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A bioactive polymer grafted on titanium oxide layer obtained by electrochemical oxidation. Improvement of cell response

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Abstract The anchorage failure of titanium implants in human body is mainly due to biointegration problem. The proposed solution is to graft a bioactive polymer at the surface of the implant in order to improve and control the interactions with the living system. In this paper, we describe the grafting of poly sodium styrene sulfonate on titanium surface by using a silanization reaction. The key point is to increase the TiOH content at the surface of the implant which can react with methoxy silane groups of 3-methacryloxypropyltrimethoxysilane (MPS). Two procedures were used: chemical oxidation and electrochemical oxidation. The last oxidation procedure was carried out in two different electrolytes: oxalic acid and methanol. These different oxidation methods allow controlling the roughness and the depth of the oxide layer. The methacryloyl group of MPS grafted at the titanium surface by silanization reaction is copolymerized with sodium styrene sulfonate using a thermal initiator able to produce radicals by heating. Colorimetric method, ATR-FTIR, XPS techniques and contact angle measurements were applied to characterize the surfaces. MG63 osteoblastic cell response was studied on polished, oxidized and grafted titanium samples. Cell adhesion, Alkaline Phosphatase activity and calcium nodules formation were significantly enhanced on grafted titanium surfaces compared to un-modified surfaces.

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1 Introduction

Osteointegration of titanium implants is weakened by the absence of strong chemical bonding at the bone-implant interface. This is due to the natural oxide layer formed on the titanium surface which is bioinert and cannot directly bond to bone. Several years after implantation, titanium implant may become encapsulated by a fibrous tissue that isolates it from the healthy bone tissue [1]. Consequently, implants will fail under shear stress, requiring revising surgery.

For this reason, surface modification of titanium is still a very active area of research. Various surface modifications, including chemical treatment [2–5], thermal treatment [6, 7], electrochemical method [8] and anodization [9, 10] have been applied to form a bioactive titanium oxide layer on the metal surface. An alternative method to control the tissue-implant interface consists in grafting peptides such as RGD sequence [11, 12] or biomolecules such as collagen [13] to the titanium surface. Surface functionalization of the material by pro-adhesive ligands would improve cell adhesion and enhance osseointegration on these materials. Cell response is controlled by intracellular signalling pathways that are originally triggered by transmembrane proteins interacting with the modified surface. However, if improvements have been obtained with these materials of new generation, the problem is on one hand the cost of the biological products from an industrial point of view and on the second hand the lost of activity for long period of time.

Our approach was to graft a "model" polymer at the surface of the titanium implant by covalent bonding. Studies carried out in our laboratory have shown that anionic polymers or copolymers such as poly(sodium styrene sulfonate) (pNaSS), poly(methacrylic acid) (pMA),

poly(methacryloyl phosphate) (pMP) can favour osteoblast cell adhesion and differentiation [14, 15]. Recently, the grafting of pNaSS was successful by using radicals issued from titanium peroxides able to initiate the radical polymerization of sodium styrene sulfonate monomer [16]. Titanium peroxides were generated at the titanium surface of implants by immersing them in a solution of pure sulphuric acid and hydrogen peroxide. The inconvenience of this oxidation method is the quality of the obtained titanium oxide layer with many defects and uncontrolled thickness.

In contrast, anodic oxidation allows controlling the oxide layer and optimizing surface density of hydroxyl groups that are propitious to the silane–derivatized spacer arms anchorage. Electrochemical oxidation allows controlling the nature of the titanium oxide layer (thickness, porosity, crystallinity) by modifying parameters such as potential, nature of electrolyte solution, temperature, current density. Hydroxyl-rich oxide layers react with trialkoxysilane coupling agent with an organofunctional substitute as methacrylic group 3-methacryloxypropyltrimethoxysilane (MPS). The radical polymerization of monomers bearing bioactive ionic groups in the presence of the modified titanium surface with pendant methacrylic function allows grafting these ionic groups to the titanium implant.

In this paper, grafting of sulfonate groups to the titanium implant surface was obtained by radical copolymerization of sodium styrene sulfonate NaSS and pendant methacrylic functions linked to the titanium oxide layer. To ensure the grafting success, modified surfaces were characterized by various techniques such as ATR-FTIR, X-ray photoelectron spectroscopy XPS, colorimetric method, contact angle analysis.

The goal of this work was to control the cell response as a function of the chemical surface modification. This will be assessed through cell attachment, alkaline phosphatase activity, morphological studies and calcium formation.

2 Materials and methods

2.1 Preparation of titanium samples

A pure titanium rod (Alfa Aesar) of 12.7 mm in diameter was cut in discs of 2 mm thickness. The samples were ground sequentially using paper from 800 to 1200. All the samples were ultrasonically washed in pure acetone for 10 min, and then dried for more than 24 h. In order to remove contaminants and the natural oxide layer, samples were immersed in a mixture of $H_2O/HNO_3/HF$ (88/10/2 in volume) for 30 s at ambient temperature. After water washing, samples were kept under inert gas. Samples were referenced as Ti_{1200}.

2.2 Oxidation of metal surfaces

Two different ways were used to oxidize Ti_{1200}:

- Chemical oxidation
- Electrochemical oxidation

(a) Chemical oxidation was carried out by immersing Ti_{1200} in sulphuric acid during 1 min in order to remove contaminants. Then, an identical volume of hydrogen peroxide (30wt%) was added to the pure sulphuric acid and titanium samples were let in contact with this solution during 3 min. Then, samples were extensively rinsed with water and immediately used for silanization reaction. Samples were referenced as Ti_{oxc}.

(b) Electrochemical oxidation was performed under galvanostatic condition at temperature of 25°C according to two procedures:

- Aqueous electrolyte (oxalic acid 0.5 M).
- Organic electrolyte (methanol/$NaNO_3$ 10 g/l).

Titanium sample (1 cm^2) and graphite (50 cm^2) were used as anode and cathode, respectively.

