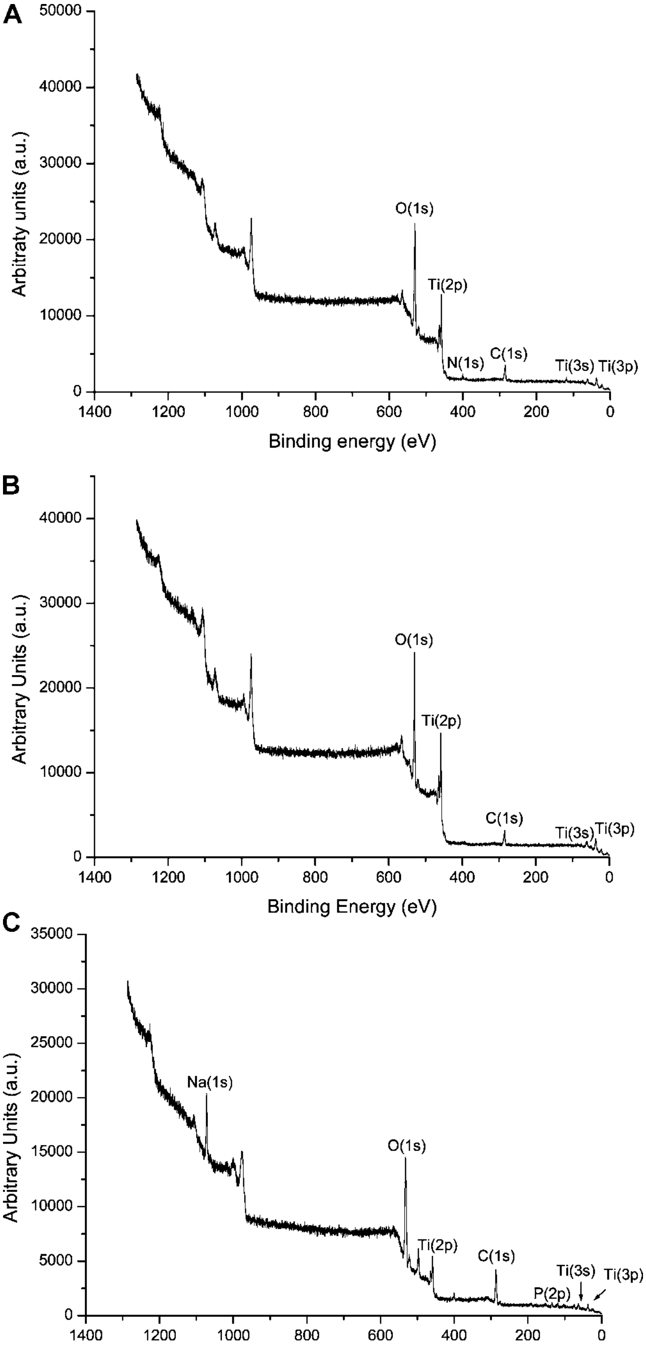

Figure 2. XPS survey spectra of (A) native TiO_2 surface, (B) etched TiO_2 surface, and (C) CMCP-functionalized TiO_2 surface.

Table 1. Surface Elemental Composition (Atomic Percentages) for Native TiO_2, H_2SO_4/H_2O_2 Treated TiO_2 Surface, and CMCP-Functionalized TiO_2 Surface

element	assignment	binding energy (eV)	normalized intensities (%)
(a) Native TiO_2			
O(1s)	TiO_2	530.1	51.5
Ti($2p_{1/2}$)	TiO_2	458.6	38.5
Ti($2p_{3/2}$)	TiO_2	464.4	
C(1s)	CH_2, CH_3	285.0	7.8
N(1s)	N−H, C−N	400.1	1.2
(b) H_2SO_4/H_2O_2 Treated TiO_2 Surface			
O(1s)	TiO_2	530.1	52.0
Ti($2p_{1/2}$)	TiO_2	458.6	41.8
Ti($2p_{3/2}$)	TiO_2	464.3	
C(1s)	CH_2, CH_3	285.0	6.2
(c) CMCP-Functionalized Surface			
O(1s) (1)	TiO_2	530.1	50.0
O(1s) (2)	P−O−R P−O−H	532.6	
Ti($2p_{1/2}$)	TiO_2	458.5	12.2
Ti($2p_{3/2}$)	TiO_2	464.4	
C(1s) (1)	CH_2, CH_3	285.0	16.7
C(1s) (2)	C−O−C, C−O−P	286.3	
C(1s) (3)	O−C−O, C=O	287.9	
P(2p)	$C{-}O{-}P{=}O(O)^-{}_2$	134.1	1.9
Na(1s)	PO_4^{3-} Na^+	1071.9	19.1

was obtained by atomic force microscopy (AFM) measurements. Stability of the CMCP coating has been evaluated under physiological conditions at 37 °C for 7 days. In vitro biological tests using osteoblast-like cells were performed in order to assess the cell behavior compared to nonfunctionalized TiO_2 surfaces. The surface modification yielded a CMCP-coated surface with a well-defined chemistry and a good biological performance.

