

Effect of Poly-L-Lysine Coating on Titanium Osseointegration: From Characterization to In Vivo Studies

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Dental implant prostheses cannot preclude a correct and stable implant osseointegration, which is still a challenge and greatly depends on biomaterial-cell interface. Titanium (Ti) coating using polyelectrolyte poly-L-lysine (PLL) may represent an interesting and simple approach, to provide a charged surface net able to improve cell adherence. However, in vitro and in vivo effects of Ti coated with PLL have been poorly investigated. The aims of the present study are (1) to obtain and characterize, chemically and physically, Ti disks coated with PLL (TiPLL); (2) to perform in vitro studies on osteoblast cell lines' cytocompatibility and functionality (alkaline phosphatase [ALP] activity, calcium deposition, proinflammatory interleukin 6 production); (3) to obtain in vivo evidence of osseointegration, using a sheep animal model. XPS, AFM, and contact-angle analyses demonstrated that the Ti disk was successfully covered with PLL, providing higher hydrophilicity to the Ti disk. No cellular toxicity, enhanced calcium deposition, and a decreased tendency toward interleukin-6 production were observed in the osteoblast seeded onto TiPLL. In vivo experiments showed cortical bone microhardness at 3 months significantly improved in the presence of the PLL coating. PLL coating on Ti implants seemed to safely enhance calcium deposition and implant early osseointegration in animals, suggesting promising evidence to optimize the surface properties of dental implants.

Key Words: titanium implant, poly-levo-lysine coating, calcium deposition, osseointegration

INTRODUCTION

Titanium (Ti) metal is the most widely used biomaterial, representing the “gold standard” for orthopedic and dental implant rehabilitation because of its high strength, stress resistance and relatively low elastic properties as compared to other biometals.^{1,2}

Indeed, Ti surface roughness, together with energy and chemistry, strongly influences bone formation.³ A sand-blasted Ti implant permits a greater amount of bone-to-implant contact surface and more strength is needed to remove it from the bone, if compared to a smoothed one.⁴ Moreover, roughened Ti implants have been shown to enhance osteoblast differentiation in vitro.⁵ On the other hand, surface chemistry

regulates the signaling pathways and downstream cell differentiation.⁶ Different surface coatings have been proposed to increase cell adhesion, proliferation, and differentiation on the implant materials and to reduce healing time, with the final aim to promote osseointegration in patients. In fact, the principal biomechanical requirements of a biomaterial, to be bio-inert or biotolerant,⁷ lead to a more difficult osseointegration on account of the low interaction with the biological environment.

On this perspective, the biomaterial-cell interface is the cornerstone for biocompatibility and functionality of biomedical implants.

Recently, Park et al described a Ti surface coating of polyelectrolyte as an interesting method to obtain a strong charged surface net using electrostatic forces² to improve cell adherence.

A polyelectrolyte is a polymer with an electrolyte group that will dissociate in aqueous solutions to form poly-ions (positive or negative, depending on the chemical nature of their repeating units and the pH of the aqueous solution). Poly-L-lysine (PLL) is a positively charged poly-amino acid, able to improve cell and tissue adhesion on different substrates.^{8,13} On the other hand, Hwang and colleagues showed how PLL could inhibit apatite growth on a Ti alloy surface,¹⁴ supposing that the presence of amino molecules in a crystallizing solution

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profoundly influences the crystallization of inorganic salts, including calcium phosphates. To date, the outcome of a PLL coating on bone deposition is still controversial. Moreover, in vitro and in vivo effects of PLL on Ti surface, in terms of calcium phosphate deposition, bone formation, and osseointegration, have been poorly investigated.

The aims of this study are: (1) to characterize PLL coating onto Ti disks (TiPLL); (2) to perform in vitro studies on osteoblasts, in order to assess cytocompatibility, cytodifferentiation, calcium deposition, and proinflammatory interleukin-6 production; and (3) to investigate in vivo mandibular osseointegration.

MATERIALS AND METHODS

Sand-blasted titanium disks coated with poly-L-lysine layer (TiPLL) were compared to the same disks without coating (Ti).

Materials and characterization

Sand blasted titanium disks. Titanium grade 4 was used to obtain disks for in vitro tests (\varnothing 10 mm) and oral implants for in vivo tests (length 8 mm, \varnothing 3.5 mm), both sand-blasted using $MgCO_3$ powder and, then, etched with 10% aqueous citric acid solution. Sterilization was carried out using γ -rays at 200 MGy.

PLL coating. The experimental material was prepared by incubating Ti disks/implants in 0.01% aqueous solution of PLL, for 10 minutes at 37°C, under sonication.