The oxidation in aqueous electrolyte was conducted with a low current density of 1 mA/cm^2 for 1 min. These conditions are assumed to give a thin titanium oxide layer. Samples were referenced as $Ti_{ox/aq.}$ At the inverse, a thick unorganized titanium oxide layer could be obtained in organic electrolyte with a high current density of 20 mA/cm^2 for 12 min. Samples were referenced as $Ti_{ox/org}$

After anodizing, samples were either washed with distilled water (aqueous electrolyte) or methanol (organic electrolyte), then whatever the procedure, washed with a 50/50 v/v mixture of water/methanol and finally extensively with water. A Hokuto HA-3001 power supply was used.

2.3 Silanization of metal surface

3-methacryloxypropyltrimethoxysilane (MPS) from ABCR firm was used as received. MPS-coating was performed in a flask containing oxidized titanium samples immersed in a 5% v/v xylene solution of MPS. Silanization occurs at 140°C during 5 h. The coated samples were subsequently washed in xylene and dried in vacuum before use. Samples were referenced as Ti_{sil}.

2.4 Grafting of bioactive polymer

Sodium styrene sulfonate (Aldrich) (NaSS) was purified as previously described. Radical copolymerization of NaSS

and methacrylic group linked to titanium oxide was carried out at a NaSS monomer concentration of 0.7 M in DMSO as solvent under inert gaz. 1-1′-azobis(cyclohexane-carbonitrile) from Aldrich was used as initiator at a concentration of 7×10^{-3} M. After elimination of oxygen, polymerization solution was heated at 70°C overnight under stirring. Then samples were washed with DMSO and water to remove un-reacted monomer. Samples were references as Ti_{graft}.

2.5 Surface characterization

a. FTIR-ATR

IR spectroscopic investigations were carried out with a Thermo Nicolet Avatar 370 Fourier transform infrared (FTIR) spectrometer. Spectra are recorded in air in the attenuated total reflection mode (ATR) using a Ge-crystal

b. Toluidin blue

Measurement of amount of grafted polymer was carried out by using the toluidin blue as complexing agent according to the method described by Helary et al. [16]

c. X-ray photoelectron spectroscopy (XPS)

A VG Scientific ESCALAB photoelectron spectrometer was used for the surface analysis with a monochromatic AlKα source at 1486.6 eV of 70 W. The area of the analytical X-ray spot on the sample surface is about 200 μm. Fitting was then realized with software provided by VG scientific, each spectrum being referenced to carbon at 284.8 eV. Binding energies values are given with a precision of ±0.1 eV.

d. Contact angle measurement

The contact angles of various solvents droplets on the different surfaces were measured using a DSA10 contact angle measuring system from KRUSS GmbH. Static solvent contact angles were measured by deposing a 0.5 μl droplet of solvent on Ti_{1200}, Ti_{ox2}, Ti_{sil} and Ti_{graft}. The dispersive (γ^D) and polar (γ^P) components of the surface energy were calculated from the contact angle measurements using the Owens–Wendt method. Surface energy γ was calculated by addition of γ^D and γ^P.

2.6 Cell-materials interactions studies

MG63 osteoblast-like cells from the American Type Culture Collection (ATCC N° CRL 1427) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2 and 100% humidity. 2 ml containing 10^5 cells were seeded on titanium samples placed on the bottom of 24-wells culture plate. Cells were cultured at 37°C under a humidified atmosphere in 5% CO_2 incubator during different periods of time according to the study on the way. After an incubation time of 30 min or 4 h, cells were submitted to a shear stress during 15 min. The shear stress was applied using a rotary stirring device. According to the equation proposed by Garcia [17] $\tau = 0.8r\sqrt{\rho\mu\omega^3}$, where r is the radial distance from the centre of the disc and the symbols ρ, μ, ω are respectively the fluid density, fluid viscosity and the angular velocity of the disc, it is possible to define a shear stress τ of 8 dynes/cm^2. Then, the solution containing non-adhered cells was removed and adhered cells were detached by trypsin digestion. Number of adherent cells on each titanium sample was quantified using a cell counter (Multisizer III—Coulter Counter from Beckman). Resulting cell number was expressed as a percentage of the cells originally seeded on the assessed surface. Experiments were performed in triplicate in three separate experiments.

For each support (Ti_{1200}, Ti_{ox} and Ti_{graft}), the following function defined as (Ncwa—Ncaa)/Ncs is calculated where Ncaa and Ncwa are the number of adhered cell submitted or non submitted to the shear stress of 8 dynes/cm^2 and Ncs is the number of seeded cells. This function is representative of the cell detachment.

Adherent cells were stained with phalloidin 4 h after seeding onto titanium samples. Pictures of stained cells observed under an inverted fluorescence microscope (Axiolab, Zeiss, Germany) were taken with a digital camera (Diagnostic Instruments, USA).

(a) Alkaline phosphatase (ALP) biochemical activity

After 14 days, ALP activity was evaluated according to the quantitative method previously described [16]: briefly *p*-nitrophenylphosphate is quantitatively transformed into *p*-nitro phenol by ALP enzyme extracted from cells at 37°C and pH 10.2. Enzymatic activity expressed in nanomoles of *p*-nitrophenol/min was standardized to the mass of protein (expressed in mg). Protein content was measured using a commercially available colorimetric assay (BCA assay, Interchim). Thus, enzymatic activity is expressed in nmol of *p*-nitrophenol/min/mg of protein.

(b) Calcium incorporation assays

During the culture of MG63 osteoblast-like cells on samples, alkaline phosphatase enzyme activity allows the liberation of phosphate in the extracellular matrix which forms by complexation solid calcium phosphate nodules. To determine the quantity of liberated phosphate, an easy way was to measure calcium content by titration after 28 days of culture on the various supports [16]. After this period of time, the supports were rinsed with PBS at 37°C and then calcium phosphate nodules were dissolved with

400 µl of trichloroacetic acid during 60 min. 5 µl of the solution was added to 300 µl of arsenazo III-containing Calcium Reagent (Diagnostic Services Ltd). The absorbance of the resulting samples was measured at 650 nm and compared to a linear standard curve of $CaCl_2$ from 50 to 1000 µg/l in trichloroacetic acid (5% w/v).