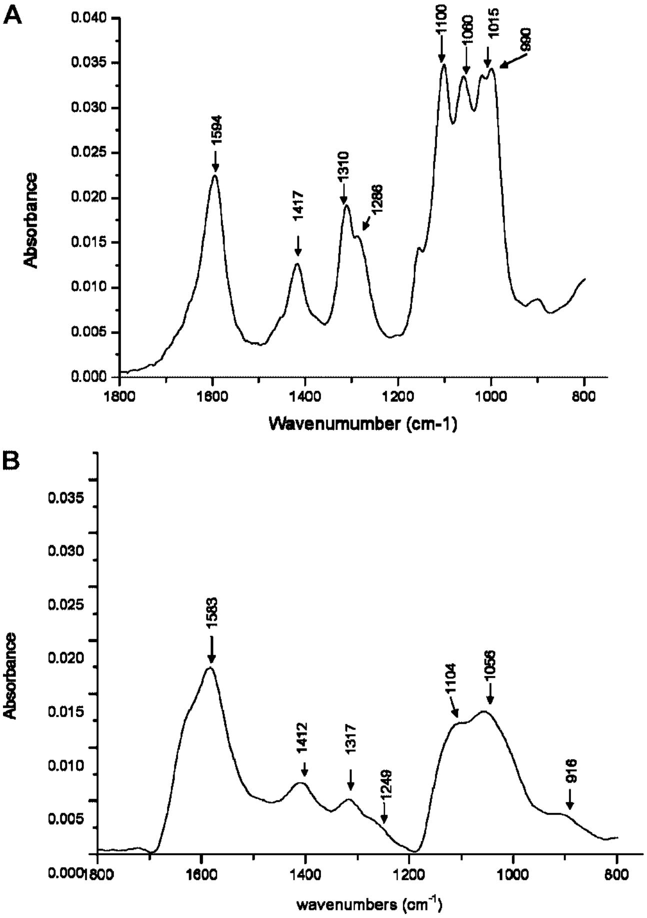

Figure 3. FT-IR spectra of (A) CMCP sodium salt (lyophilized state: content of water ~10–15% w/w) and (B) CMCP-TiO_2 surface. Spectral region: 2000–750 cm^{-1} for parts A and B.

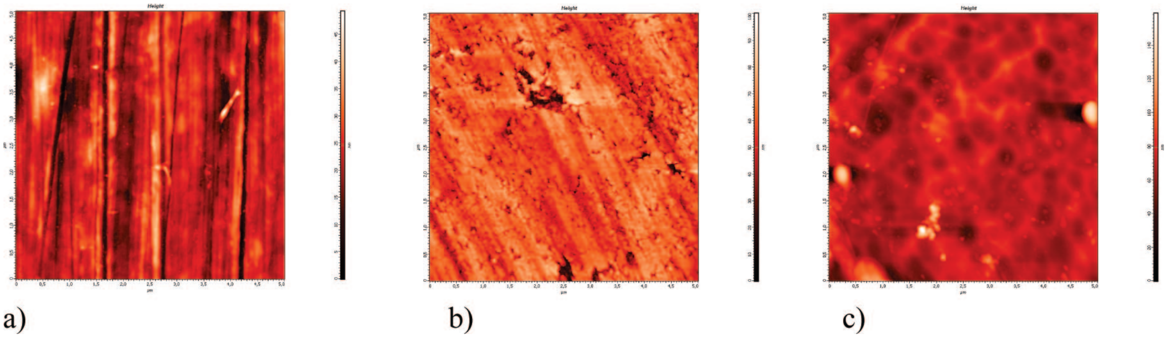

Figure 4. AFM scans (scan size 5 × 5 μm^2) of (a) TiO$_2$ native substrate, (b) etched TiO$_2$, and (c) CMCP-functionalized TiO$_2$ surface.

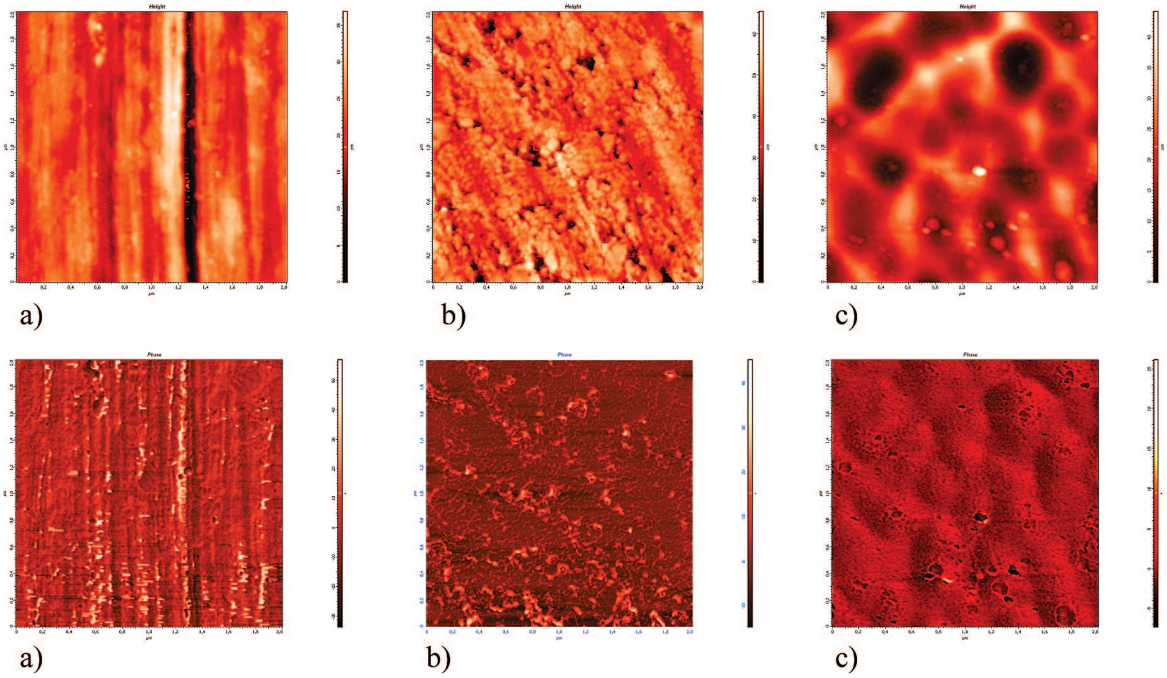

Figure 5. AFM scans (scan size 2 × 2 μm^2) of topography (top line) and phase mode (bottom line) of (a) TiO$_2$ native substrate, (b) etched TiO$_2$, and (c) CMCP-functionalized TiO$_2$ surface.