Surface characterization. The surface chemical, to ascertain the presence of PLL, was investigated by means of X-ray photoelectron spectroscopy (XPS) using a PHI 5600-ci spectrometer (Physical Electronics, Eden Prairie, Minn). A standard aluminum X-ray source (1486.6eV) was used at 300W with a neutralizer to record the survey spectra, and the high-resolution spectra were obtained using a standard magnesium X-ray source (1253.6eV) at 300W with no charge neutralization. Photoelectron detection was generally performed at 45° with respect to the surface plane. The analyzed area was 0.005 cm²; the depth analysis was 5 nm.

Surface wettability was investigated by static contact angle measurements of the samples, which were recorded using a VCA 2500 XE system (AST, Billerica, Mass). A drop of 40 μ l milliQ water was deposited on the surface.

PLL presence on disks and surface topography were further evaluated using an atomic force microscope (AFM), (Digital Instruments, Santa Barbara, Calif) in tapping mode with an etched silicon tip (OTESPATM, tip radius < 10 nm, aspect ratio \approx 1.6/1). The surface roughness for areas of 1 \times 1 μ m² was calculated using the root mean square roughness parameter). Visualization and analysis of the morphology were performed using WsXM 5.0 software (Nanotec, Madrid, Spain).¹⁵

Cells and Media

Cell cultures and media. Mouse osteoblast-like cells MC3T3-E1 (ATCC 2593) were maintained in α -MEM (Gibco, Life Technologies, Monza, Italy), supplemented with 10% fetal bovine serum (FBS-Gibco, Life Technologies), 100 U/mL of penicillin (Gibco, Life Technologies) and 100 μ g/mL of streptomycin (Gibco, Life Technologies), at 37 °C in 5% CO₂.

Osteogenic differentiation was induced by adding β -glycerol phosphate 10⁻³ M, ascorbic acid 50 μ g/mL and dexamethasone 10⁻⁸ M to the maintenance medium (osteogenic medium).

In Vitro Studies

All the experiments were done in triplicate and repeated 4 times, using 24-multiwells plates.

Cell viability. MC3T3-E1 were seeded onto titanium disks in a small volume (20 μ L) of maintenance medium at a density of 1 \times 10⁴. After seeding, the cultures were left untouched for 2 hours at 37°C to promote cell attachment and then the medium was added. Cell viability was evaluated by MTT assay (Sigma Aldrich, St. Louis, Mo) at days 3 and 7, after osteogenic induction. Four hours after MTT addition at a concentration of 100 μ L/mL, formazan salt crystals were solubilized with 70 μ L isopropanol and the absorbance was read at an optical density of 570 nm with a spectrophotometer plate reader (BS100 Spectra Count, San Jose, Calif). Cell viability was expressed in percentage, using values corresponding to Ti disks, without PLL, as control.

Cytodifferentiation. Cells were seeded at a density of 1 \times 10⁴ and were treated using Lowry's method.¹⁶ Alkaline phosphatase (ALP) activity, which is considered an early osteogenic marker, was evaluated using pNPP (4-nitrophenylphosphate disodium salt hexahydrate; Sigma-Aldrich) as substrate at days 7 and 14. ALP was evaluated on the total protein amount obtained using BCA assay for normalization. Briefly, cells were lysed on ice for 15 min with 100 μ L of Triton X-100 0.2% in Tris-HCl 50 mM (pH 8). The cell lysate was centrifuged at 14 000 rpm. at 4°C for 10 minutes and the supernatant (30 μ L) was incubated with 30 μ L of assay mixture (Glycine 50 mM, MgCl₂ 1 mM and pNPP 8 mM, pH = 10) for 30 minutes at 37°C. The reaction was stopped by adding NaOH 1 M and the absorbance was read at 405 nm with a spectrophotometer plate reader (BS100 Spectra Count).

Calcium deposition. Cells were seeded at a density of 1 \times 10⁴ and, after 28 days, matrix calcification, late marker of differentiation, was assessed with Alizarin Red S assay. Staining was performed with a 2% solution of Alizarin Red S (Sigma-Aldrich) in distilled water for 10 minutes. After aspiration of the unincorporated dye, the specimens were washed with distilled water. For quantification of staining, the incorporated dye was extracted with a 10% solution of CPC (Sigma-Aldrich) for 15 minutes at room temperature and the absorbance was read at 570 nm with a spectrophotometer plate reader (BS100 Spectra Count).

Proinflammatory interleukin 6 (IL-6) production. MC3T3-E1 were seeded onto titanium disks in a small volume (40 μ L) of maintenance medium at a density of 1 \times 10⁴. The cells adhered for 3 hours, afterwards α -MEM (FBS 10%) cellular medium was added, at the following experimental times: 1, 7, and 14 days.