Calcein labelling was performed after 4 weeks of cell culture. Briefly, the cells were incubated with calcein at a concentration of 25 mg/ml for 4 h at 37°C. After this period, cells were twice washed with phosphate buffered saline, fixed in 3.7% paraformaldehyde in 0.1 M Na-cacodylate buffer, pH 7.4. After rinsing three times with PBS, pictures of stained cells observed under an inverted fluorescence microscope (Axiolab, Zeiss, Germany) were taken with a digital camera (Diagnostic Instruments, USA).

3 Results and discussion

3.1 Oxidation of titanium samples

Two ways were used to increase the content of TiOH at the surface of titanium implants Ti_{1200}: chemical and electrochemical oxidation.

Chemical oxidation was obtained by immersing Ti_{1200} in a mixture of sulphuric acid and hydrogen peroxide during 5 min. A dark oxide layer of high porosity (diameter of around 5 µm) was observed on SEM images (see Fig. 1).

For electrochemical oxidation according to the method described by Shirkhanzadeh [18, 19], two different electrolytes were used: oxalic acid and methanol. Oxidation in aqueous solution with a current density of 1 mA/cm^2 for 1 min gives a yellow oxide layer with a thickness of 21 nm measured by ellipsometry. Raman spectra of the oxide layer show a peak of weak intensity, at 608 cm^{-1} characteristic of rutile phase (Fig. 2).

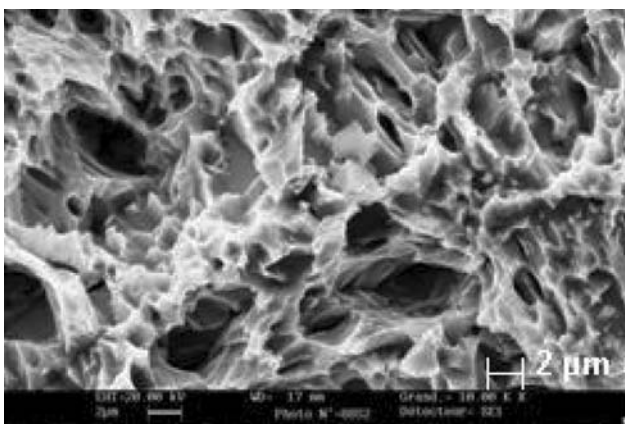

Fig. 1 Oxide layer of high porosity on Ti1200 immersed during 5 min in a mixture of sulphuric acid and peroxide hydrogen (×10000)

Oxide layer thickness formed by anodic oxidation in methanol, with a current density of 20 mA/cm^2 for 12 min is of about 8 µm, the same order of magnitude as the values mentioned in the literature [18, 20]. Raman spectra (Fig. 2) demonstrated a mixture of anatase (bands at 144 and 513 cm^{-1}) and rutile phases (bands at 440, 608 cm^{-1} and a shoulder at 245 cm^{-1}). A mixture of anatase and rutile phases was already cited by Sul and co-workers [21] for electrochemically oxidized titanium implants. The crystallinity of the titanium oxide varied substantially with the oxide thickness. The analysis of the oxide layer (10 µm) obtained after an oxidation period of 1 h 30 only shows peaks of anatase at 144, 513 and 640 cm^{-1}.

However, the weak intensities of bands obtained with our experimental procedure suggest that oxide layer, whatever the mode of electrochemical oxidation, is mainly amorphous with some scattered crystallites.

3.2 Silanization reaction

Coating of the oxidized titanium surfaces with methacrylic-functionalized silane MPS was carried out in anhydride xylene in order to prioritize the reaction between Ti–OH and silanol groups. MPS concentration in xylene was of 4×10^{-2} M, in the same order of magnitude as concentrations reported in the literature for silanization of titanium oxide surfaces [22]. The immobilization of methacryloyl group containing silane coupling agents on the surface of titanium was proved by FTIR-ATR and XPS analyses. IR spectrum shows characteristic bands at 1715 cm^{-1} (C=O of methyl ester) and 1080 cm^{-1} (Si–O–C) of MPS grafted to titanium surface (Fig. 3).

On Table 1 are reported the atomic compositions on the upper surfaces after oxidation and silanization for Ti_{oxc}, $Ti_{ox/aq}$ and $Ti_{ox/org}$ samples. Whatever the oxidation procedure (chemical or electrochemical), carbon, oxygen and

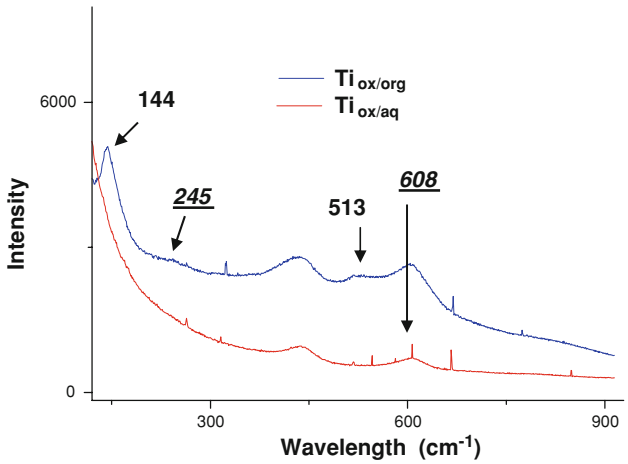

Fig. 2 Raman spectra of the oxide layer of $Ti_{ox/aq}$ and $Ti_{ox/org}$

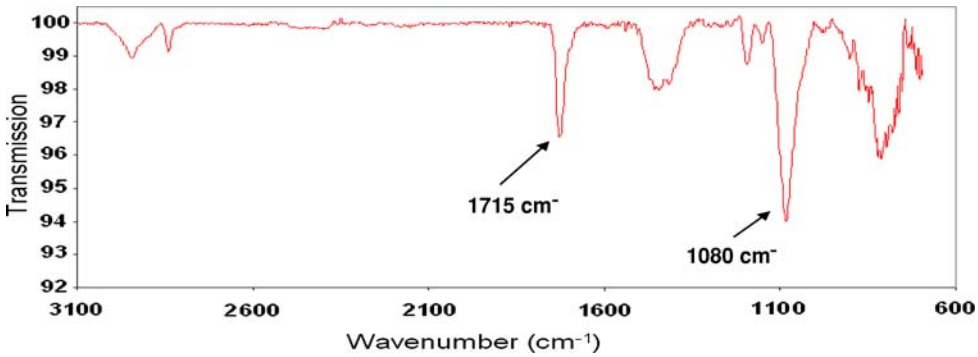

Fig. 3 IR spectrum of the MPS grafted to titanium surface

Table 1 Atomic compositions on the titanium surface after oxidation and silanization for $Ti_{ox}c$, $Ti_{ox/aq}$ and $Ti_{ox/org}$