Materials and Methods

Materials. Carboxymethylcellulose sodium salt (CMCP-Na) containing one phosphate group for each disaccharidic unit was allowed to adhere onto freshly cleaned titanium oxide surface. The introduction of phosphate groups into CMC-Na (Hercules SpA, Italy) was obtained as already described elsewhere,[17] and the determination of the amount of phosphate groups was performed by elemental analysis.[17] The structure of the disaccharidic unit of CMCP-Na is shown in Figure 1. Commercial titanium oxide disks (grade 2, diameter 12 mm, thickness 1 mm) were supplied by L.I.M.A-Lto SpA (Italy). All the chemicals were purchased from Sigma-Aldrich.

Titanium Oxide Surface Functionalization. Prior to the surface modifications, the TiO$_2$ disks were ultrasonicated (CP-104 Bio-Class) in 2-propanol for 10 min at room temperature twice in order to remove macroscopic contaminant. Then the disks were dipped into a H_2SO_4/H_2O_2 mixture 1:1 vol/vol for 5 min and then rinsed with plenty of double distilled water. After acid mixture dipping, the disks were referred to as etched TiO$_2$. The disks were finally dried with filtered (0.22 μm) nitrogen flow and immediately dipped into a CMCP-Na aqueous 5 mM solution. The surfaces were maintained for 48 h at room temperature to allow the polymer to bind to the surface, then they were rinsed with double distilled water (even under flow) and dried with filtered nitrogen flow.

Surface Characterization. *X-ray Photoelectron Spectroscopy (XPS).* The surface chemical composition of native, treated with acid mixture and CMCP-functionalized TiO$_2$ disks, was investigated by XPS analysis. Three samples for each surface type were analyzed in order to guarantee a reproducible measurement. XPS spectra were recorded in an ultrahigh vacuum (UHV) chamber equipped with a hemispherical electron energy analyzer (VSW HA150) and a nonmonochromatized Al Kα (1486.6 eV) X-ray source. The X-ray gun was operated with a power of 180 W. The electron takeoff angle was 90°. Low-resolution survey spectra were collected from 400 to 1490 eV of electron kinetic energy with a constant pass energy of 90 eV. Higher resolution spectra of the most relevant peaks were acquired with a constant pass energy of 44 eV. The analyzed area was a 5 mm wide circle. All binding energies were referenced relative to the main hydrocarbon peak (from residual hydrocarbon contamination and from the polymer in the case of CMCP-coated surfaces) set at 285.0 eV. The data analysis was performed by Casa-XPS software.

Grazing Angle Ft-IR Analysis. Infrared analysis was performed by FT-IR spectrometer, Bio-Rad FTS 6000, purged with nitrogen. Spectra of CMCP-functionalized surfaces (at least three samples) were acquired with the help of grazing angle specular reflectance accessory (80-spec) which allows us to get information about the vibrational modes of relatively thin layers equipped with a 45° Ge ATR crystal and a mercurium cadmium telluride (MCT) detector. Measurements were performed with a manual p-polarizer mounted on incoming beam to enhance the signal with a transmission efficiency of 90% and a theoretical degree of polarization of 99%. A total of 512 scans at a resolution of 4 cm^{-1} were averaged for each spectrum. Bare TiO$_2$

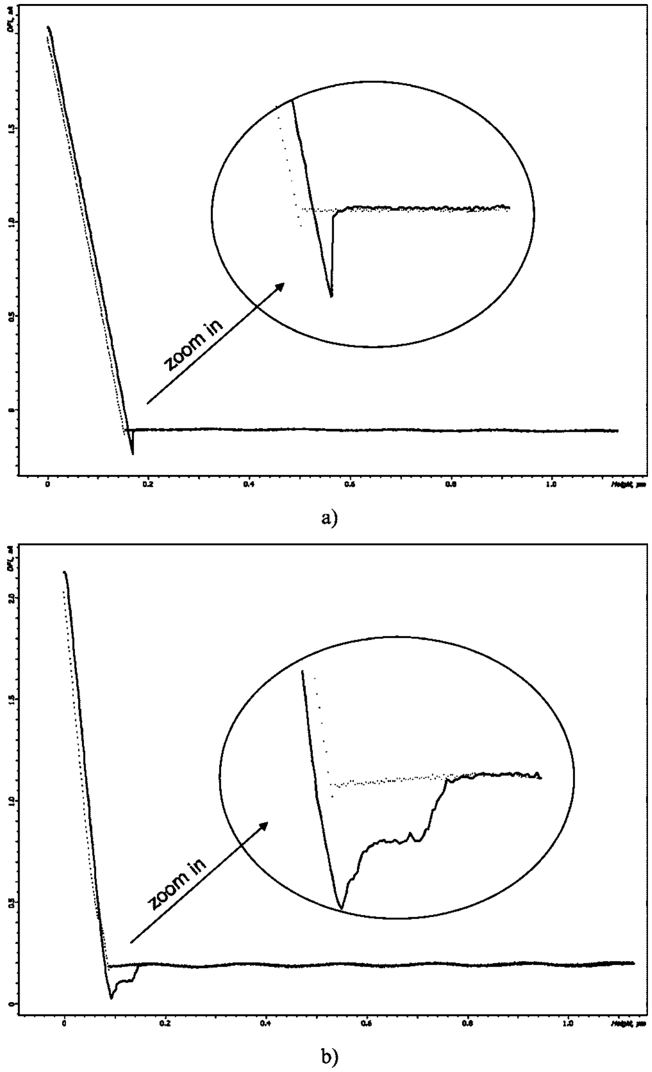

Figure 6. Force vs distance curve for (a) TiO_2 and (b) CMCP−TiO_2 surfaces. Dotted line represents the approaching curve and straight line represents the retraction curve.

substrates were used to acquire the background spectrum. Spectra were elaborated by WIN-IR PRO version 2.6, recorded by baseline correction and smoothing (boxcar function; 9 N. of P.).