A stimulation experiment was also performed: after 24 hours from the seeding, α -MEM fresh medium was used, containing FBS 0.1% and lipopolysaccharide (LPS; Gibco, Life Technologies) at a concentration of 50 ng/mL. Cells remained under stimulation for 24 hours. FBS 0.1% α -MEM fresh medium was used, without LPS, was used as control.

At each experimental time, supernatants were collected to test IL-6 production, which is considered a marker of bone

remodeling and desorption. ELISA test was applied (Mouse IL-6 Quantikine ELISA, R&D Systems, Minneapolis, Minn) measuring the absorbance at 405 nm by means of a spectrophotometer plate reader (SpectraCount, Packard Bell, Los Angeles, Calif).

In Vivo Experiments

This study was performed according to European and Italian law on animal experimentation, whose principles are stated in the "Guide for the Care and Use of Laboratory Animals" and the Animal Welfare Assurance No A5424-01 by the National Institute of Health (NIH, Rockville, Md). Five Appenninica adult female sheep, 3.5 ± 0.5 years old and 80 ± 5 kg b.w., were submitted to bilateral screw implantation in the mandible. Surgical procedures were performed in a strict sterile environment under general anesthesia. Sedation was achieved with xylazine, 0.2 mg/kg i.m. (Rompun, Bayer, Barmen, Germany), followed after 10 minutes by diazepam, 0.2 mg/kg i.v. (diazepam, Intervet, Peschiera Borromeo, Milano, Italy) and atropine sulfate, 6 mg i.m. (Atropina Solfato, Fort Dodge, Bologna, Italy). Induction of anesthesia was obtained with ketamine 10 mg/kg i.m. (Ketavet 100, Intervet). The sheep were intubated, and general anesthesia maintained by inhalation of isoflurane (Isoflo, Esteve, Milano, Italy) in a mix of oxygen.

Surgical protocol. The surgical field was prepared to include both ventral mandibles. A 12–15-cm skin incision was performed along the ventral ramus of each mandibula from the notch for facial vessels rostrally. The subcutis, together with the periosteum, were sharply divided. Three implants were positioned on each ventral mandible, 4 cm apart from one another. Skin flaps were elevated on the inferior mandibular margins and the bone was exposed. On the ventral mandibular margin 3.5-mm diameter holes were drilled at low speed. The holes were then flushed and cooled with sterile 0.9% NaCl solution to remove bone debris and the screws, randomly chosen, were placed and tightened. The subcutis was closed in a simple continuous pattern using a 2/0 USP monofilament absorbable suture (Biosyn, Syneture, Covidien, Dublin, Ireland). The skin was closed in a simple interrupted pattern using a 2/0 USP monofilament non absorbable suture (Novafil, Syneture, Covidien). Postoperatively, antibiotics and analgesics (cephalosporin, 1 g, q12 h i.m. for 5 days and ketoprofen, 500 mg/d for 3 days) were administered.

The animals were pharmacologically euthanized 12 weeks (3 months) after surgery using an overdose of thiopental (pentothal sodium, Intervet) and embutramide (Tanax, Intervet).

Histologic preparation, microhardness, and histomorphometric measurements. After euthanasia each maxilla was separated from the skull and the mandibula were excised, cleaned of soft tissue, and fixed in 4% paraformaldehyde for histomorphometric and microhardness evaluations, which were carried out by blinded operators.

The biopsies obtained were dehydrated in ethanol series (70%, 80%, 90%, 96%, 100%), and then embedded in polymethyl-acrylate (Kulzer Technovit 7200 VLC, Bio-Optica, Milano, Italy) under gentle stirring and vacuum. Each biopsy was divided into 2 parts using a cutting system (Micromet Remet, Bologna, Italy) along the long axis of the screw. The 2 parts were reduced and polished with abrasive paper using a

grinding apparatus (LS2, Remet) till reaching the thickness of $100 \pm 20 \mu\text{m}$. The bone–material interface was observed using an optical stereo microscope (Wild Heerbrugg, Switzerland), and the microhardness measurements were performed using a Leitz micro-durometer tangentially to the interface with a Vickers indenter applied to the cancellous bone at a load of 0.05 kgf and dwell time of 5 seconds. The indentations observed with optical stereo microscope were measured and recorded with image analyzer software (Q-win-Leika SpA, Milano, Italy). The average value for each sample, expressed in Vickers unit or Vickers hardness degrees (HV), was calculated on a mean of 5 measurements for each examined area at the following sites: (1) within 200 μm from the interface and inside the thread depth (HV200 μm), and (2) at 2000 μm from the top of the screw thread in the host bone (HV2000 μm).