	C	O	Ti	Si	S	Na	Contaminants
Ti_{1200}	33.2 ± 4.4	46.4 ± 2.1	14.7 ± 0.4	–	–	–	5.4 ± 2.2
Ti_{oxc}	32.2 ± 4.4	47.4 ± 2.1	14.7 ± 0.4	–	–	–	5.7 ± 2.2
$Ti_{ox/aq}$	37.0 ± 2.4	45.8 ± 0.2	14.6 ± 0.1	–	–	–	2.6 ± 0.4
$Ti_{ox/org}$	27.2 ± 0.2	52.7 ± 0.2	16.7 ± 0.3	–	–	–	3.4 ± 0.3
$Ti_{ox/aq/sil}$	56.4 ± 1.3	33.3 ± 1.50	2.2 ± 0.3	7.5 ± 0.8	–	–	0.6 ± 0.3
$Ti_{ox/org/sil}$	55.8 ± 0.2	32.0 ± 0.8	1.8 ± 0.2	9.2 ± 0.3	–	–	1.2 ± 0.2
$Ti_{ox/aq/sil/graft}$	63.2 ± 1.6	23.4 ± 1.0	0.1 ± 0.2	1.7 ± 0.6	7.1 ± 0.2	1.5 ± 1.1	3.0 ± 1.2
$Ti_{ox/org/sil/graft}$	61.5 ± 0.9	23.7 ± 0.3	0.1 ± 0.1	2.3 ± 0.4	6.2 ± 0.7	1.3 ± 0.8	4.9 ± 0.5

$Ti_{ox/aq/sil}$ and $Ti_{ox/org/sil}$ correspond to the titanium surfaces grafted with MPS according to the nature of the electrolyte. $Ti_{ox/aq/MPS/pNaSS}$ and $Ti_{ox/org/MPS/graft}$ are the references of titanium surfaces grafted with poly NaSS

titanium are detected with similar compositions. Carbon components are characteristic of pollution contribution.

As expected, an increase of carbon content and a decrease of oxygen and titanium contents appear after MPS grafting. The value of around 55% of carbon after silanization is due to the increase of a peak at 285.2 eV characteristic of carbon linked to CO group. The presence of MPS at the titanium surface is also proved by the apparition of a peak at 283.8 eV of small intensity attributed to the carbon linked to the group Si–O. Another proof of the presence of MPS is the decrease of the peak O^{2-} at 530.2 eV on the O_{1s} spectrum due to the titanium oxide layer. The percentage of oxygen due to O^{2-} decreases from 70 to 14% after silanization of the electrochemical oxide layer. Grafting of MPS hides the titanium atoms confirmed by a titanium component decrease by a factor 7 or 8 according to the electrochemical procedure. Finally, a peak due to Si_{2p} component at around 102 eV appears which corresponds to Si–O group.

3.3 Grafting of polyNaSS

After copolymerization of NaSS and the double bond of the methacrylic group linked to the titanium surface, XPS analysis show the disappearance of the peak at 288.8 and 283.8 eV, respectively, characteristic of COOR and Si–O–C groups. It is noteworthy that titanium component is not detectable (less than 0.1%). The apparition of S and Na components confirms the grafting of polyNaSS at the titanium surface (Table 1).

The IR spectrum shows peaks at 1008 and 1040 cm^{-1} corresponding to the sulfonate groups SO^{3-} whereas they are not detectable on the IR spectra of the samples Ti_{oxc}, $Ti_{ox/org}$, $Ti_{ox/aq}$ and Ti_{oxc} considered as reference.

Toluidin blue was used to evaluate the density of sulfonate groups from the pNaSS macromolecular chains linked to the titanium surface. No coloration was observed with $Ti_{ox/aq}$ and Ti_{oxc}. As earlier mentioned, oxide layer thickness with methanol electrolyte is important and highly porous retaining the dye even after many washings. For this reason, quantification of grafted polyNaSS was carried out with Ti_{oxc}, $Ti_{ox/aq}$ and $Ti_{ox/org}$ but values mentioned for the last reference should be considered as information. Amounts of grafted polymer on Ti_{oxc}, $Ti_{ox/aq}$ and $Ti_{ox/org}$ were found to 1.9 ± 0.3, 7.2 ± 1.1 and 8.5 ± 1.2 μg/cm^2, respectively. It is difficult to give an explanation to the different amounts of grafted polyNaSS measured onto Ti_{oxc} and $Ti_{ox/aq.}$ Electrochemical oxidation drives to high concentration of Ti–OH groups which can easily react with MPS during the silanization reaction. According to the literature [23], the density of Ti–OH groups is of 1.8 10^{-5} mol/m^2

which would correspond for each sample to 4.3 10^{-9} mol of MPS grafted in the case of a yield of 100%. The assumption of a complete reaction can be considered as accurate considering that the concentration of cysteine grafted [24] to titanium disks by silanization reaction was estimated to 2 × 10^{-5} mol/m^2, value similar to the TiOH density. From the value of 4.3·10^{-9} mole of MPS grafted onto one titanium sample and the amount of grafted polyNaSS, it is possible to calculate a virtual degree of polymerization of around 15. This degree of polymerization could be higher assuming that the copolymerization of NaSS and the double bond of the methacryloyl group linked to the titanium sample can be made hindered due to the formation of a network of polysiloxane by reaction of Ti–OMe during the silanization or polymerization reactions. However, this hypothesis is confirmed by Durrieu et al. [24] which show that during silanization reaction of 3-aminopropyltriethoxysilane APTES, each TiOH reacts with only one SiOMe of APTES on the fact that the density of APTES is equal to that of TiOH. The probability that the SiOMe groups linked to each MPS react with those linked to other MPS molecules is high driving to the formation of a polysiloxane network