Atomic Force Microscopy (AFM). Freshly cleaned, H_2SO_4/H_2O_2 mixture treated, and CMCP-functionalized TiO_2 surfaces were viewed by AFM (Solver Pro, NT MDI Instruments, Russia). AFM images were acquired in air in noncontact mode on five different areas for sample (three samples for type) with a sharpened gold-coated silicon tip with a spring constant of 2.5–10 N/m and using a nominal resonance frequency between 120 and 180 kHz.

Surface roughness parameters, (R_a) and (R_q) of five characteristic scan areas (scan size $5 \times 5 \mu m^2$) of the different substrates were determined using SPLM-Laboratory version 5.01 software. The arithmetic average (R_a) and the root mean squares (R_q) roughness are defined as follows:

R_a is the arithmetic average of the absolute height values of the m points of the profile and determined by the following equation:[19]

$$R_a = \frac{1}{m}\sum_{i=1}^{m} |z(x_i)| \tag{1}$$

R_q, also called rms (root mean square), is the root mean square of all the values of the profile and defined by the following equation:[19]

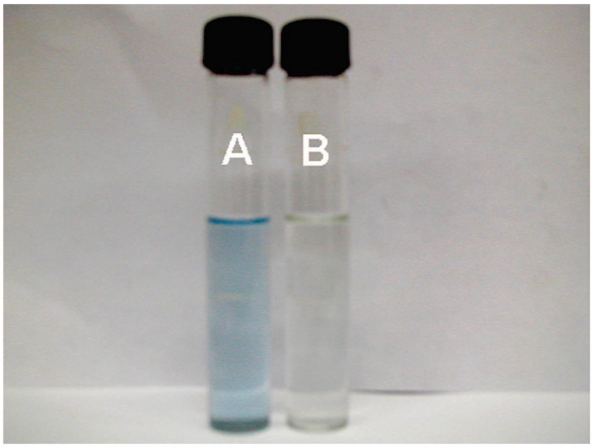

Figure 7. Results of the test for the determination of the phosphate groups: test tube A, solution containing CMCP detached from the TiO_2 disks; test tube B, water (control solution).

$$R_q = \sqrt{\frac{1}{m}\sum_{i=1}^{m} z^2(x_i)} \tag{2}$$

Force curves between the AFM probe and the TiO_2 and CMCP-modified TiO_2 surface were measured at room temperature. Force was measured in spectroscopy force mode by collecting a force curve which is the plot of the cantilever deflection as a function of sample position along the z axis. Silicon cantilevers coated with gold with a spring constant of 5 N/m (provided by the manufacturer) were used. Force measurements were performed on the different positions (ten curves for each spot).

Stability of the CMCP Coating. The coating stability was investigated by maintaining the CMCP-functionalized TiO_2 disks (three samples) immersed in phosphate buffer solution (PBS, pH 7.4) at 37 °C in controlled atmosphere (air 95%, CO_2 5%) for 7 days. After that time, the disks were rinsed with plenty of double distilled water to remove all the PBS traces, dried with filtered (0.22 μm) nitrogen flow, and finally dipped in 0.5 M NaOH solution to detach all the CMCP bound to the TiO_2. The presence of the polymer in the solution was verified by determining the amount of phosphate groups by using a AQUANAL-PLUS test (Riedel-de-Häen). The test is based on the formation of a blue-colored complex between the phosphate groups of the polysaccharide and the ammonium molybdate (phosphomolybdenum blue). The measured range of the test is between 0.02 and 0.4 mg of phosphate groups mg/L. The amount of phosphates was determined by spectrophotometric analysis (UV/visible spectrophotometer UltroSpec 3300, Biosciences) on the basis of a calibration curve obtained using solutions at a known concentration. The same test was performed on three TiO_2 disks on which the CMCP was allowed to adhere without the acid/hydrogen peroxide etching in order to verify if the surface pretreatment is necessary for the polymer binding.

Biological Tests. *Cell Cultures.* Cell behavior was studied on bare and functionalized TiO_2 disks using a line of osteoblast-like cells (MG-63) derived from a human osteosarcoma. Cell medium was Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% of heat-inactivated fetal calf serum, 1% of antibiotic−antimycotic solution (10.000 U of penicillin, 10.000 μg of streptomycin, 25 μg of amphotericin B/mL), 1% of vitamin solution, 1% of nonessential amino acids, and 2 mM of L-glutamine. Cells were plated at the concentration of 1.5×10^4 cells/cm^2/100 μL on three samples for each type (TiO_2, etched TiO_2, and CMCP TiO_2). Disks were previously ethanol-sterilized and rinsed with PBS. When cells had supposedly attached to the surface, the well was filled with fresh medium.