After microhardness evaluation the specimens were further grinded and, finally, stained with toluidine blue and pironine G (Sigma-Aldrich).

The sections were observed with an optical microscope (Eclipse E600, Nikon, Tokyo, Japan), stereomicroscope (SMZ800i, Nikon) and digitalized images were obtained (DXM1200, Nikon).

Bone in growth and bone implant contact index (BIC) was evaluated on the obtained images observed at 100X using an images analyzer software (NIS-Elements; Nikon). BIC values were obtained by calculating the ratio between the bone in contact with the implant and the total perimeter of the implant.

Statistical Analysis

For statistical analysis, OriginPro8 software (OriginLab Corporation, Northampton, Mass) was used. Mean values and Standard Deviation (SD) were calculated. Paired-sample Student's t test was used to compare 2 different means. In both tests, significance level was considered for $P < .05$.

RESULTS

Surface characterization

An electrostatically adsorbed PLL coating was obtained.

XPS survey spectra (Table) revealed the presence of 3.5% Ti on the PLL-coated sample, whereas it was 5.9% for the uncoated one. The presence of PLL coating was clearly shown by the amounts of N (3.3%) and the N/C ratio (~ 0.07) compared to the Ti disc (0.9% and 0.02, respectively). The nitrogen species were due to the PLL chemical composition and were revealed by high resolution spectra (Figure 1). High-resolution spectra exhibited an important peak at 400 eV, and the C1s high resolution spectra showed 3 main bands at 285, 286.2, and 288.4 eV assigned to C-C/C-H, C-O/C-N and C=O(NH)/C=O(O), respectively. For the high resolution of O1s, 3 main bands were also detected: metallic oxide at 529.8eV, C-O at 531.7eV, and C=O at 533.3eV, coming mainly from peptide structure and metallic substrate. It should be emphasized that Ti deconvolution exhibited a single peak at 458 eV (Table), attributed to TiO₂, which is the native oxide layer of titanium. Indeed, titanium is very sensitive to chemical environment, and its chemical nature can be therefore easily

TABLE

Surface chemical composition obtained from XPS survey spectra and water contact angle of Ti with and without PLL (n = 3). High-resolution spectra of titanium exhibited 1 peak centered at 458eV assigned to TiO₂ structure.

PLL	C (%)	O (%)	N (%)	Ti (%)	N/C	Contact Angle (°)
Without†	38.1 ± 0.4	41.6 ± 0.4	0.9 ± 0.1	5.9 ± 0.1 TiO ₂ – 100%	0.024 ± 0.001	88.6 ± 2.7
With†	48.4 ± 2.4	36.7 ± 1.2	3.3 ± 0.2	3.5 ± 0.6 TiO ₂ – 100%	0.067 ± 0.003	45.6 ± 0.9

†The balance in XPS for Ti with and without PLL were Ca, Na, and Al.

assigned: 454eV is associated to metallic Ti, 455-456eV TiO and 458eV TiO₂.

In order to study wettability/roughness, milliQ water contact angle measurement was performed, obtaining the sequence of values reported in Table.

The contact angle values were lower for the PLL-coated sample compared to the uncoated one. In order to screen surface morphology, AFM images of 1x1µm² were made: the Ti disk had an average roughness of about 95 nm², the Ti-PLL disk of 292 nm² (Figure 1s; <http://dx.doi.org/10.1563/AAID-JOI-D-13-00036.S1>).

In vitro experiments

Cell viability. MTT assays (Figure 2sa; <http://dx.doi.org/10.1563/AAID-JOI-D-13-00036.S2a>) [a] showed an higher osteoblasts viability on TiPLL (116% ± 2.8) than Ti (P < .05), at 3 days after osteogenic differentiation induction. No significant difference among Ti and TiPLL was detectable at 7 days, showing high cytocompatibility.

Cytodifferentiation. ALP concentration analysis (Figure 2sb; <http://dx.doi.org/10.1563/AAID-JOI-D-13-00036.S2b>) pointed out a similar trend in ALP activity of osteoblasts cultured onto Ti and TiPLL disks, after 7 and 14 days of osteogenic differentiation induction, with a peak at 7 days, then reduced after 14 days.