An important parameter is the stability of grafted polyNaSS at the surface of titanium sample in water. To check this parameter, grafted titanium samples were immersed in water at a temperature of 37°C under stirring. At various period of time, the amounts of polyNaSS on grafted titanium samples were determined using the toluidin blue method. As reported on Table 2, we can notice a decrease of the amount of grafted polymer which varies from 7.2 ± 1.1 to 5.5 ± 0.6 μg/cm^2 after 1 month for the system $Ti_{ox/aq/sil/graft}$. After this period, there is no significant variation of the amount of grafted polyNaSS which can be considered as constant. In the case of polyNaSS grafted onto the chemically oxide layer or $Ti_{ox/org}$, a decrease is rapidly observed. After 2 months, the amount of grafted polyNaSS is low 1.0 μg/cm^2 ± 0.3 for the system $Ti_{ox/org/sil/graft}$ and 0.2 ± 0.1 μg/cm^2 for the system $Ti_{oxc/sil/graft}$. The explanation could be a weathering of the oxide layer which is porous and heterogeneous for the two systems studied and in fact, small wear debris in shape of particles were found in the washing solution.

Table 2 Stability of polyNaSS layer grafted to titanium surface

	1 day	2 months	2 months
$Ti_{oxc/sil/graft}$	1.9 ± 0.3		0.2 ± 0.1
$Ti_{ox/aq/sil/graft}$	7.2 ± 1.1	5.5 ± 0.6	5.2 ± 0.6
$Ti_{ox/org/sil/graft}$	8.5 ± 1.2		1.0 ± 0.3

Values reported on the table are expressed in μg/cm^2

3.4 Biological tests

In order to briefly check the efficiency of the polyNaSS grafted to titanium implants according to the different above described methods, three current biological tests were carried out: cell attachment, alkaline phosphatase activity and calcification.

- As mentioned in a previous paper [16], application of a shear stress of 8 dynes/cm^2 during 15 min allows evidencing the different cell behaviour at the level of cell adherence on Ti_{1200}, Ti_{ox} and Ti_{graft}. The values calculated for each support from the function defined in Sect.2 representative of the cell detachment are reported on Figs. 4 and 5. The shear stress of 8 dynes/cm^2 was applied after cells have been incubated on each support during 30 min and a longer period of time: 4 h. After 30 min of incubation (Fig. 4), grafting of polyNaSS favours the cell adherence by a factor of 3 or 2 depending of the electrochemical oxide layer used for the grafting ($Ti_{ox/aq}$ and $Ti_{ox/org}$). For a longer period of time (4 h), the improvement of cell adhesion is less important: values are similar for the systems $Ti_{ox/org/graft}$, $Ti_{oxc/graft}$ and increase by a factor 2 for the system $Ti_{ox/aq}$ (12%)/Ti_{graft} (6%) (Fig. 5). The improvement of cell adherence for a short period of incubation has been already observed by Kowalczynska et al. [25] who have

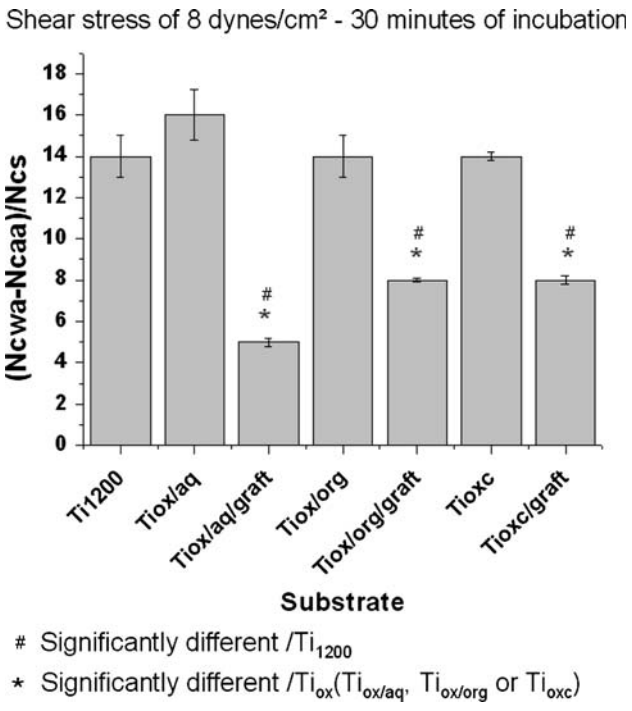

Fig. 4 Cell detachment of MG63 osteoblast-like cells on Ti_{1200}, Ti_{ox} and Ti_{graft}. N_{cwa} and N_{caa} are the number of adhered cell submitted or non-submitted to the shear stress of 8 dynes/cm^2 after 30 min of incubation Nsc is the number of seeded cells

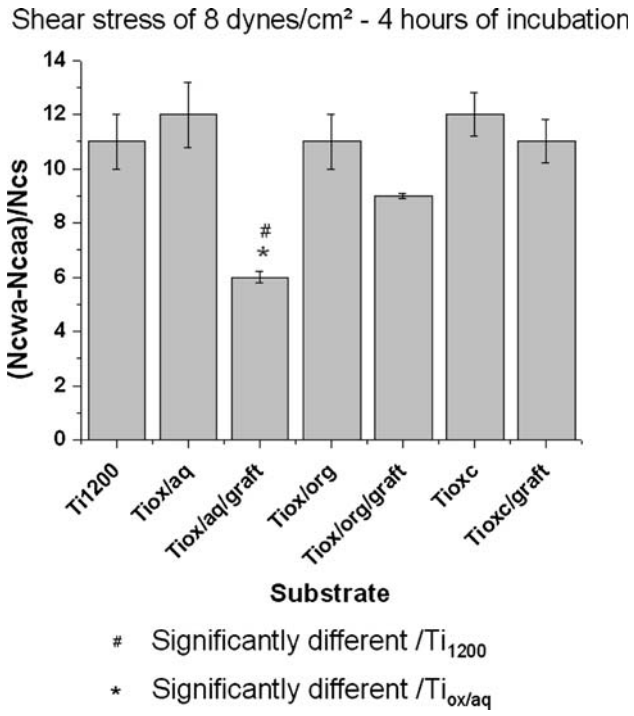

Fig. 5 Cell detachment of MG63 osteoblast-like cells on Ti_{1200}, Ti_{ox} and Ti_{graft}. N_{cwa} and N_{caa} are the number of adhered cell submitted or non-submitted to the shear stress of 8 dynes/cm^2 after 4 h of incubation. Nsc is the number of seeded cells