Scanning Electron Microscopy (SEM) Analysis. Disks were washed in phosphate buffer solution (PBS) to remove any nonadhering cells and fixed with 4% paraformaldehyde in 0.1 M PBS for 10 min at room

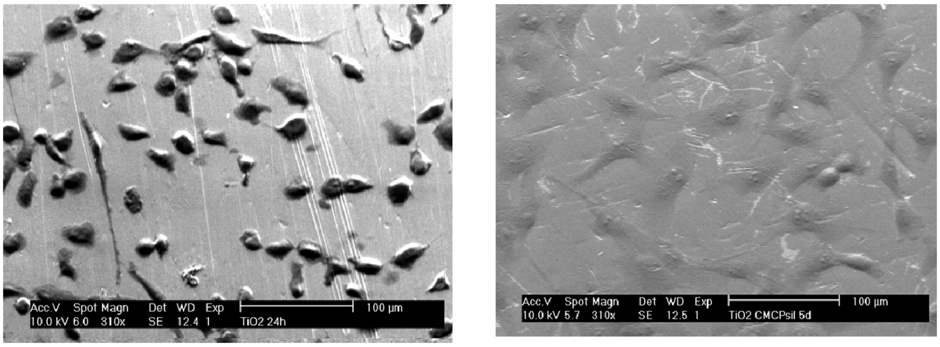

Figure 8. SEM micrographs showing MG-63 cells cultured on (left) TiO_2 and (right) CMCP TiO_2 surfaces at 24 h of culture. The cells seeded on CMCP TiO_2 are more spread.

temperature, dehydrated, desiccated overnight, gold sputtered in a sputter coater (BAL-TEC SCD 050, Balzer, Germany), and finally analyzed by SEM (XL20 Philips, The Netherlands) at 15 kV accelerating voltage.

Evaluation of Focal Adhesion Molecules (Integrins and Focal Adhesion Kinase). A double staining was performed in order to determine whether focal adhesion kinase pp125 ^{FAK} (FAK) colocalized with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. For FAK$-\alpha_v\beta_3$ double staining, cells were fixed with acetone at −20 °C for 10 min whereas for FAK$-\alpha_5\beta_1$ double staining cells were fixed with 4% paraformaldehyde in PBS. First labeling with monoclonal antibodies to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins (Chemicon, 1:30 in 0.5% BSA in PBS and 0.1% Triton in PBS, hereafter referred to as buffer) was performed overnight at 4 °C. The reaction was revealed with a TRITC (tetramethylrhodamine isothiocyanate) conjugated antimouse IgG secondary antibody (Dako, 1:100 in buffer) at room temperature for 2 h. Second labeling with a polyclonal primary antibody to FAK (Sigma, 1:20 in buffer) was done for 2 h at room temperature. The reaction was revealed with a FITC (fluorescein isothiocyanate) conjugated polyclonal anti-rabbit IgG secondary antibody (Sigma, 1:200 in buffer) for 2 h at room temperature. The surfaces were viewed by fluorescence microscopy (Nikon ECLIPSE E600 equipped with Digital Still Camera DMX 1200).

Cell Counts. Since TiO_2 disks are not transparent, the number of adherent cells was evaluated by labeling cell nuclei with a fluorescent dye (Hoechst, 1:1000 in phosphate buffered solution, PBS). Ten randomly selected areas were photographed for each sample (all samples were in triplicate) and the nuclei counted on photographs (the area of the photographic field at the magnification used of 40× was 3.5×10^{-4} cm^2). Statistical evaluation was performed with Student's *t* test.

Results and Discussion

Characterization of Bare, Etched, and Functionalized TiO_2 Surfaces. *XPS Analysis.* The survey XPS spectra of native, H_2SO_4/H_2O_2 etched and CMCP-functionalized TiO_2 surfaces are reported in Figure 2. The XPS analysis of the native (cleaned with 2-propanol in u.s bath) TiO_2 substrate showed the presence of titanium, oxygen, carbon, and nitrogen (Table 1a). The C and N are due to adventitious contamination which cannot be completely removed especially for samples which are exposed to ambient air. However, this degree of contamination is conformal to the percentages found after a common process of surface cleaning.[20] No presence of Ti metal was detected meaning that all the surface is covered by an oxide layer thicker than 10 nm (XPS penetration depth). However the spectrum is perfectly consistent with those of titanium oxide flat surfaces reported in literature.[20]

The XPS of H_2SO_4/H_2O_2 treated TiO_2 showed that the acid treatment caused a decrease of the percentage of carbon and disappearance of N(1s) signal due to the removal of any residual organic traces (Table 1b). This is due to the etching caused by the contact with the acid solution. Table 1c summarizes the experimental binding energies and relative assignment of O(1s), Ti(2p), C(1s), P(2p), and Na(1s) peaks after the CMCP coating. The increase of C(1s) signal together with the appearance of P(2p) provides evidence that the CMCP polymer adhered to the TiO_2 surface. The peak fitting of the O(1s) shows that there are at least two components of the O(1s) signal, one at 530.1 eV, typical of metal oxide, and another at 532.6 eV due the polymer binding to the surface. The peak fitting of C(1s) shows that besides the contribution of adventitious carbon, the main peak components located at 286.3 eV can be attributed to carbon singly bonded to oxygen, while the other components at 287.9 eV to carbon double bonded to oxygen ($-C-O-C-$ and $-C{=}O$ groups).

Grazing Angle Ft-IR Analysis. Surface infrared analysis evinced the presence of a thin layer of CMCP onto TiO_2 surface (see Figure 3). The spectrum of CMCP polymer is reported in Figure 3A. The presence of phosphate groups was revealed from the peaks in the region between 900 and 1100 cm^{-1}. In particular, the double peak at 990–1015 cm^{-1} is ascribed to the symmetric stretching of the $P-O-C$ bonds and the peak at 1060 cm^{-1} is attributed to the asymmetric stretching of the same bond. The peaks ascribable to the polysaccharidic ring are overlapped in the same region (1100–900 cm^{-1}) making the definitive assignment of these bands quite difficult. The double bands at 1286 and 1319 cm^{-1} were attributed to the $P{=}O$ and $P-O-H$ stretching vibrations. Moreover, the typical bands of CMC of carboxylate groups are however present in their typical position (1594 cm^{-1} asymmetrical stretching, 1417 cm^{-1} symmetrical stretching).

Figure 3B shows the spectrum of TiO_2 surface after CMCP coating. The spectrum is very similar to that of the CMCP polymer before the surface grafting (Figure 3A). The peaks at 1104 and 1056 cm^{-1} which were assigned to the $O-P-C$ stretching, are broadened and slightly shifted with respect to those of the dry polymer which are located at 1110 and 1060 cm^{-1}. The peak at 916 cm^{-1} (attributed $P-O-C$ stretching) is decreased and shifted with respect to that of unbound CMCP. The characteristic double band located at 1286–1319 cm^{-1} (ascribed to $P{=}O$ and $P-O-H$ groups) is broadened and shifted to 1317 and 1249 cm^{-1} after the CMCP surface binding, as it happens for the one belonging to $O-P-C$ stretching vibration. The shift of these two bands was interpreted as evidence for the fact that the phosphate groups of CMCP are involved in the bond with titanium oxide surfaces. The shift and in some cases the disappearance of the double peak in the region 1280–1320 cm^{-1} were also observed in previous works[15,21] and considered as evidence for the binding of phosphate groups with a metal oxide surface.

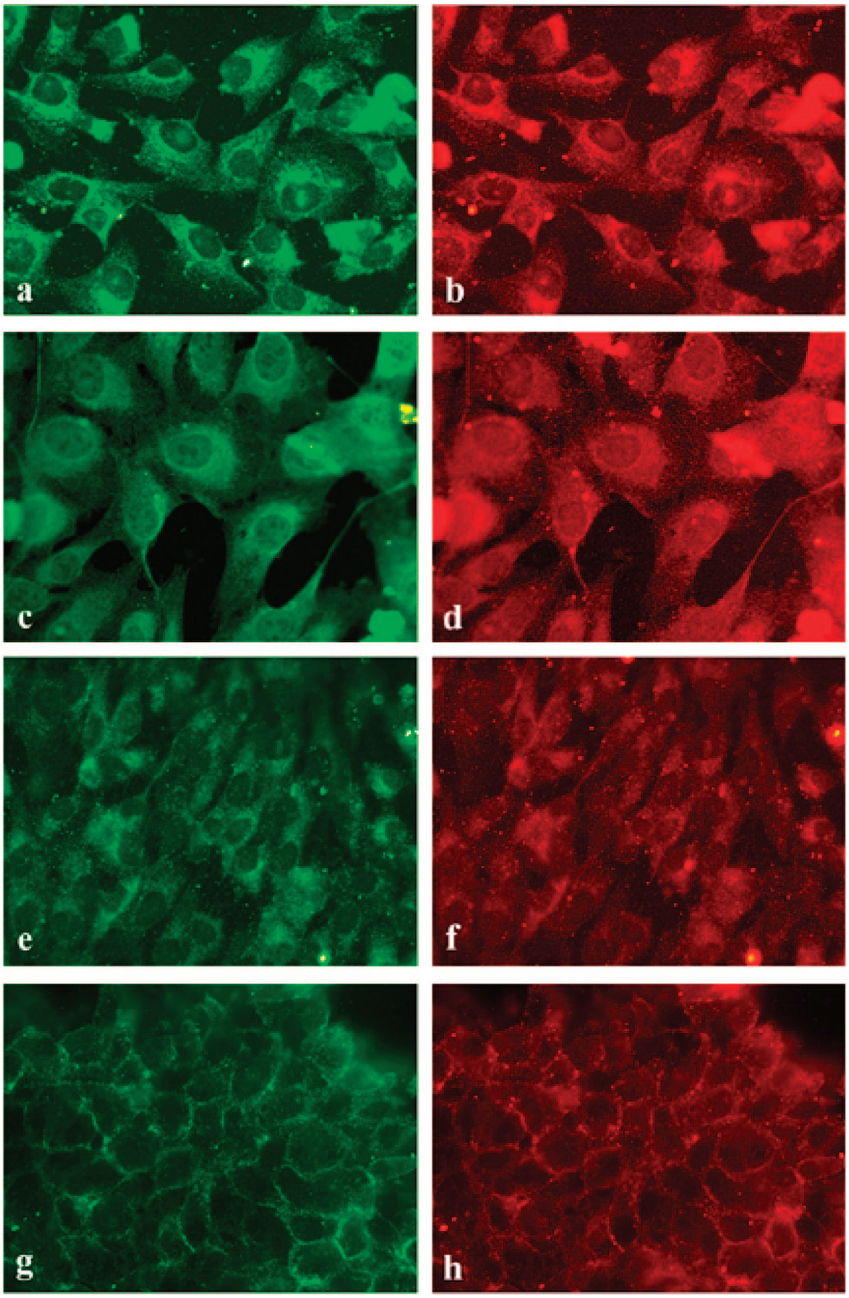

Figure 9. Immunostaining for FAK (left column) and integrins (right column): (a) FAK and (b) $\alpha v\beta 3$ staining of MG-63 osteoblasts cultured on etched TiO_2; (c) FAK and (d) $\alpha 5\beta 1$ staining of MG-63 osteoblasts cultured on treated TiO_2; (e) FAK and (f) $\alpha v\beta 3$ staining of MG-63 osteoblasts cultured on CMCP TiO_2; (g) FAK and (h) $\alpha 5\beta 1$ staining of MG-63 osteoblasts cultured on CMCP TiO_2. FAK and integrins colocalized on all substrates. Original magnification 40×.