shown that for a density of sulfonate groups of $3.3{\cdot}10^{-6}$ mol/m^2, the rate and strength of the early phase of cell adhesion is favoured. In our case, the density of sulfonate groups on titanium samples was found in a domain between $9.2{\cdot}10^{-6}$ and $3.5{\cdot}10^{-5}$ mol/m^2, higher than the value reported by these authors. The sulfonate groups stimulate cell spreading and the cell average surface is increased by a factor of 1.7 for the system $Ti_{ox/aq/graft}$ (1426 $\mu m^2 \pm 58$) after 4 h of incubation when compared to $Ti_{ox/aq}$ (838 $\mu m^2 \pm 25$). It is noteworthy that the cell average surface is lightly increased for the system $Ti_{ox/org/graft} = 1326\ \mu m^2 \pm 52$ compared to $Ti_{ox/org} = 1172\ \mu m^2 \pm 52$) which is correlated to the weak improvement of cell adhesion after 4 h of incubation. One explanation could be the highest roughness of the system $Ti_{ox/org/graft}$ compared to $Ti_{ox/aq/graft}$ as early mentioned. But the other is that surface chemistry of pNaSS grafted Ti samples differs significantly from non grafted ones, the presence of this thin surface layer of grafted pNaSS is expected to have a pronounced effect on protein adsorption and cell adhesion. For example, we have previously shown that poly(methyl methacrylate) (PMMA) surfaces with sulfonate and carboxylic acid groups showed an enhanced affinity for fibronectin over albumin, resulting in adsorbed protein film that were significantly enriched in fibronectin compared to the PMMA surface without anionic groups [26–32]. These differences in the concentration and conformation of adsorbed proteins can have a significant influence on cell interactions with biomaterials surface since protein adsorption is one of the first events that occur when a biomaterial is placed in contact with biological fluids. It is well documented that the adhesion of cells is mediated by adhesion proteins such as fibronectin, vitronectin, collagen and fibrin. Among these proteins, fibronectin and vitronectin are two ubiquitous glycoproteins that are synthesized by a large variety of cell types. These two proteins have been proposed to play a major role in the cell adhesion process on biomaterials, even when present at low concentration in plasma. Both proteins were present in the 10%FCS-DMEM solutions used to precoat the samples surfaces prior to the cell adhesion experiments.

- Alkaline phosphatase ALP, an early marker of osteoblast differentiation relating to the production of a mineralized matrix, was measured after 14 days of osteoblasts seeded on Ti_{1200}, $Ti_{ox/org}$, $Ti_{ox/aq}$, Ti_{oxc} and Ti_{graft}. ALP activities are close to 1.4 nmol/min/mg of proteins, whatever the titanium surfaces, except for $Ti_{ox/aq/graft}$ and $Ti_{ox/org/graft}$ where ALP is increased of 19 and 26%, respectively (Fig. 6). For the system $Ti_{ox/aq/graft}$, ALP activity was found to 1.68 nmol/min/mg of proteins, value comparable to those reported in the literature with MG63 cells. For instance, Ramires et al [33] have found a value of 1.5 nmol/min/mg of proteins with MG63 cells cultured on titania/hydroxyapatite composite coatings.
- Calcium contents of 4 weeks cultured MG63 cells on titanium samples are reported on Fig. 7. The hypothesis that precipitation of calcium could be formed independently of the cell activity was rejected by leaving on

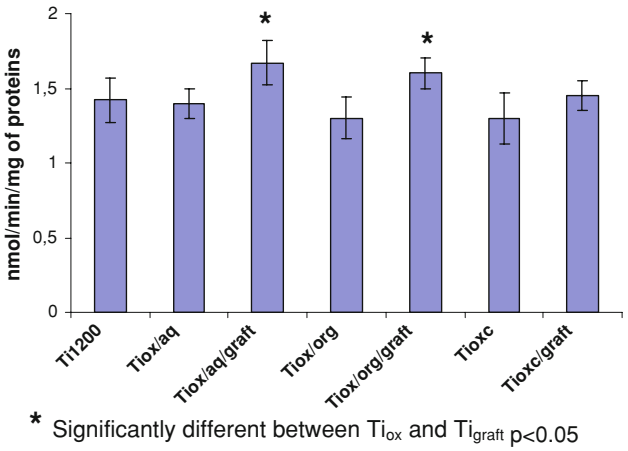

Fig. 6 ALP activity of MG63 osteoblast-like cells after 14 days of proliferation on various supports

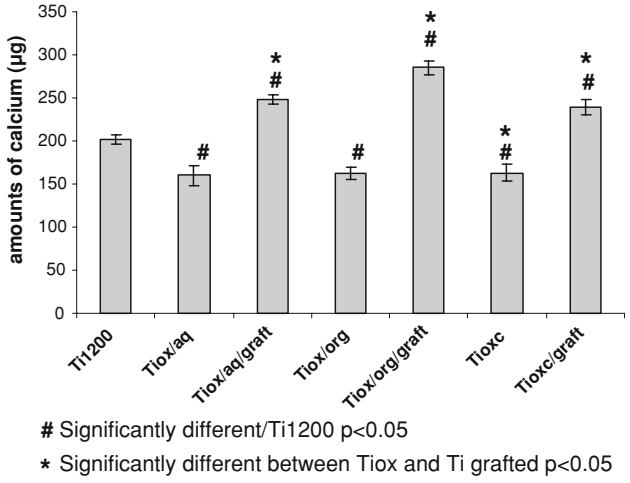

Fig. 7 Calcium content of MG63 osteoblast-like cells matrix at 4 weeks of proliferation

titanium, during 4 weeks, Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum (FBS). No calcium nodule was detected indicating that precipitation of calcium is due to MG63 cells. Amounts of calcium increased on all pNaSS grafted titanium surfaces which confirm previous results [16].