AFM Analysis. The surface topography and surface roughness of native, etched and CMCP-functionalized TiO_2 surfaces were evaluated by AFM. AFM scans of the different surfaces at two different scan sizes are reported in Figure 4. The images are representative of the whole surface. The native TiO_2 surface (commercial disks) was characterized by the presence of parallel lines and scratches (due to the fabrication procedure) with a surface average roughness (R_a) of 4.30 ± 0.4 nm ($R_q = 5.6 \pm 0.6$ nm) (see Figure 4a). After the acid/hydrogen peroxide etching, the surface roughness value increased ($R_a = 10.9 \pm 0.5$, $R_q = 14.0 \pm 0.9$ nm) (Figure 4b). The use of hydrogen peroxide induced the formation of submicrometer porous structure of the TiO_2 layer.[6,7] The topography of CMCP-functionalized TiO_2 surfaces is completely different from the uncovered polymer ones (Figure 4c). The presence of the polysaccharide coating was observed all over the surface. Roughness analysis showed that TiO_2–CMCP surfaces are smoother than the native and treated TiO_2 ($R_a = 8.0 \pm 0.4$, $R_q = 11.2 \pm 0.7$ nm). Phase analysis confirmed the presence of a homogeneous layer of CMCP on the TiO_2 surface (Figure 5 a–c line bottom). In fact, no differences in the phase mode signal were noticed meaning that the surface chemical composition is uniform: TiO_2 for native and etched surfaces, and polysaccharide for CMCP-coated surfaces.

The force versus distance curves of native TiO_2 and TiO_2–CMCP surfaces are plotted in Figure 6. Force versus distance measurements on TiO_2 surfaces showed the typical trend of force–distance curve of a hard material (see Figure 6a). The force versus distance curved measured on CMCP-functionalized TiO_2 surfaces is shown in Figure 6b (approaching

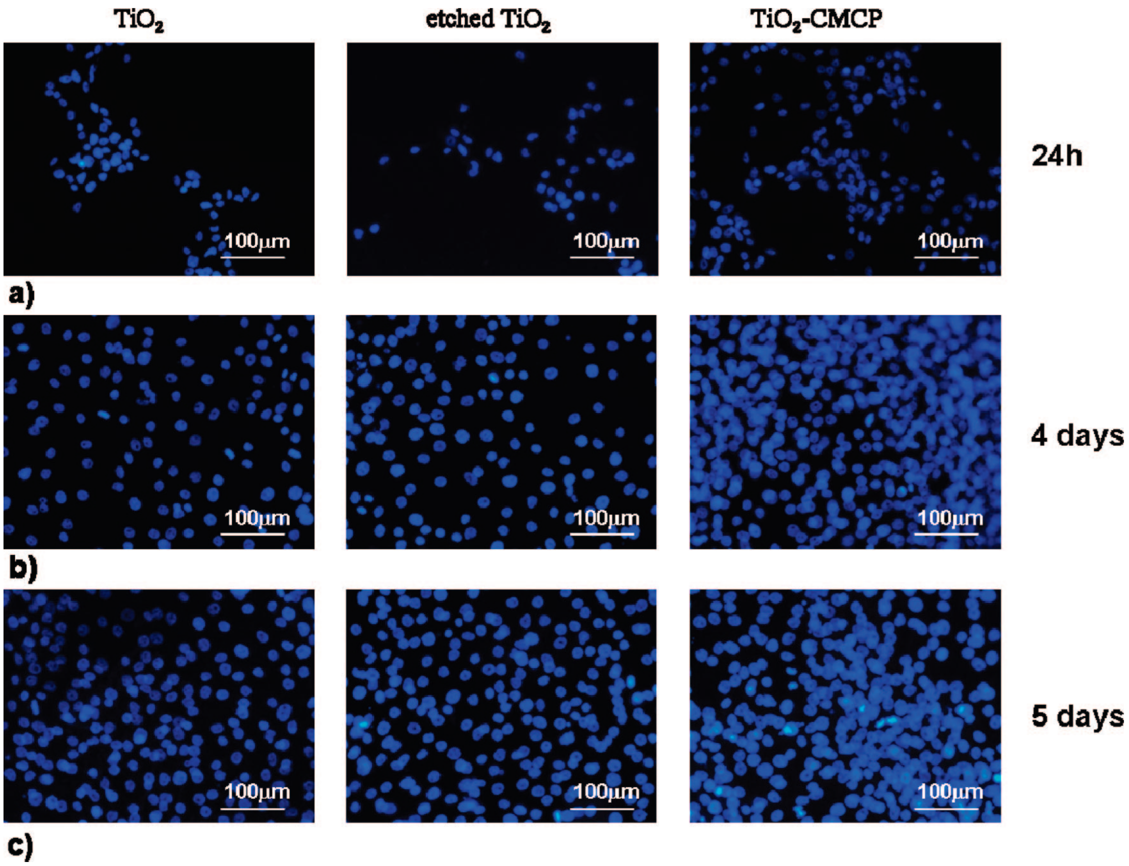

Figure 10. Fluorescent microscopy showing nuclear staining with DAPI of MG-63 cells adhered on the different surface types at (a) 24 h, (b) 4 days, and (c) 5 days of culture.

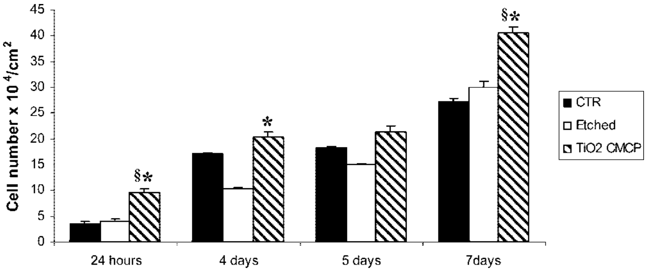

Figure 11. Number of cells × 10^4/cm^2 on the different surfaces at 24 h and at 4, 5, and 7 days of seeding. Data are reported as mean ± standard error. Controls are cells seeded on bare TiO_2 in all cases except at 7 days when cells grown on plastic were used as controls. §*$P < 0.0001$ vs control and etched TiO_2 at the same time; *$P < 0.0001$ vs treated TiO_2 at the same time.