As mentioned earlier [16], the hydrophilic character of polyNaSS surface is not sufficient to explain our results. From contact angles determined on each surface using solvents with various polarity (water, formamide, ethylene glycol, diiodomethane), the surface energies were calculated and reported on Table 3. Whatever the mode of oxidation and treatment, values of surface energy are close. For instance, a surface energy was found to 60.9 ± 2.6 mN/m for $Ti_{ox/aq}$ and 67.3 ± 1.4 mN/m for $Ti_{ox/org}$. Electrochemical oxidation induce an increase of the hydrophilic character as expected due to the increase of the polar surface tension component (49.5 ± 0.7 mN/m) by a factor 3 compared to the value for Ti_{1200} (16.5 ± 1.2 mN/m). On the contrary, MPS grafting induces an increase of the hydrophobic character of the titanium surface as revealed by the decrease of the surface energy from 60.9 ± 2.6 for $Ti_{ox/aq}$ to 36.5 ± 0.9 mN/m for $Ti_{sil.}$ The grafting of polyNaSS provokes an increase of the surface energy mainly due to the polar component which has similar value (44.1 ± 0.9 mN/m) to that of the anodic oxide layer (49.5 ± 0.7 mN/m). The explanation to the cell behaviour between oxidized titanium surface and grafted titanium surface is not due to their hydrophilic character which is similar. In fact the difference is the presence of the ionic groups which may play a role on the conformation of the adhesive proteins such as fibronectin [26, 29, 34], vitronectin with the consequence to favor the differentiation of MG63 cells as detailed above.

4 Conclusion

A three-step reaction procedure was developed to covalently link a bioactive polymer (polyNaSS) onto titanium implant. First, chemical or electrochemical oxidation was applied to increase the amount of TiOH groups at the surface of titanium. Secondly, hydroxyl titanium groups were attached to the hetero-cross-linker: 3-methacryloxypropyltrimethoxysilane MPS. Finally, radical copolymerization of methacryloyl end group of MPS with sodium styrene sulfonate was carried out in order to immobilize cell-adhesive polymer (polyNaSS). The presence of the different molecules at each reaction step was confirmed by XPS and IR analysis. PolyNaSS density was evaluated by colorimetric method and high amounts of linked-polymer were found with oxide layer prepared by electrochemical oxidation: 7.2–8.5 ± 1.2 µg/cm^2 depending of the electrolyte nature. Cell attachment was improved on grafted titanium samples correlated with an increase of the cell average surface. The key point is the improvement of MG63 cell osseointegration as shown by a better ALP and formation of calcium nodule when these cells are seeded on polyNaSS grafted titanium samples. The stability of the polymer layer covalently linked to titanium surface with time is an important factor to decrease the anchorage failure of titanium implants in the human body. Nevertheless, various chemical compositions

Table 3 Surface free energy of Ti_{1200}, Ti_{ox} and Ti_{graft} calculated with Owens–Wendt model

	Surface energy γ (mN/m)	Dispersive component γ^D (mN/m)	Polar component γ^P (mN/m)
Ti_{oxc}	64.1 ± 1.9	33.6 ± 1.7	30.5 ± 0.9
$Ti_{ox/aq}$	60.9 ± 2.6	15.0 ± 0.9	45.9 ± 1.6
$Ti_{ox/org}$	67.3 ± 1.4	17.9 ± 0.7	49.5 ± 0.7
$Ti_{ox/aq/sil}$	36.5 ± 0.9	31.5 ± 0.6	4.9 ± 0.3
$Ti_{ox/org/sil}$	44.9 ± 1.0	41.2 ± 0.7	3.7 ± 0.3
$Ti_{oxc/graft}$	67.5 ± 2.0	22.5 ± 1.6	45.0 ± 2.3
$Ti_{ox/aq/sil/graft}$	62.3 ± 0.9	20.2 ± 0.5	42.0 ± 0.5
$Ti_{ox/org/sil/graft}$	61.0 ± 1.5	16.8 ± 0.6	44.1 ± 0.9

of appropriate ionic groups and densities of these groups grafted at the titanium surface would be biologically tested in order to find the ideal material.