curve dotted line and retracing curve straight line). The trend of the approaching curve indicated that the surface is soft and deformable as demonstrated by the absence of a sharp jump down (the probe and the surface are in contact at about 90 nm). The retraction curve shows that no sharp break occurs in the outgoing of the AFM tip. This behavior suggests that the adhesive interaction between the tip and the CMCP coating is quite strong, the probe is not able to break away form the surface in one time, but it produces a gradual detaching with a plateau part in the curve. This effect may be due to the presence of a multilayer or simply to the soft nature of the polysaccharide. The calculated maximum adhesion force for CMCP-modified surface is larger than that for native TiO_2 substrates (60 nN vs 45nN) because of the soft nature of the polysaccharide. The adhesion area for the polysaccharide-coated surfaces is larger, as expected. This may suggest a sort of indentation of the tip into the CMCP layer down to the metal oxide surface in the approaching step with a sort of compression of the polysaccharide chains. Upon retraction of the AFM tip, the polysaccharide chains, which are quite mobile, have the capability to behave like a spring and lead the system at the starting point.

Stability of the CMCP Coating. The stability of the CMCP coating was verified by spectrophotometric analysis. The presence of the polymer was supported by the determination of the phosphate groups of CMCP coating. The appearance of a light blue in the solution indicated the presence of phosphate groups belonging to the polysaccharide chain (see Figure 7). The amount of phosphate groups, and as a consequence the amount of CMCP, was determined by spectrophotometric analysis. The area density of CMCP bound to etched TiO_2 disks, after being maintained in PBS at 37 °C for 7 days, is approximately 16 ng/mm^2. The data about the surface density of the polysaccharide are conformal to previous findings reported in literature.[22] The amount of bound CMCP on the same disks without H_2SO_4/H_2O_2 etching is 0.5 ng/mm^2 indicating that the acid mixture etching is mandatory for the obtainment of a stable thick coating of the polysaccharide on TiO_2.

Biological Tests. *Cell Morphology at SEM.* SEM analysis showed that after 24 h, the spreading of osteoblast cells cultured on CMCP-modified TiO_2 was greater than that of cells plated on bare and etched TiO_2 surfaces, meaning that the presence of the polymer influenced cell response to TiO_2 substrates (Figure 8). After four days the cells reached confluence and differences in morphology were less pronounced.

Focal Adhesion Molecules ($\alpha_v\beta_3$ and $\alpha_5\beta_1$ Integrins and FAK). The distribution of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins and FAK in MG-63 cultured on treated TiO_2 and TiO_2 CMCP surfaces is shown in Figure 9. Integrin clusters, visible as fluorescent dashes, were observed along cell borders of isolated cells and where cells got in contact with each other. FAK was also characteristically localized along cell borders or at the leading edge of isolated cells or cell colonies, sites where new focal adhesions are probably formed. The colocalization of integrins and FAK suggests that (i) focal adhesions are formed when cells are cultured on bare, etched, and CMCP-functionalized TiO_2 substrates, (ii) cell adhesion of MG-63 osteoblasts to all the examined surfaces is mediated by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, and (iii) signal transduction may thus occur from the TiO_2 substrate into the cell probably promoting substrate-dependent cellular adaptations.

Cell Counts. The aspect of adhered cells after staining of nuclei with Hoechst is shown in Figure 10.

The number of cells adhered to each substrate at various times of culture is shown in Figure 11. During the first 24 h, the cells adhere to the substrate, then they started proliferating. Therefore the time point of 24 h was chosen to detect cell adhesion, whereas days 4, 5, and 7 allowed monitoring of proliferation with time. At 24 h the surface that better promoted cell adhesion was CMCP-TiO_2 ($(9.6 \pm 0.5) \times 10^4$ cells/cm^2). Both bare TiO_2 and etched TiO_2 substrates had a much lower number of adhering cells than CMCP−TiO_2 (bare TiO_2 $(3.4 \pm 0.6) \times 10^4$cells/cm^2, etched TiO_2 $(4.1 \pm 0.4) \times 10^4$cells/cm^2). A progressive increase in cell number was noticed on all surfaces with time with significantly higher levels on CMCP-functionalized surfaces (Figure 11).

Conclusions

We presented a novel method to functionalize titanium oxide surfaces with a phosphated polysaccharide (CMCP). The functionalized surfaces was completely covered by a homogeneus layer of CMCP which is bound to the titanium oxide through the phosphate groups of the polymer. The coating was demonstrated to be stable under physiological conditions at least for 7 days.

The CMCP-functionalized TiO_2 surfaces were able to promote the adhesion and the proliferation of MG-63 cells. The behavior of CMCP on TiO_2 resembled the one observed for the same polymer in a cross-linked form (i.e. hydrogel). Biological tests (i.e. production of collagen type I, osteocalcin, and alkaline phosphatase) performed on osteoblast cells cultured on such scaffolds emphasized the good performance of CMCP toward bonelike cells.[17] The coating of CMCP on TiO_2 surfaces is not tight, as demonstrated by force measurements (AFM), but on the contrary, it allows a high degree of mobility of the polymer chains (as observed in the case of hydrogels) and, as a consequence, a good integration with cells. Hence, the functionalization of TiO_2 surfaces through the bond with CMCP, increased the surface performance in terms of biocompability and bonelike cells growth potentially improving the osseointegration.

Acknowledgment. The authors gratefully thank L.I.M.A-LtO SpA for providing the titanium oxide disks and the national project "Progetti di ricerca innovativa in scienza dei materiali" (PRISMA) PC 23/2005 and MIUR Project No. 2006038548_003 for financial support.

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BM701033U