References

1. Anderson JM. Biological responses to materials. Annu Rev Mater Res. 2001;31:81–110.
2. Chen Y, Zheng X, Ji H, Ding C. Effect of Ti–OH formation on bioactivity of vacuum plasma sprayed titanium coating after chemical treatment. Surf Coat Technol. 2007;202:494–8.
3. Lewandowska M, Roguska A, Pisarek M, Polak B, Janik-Czachor M, Kurzydłowski KJ. Morphology and chemical characterization of Ti surfaces modified for biomedical applications. Biomol Eng. 2007;24:438–42.
4. Takemoto M, Fujibayashi S, Neo M, Suzuki J, Matsushita T, Kokubo T, et al. Osteoinductive porous titanium implants: effect of sodium removal by dilute HCl treatment. Biomaterials. 2006;27:2682–91.
5. Jonasova L, Müller FA, Helebrant A, Strnad J, Greil P. Biomimetic apatite formation on chemically treated titanium. Biomaterials. 2004;25:1187–94.
6. Park YJ, Song HJ, Kim I, Yang HS. Surface characteristics and bioactivity of oxide film on titanium metal formed by thermal oxidation. J Mater Sci: Mater Med. 2007;18:565–75.
7. Saldana L, Barranco V, Gonzalez-Carrasco JL, Rodriguez M, Munuera L, Vilaboa N. Thermal oxidation enhances early interactions between human osteoblasts and alumina blasted Ti6Al4V alloy. J Biomed Mater Res A. 2007;81:334–46.
8. Sul Y, Johansson CB, Jeong Y, Albrektsson T. The electrochemical oxide growth behaviour on titanium in acid and alkaline electrolytes. Med Eng Phys. 2001;23:329–46.
9. Yanga B, Uchidab M, Kimc H, Zhanga X, Kokubod T. Preparation of bioactive titanium metal via anodic oxidation treatment. Biomaterials. 2004;25:1003–10.
10. Das K, Bose S, Bandyopadhyay A. Surface modification and cell-materials interactions with anodized Ti. Acta Biomater. 2007;3:583–5.
11. Zhao B, Tian W, Feng H, Lee IS, Cui F. Effects of RGD peptides grafting to titanium dental implants on the adhesion of human gingival fibroblasts and epithelial cells. Curr Appl Phys. 2005;5:407–10.
12. Bagno A, Piovan A, Dettin M, Chiarion A, Brun P, Gambaretto R, et al. Human osteoblast-like cell adhesion on titanium substrates covalently functionalized with synthetic peptides. Bone. 2007;40:693–9.
13. Morra M, Cassinelli C, Cascardo G, Cahalan P, Cahalan L, Fini M, et al. Surface engineering of titanium by collagen immobilization. Surface characterization and in vtro and in vivo studies. Biomaterials. 2003;24:4639–54.
14. Anagnostou F, Debet A, Pavon-Djavid G, Goudaby Z, Helary G, Migonney V. Osteoblast functions on functionalized PMMA-based polymers exhibiting Staphylococcus aureus adhesion inhibition. Biomaterials. 2006;27:3912–9.
15. Poussard L, Pavon-Djavid G, Migonney V. Synthèse de copolymères ioniques porteurs de fonctions phosphate. Congrès Matériaux Dijon (France) 2006;92.
16. Helary G, Noirclère F, Mayingi J, Migonney V. A new approach to graft bioactive polymer on titanium implants. Improvement of MG 63 cell differentiation onto this coating. Acta Biomater. 2009;5:124–33.
17. Garcia AJ, Ducheyne P, Boettiger D. Quantification of cell adhesion using a spinning disc device and application to surface-reactive materials. Biomaterials. 1997;18:1091–8.
18. Shirkhanzadeh M. Fabrication and characterization of alkoxy-derived nanophase TiO_2 coating. Nanostruct Mater. 1995;5:33–40.
19. Shirkhanzadeh M. Nanoporous alkoxy-derived titanium oxide coating: a reactive overlayer for functionalizing titanium surface. J Mater Sci: Mater Med. 1998;9:355–62.
20. Shirkhanzadeh M. Electrochemical preparation of protective oxide coatings on titanium surgical alloy. J Mater Sci: Mater Med. 1992;3:322–5.
21. Sul YT, Johansson CB, Petronis S, Anatol Krozer A, Yongsoo Jeong Y, Wennerberg A, et al. Characteristics of the surface oxides on turned and electrochemicallyoxidized pure titanium implants up to dielectric breakdown: the oxide thickness, micropore configurations, surface roughness, crystal structure and chemical composition. Biomaterials. 2002;23:491–501.
22. Yoshida W, Castro RP, Jou JD, Cohen Y. Multilayer alkoxysilane silylation of oxide surfaces. Langmuir. 2001;17:5882–8.
23. McCafferty E, Wightman JP. Determination of the concentration of surface hydroxyl groups on metal oxide films by a quantitative XPS method. Surf Interface Anal. 1998;26:549–64.
24. Porté-Durrieu MC, et al. Cyclo-(DfKRG) peptide grafting onto Ti-6Al-4 V: physical characterization and interest towards human osteoprogenitor cells adhesion. Biomaterials. 2004;25:4837–46.
25. Kowalczynska HM, Nowak-Wyrzykowska M. Modulation of adhesion, spreading and cytoskeleton organization of 3T3 fibroblasts by sulfonic groups present on polymer surfaces. Cell Biol Int. 2003;27:101–14.
26. Evans DM, Pavon-Djavid G, Helary G, Legeais J-M, Migonney V. Vitronectin is significant in the adhesion of lens epithelial cells to PMMA polymers. J Biomed Mater Res. 2004;69A:469–76.
27. Helary G, Yamine P, Migonney V. Surface modification of hydrogel intraocular lenses to prevent cell proliferation. J Appl Biomater Biomech. 2005;2:183–90.
28. Helary G, Migonney V**.** Bioactive polymer coatings to improve bone repair. Bone repair Biomaterials 2009, editor: Planell JA, Woodhead publishing.
29. El Kadhali F, Pavon-Djavid G, Helary G, Migonney V. Modulating fibroblast cell proliferation with functionalized poly(methyl methacrylate) based copolymers: chemical composition and monomer distribution effect. Biomacromolecules. 2002;3:51–6.
30. Le Guillou-Buffello D, Helary G, Gindre M, Pavon-Djavid G, Laugier P, Migonney V. Estimation de l'inhibition de l'adhésion cellulaire sur des films de polymère par la technique du résonateur à quartz. ITBM-RBM. 2004;25:34–9.
31. Latz C, Pavon-Djavid G, Helary G, Evans M, Migonney V. Alternative intracellular signaling mechanism involved in the inhibitory biological response of functionalized PMMA-based terpolymers. Biomacromolecules. 2003;4:766–71.
32. Le Guillou-Buffello D, Helary G, Gindre M, Pavon-Djavid G, Laugier P, Migonney V. Monitoring cell adhesion processes on bioactive polymers with the quartz crystal resonator technique. Biomaterials. 2005;26:4197–205.
33. Ramires PA, Romito A, Cosentino F, Milella E. The influence of titania/hydroxyapatite composite coatings on in Vitro osteoblasts behaviour. Biomaterials. 2001;22:1467–74.
34. El Khadali F. Studies of the interactions between poly(methyl methactylate) based copolymers and human fibroblastic cells. Thesis, University Paris 13; (2003).