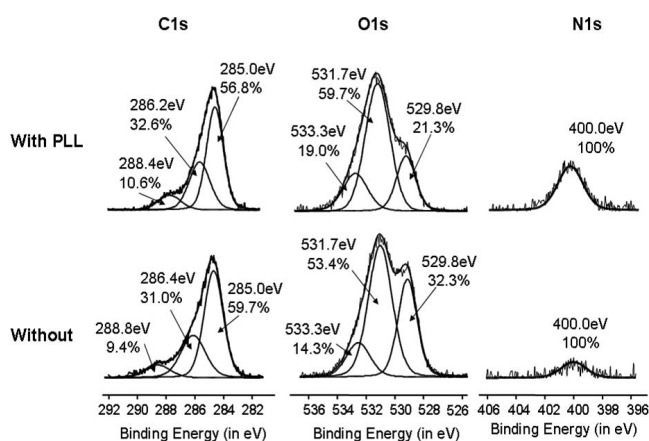


FIGURE 1. X-ray photoelectron spectroscopy (XPS). N1s, C1s, and O1s high-resolution (HR) spectra are reported for samples with and without PLL coating (upper and lower, respectively), showing the typical peaks of organic material/protein: HR-N1s showed a single peak close to 400 eV, HR-C1s had 285-286-288 eV peaks, and HR-O1s displayed 530-532-533 eV peaks, suggesting the presence of PLL coating.

Calcium deposition. As Figure 2 (a) shows, Alizarin Red assay, performed after 28 days of cell culture, revealed that TiPLL disks induced a significantly higher amount of calcified secreted matrix (Abs 570 nm: 0.299 ± 0.053) in comparison to Ti disks (Abs 570 nm: 0.577 ± 0.047) (P < .05).

Proinflammatory Il-6 production. After 1 day, the IL-6 amount was similar in all tested conditions, while at 7 and 14 days and after a day under LPS stimulation, IL-6 expression displayed a decrease in osteoblasts seeded on TiPLL disks than on Ti, even if without a statistical significance (Figure 2b).

In Vivo Experiment

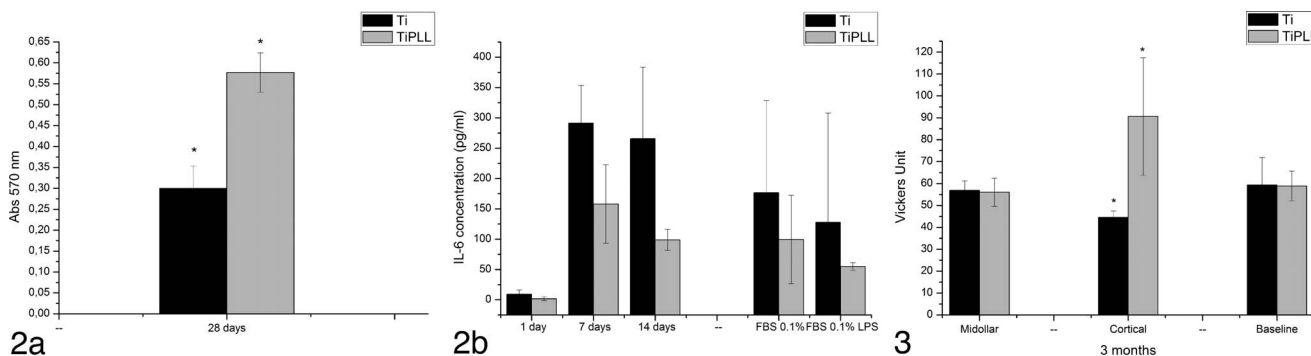
The whole hardness of the bone surrounding the implants was evaluated after 3 months from implantation by means of the Vickers test. After 3 months, as Figure 3 highlights, an increased hardness in cortical bone was obtained around TiPLL, in comparison to uncoated Ti (90.59 ± 26.8 vs 44.53 ± 2.94 Vickers, respectively, P < .05). On the other hand, no significant increase could be found in relation to the trabecular and native bone surrounding the implants. Indeed, as demonstrated by trabecular bone, microhardness was 56.00 ± 6.47 Vickers around TiPLL implants, compared to 56.90 ± 4.32 Vickers around Ti implants. For native bone, the values were 58.85 ± 6.8 and 59.29 ± 12.58 Vickers, respectively.

Moreover, despite the paucity of sample, histomorphometric analysis was conducted on peri-implant tissue surrounding uncoated and coated Ti implants. After 3 months, the bone in growth was of 91.2% ± 3.6 around Ti and 79.7% ± 17.5 around PLLTi. The bone-to-implant contact (BIC) revealed a similar trend between the 2 groups, both displaying high-contact values, 90.3% ± 4.7 for Ti implants and 84.5% ± 15.4 for TiPLL implants.

DISCUSSION

Here, we studied the coating of PLL on a titanium (Ti) implant surface, in order to elucidate PLL potential role in bone deposition. To the best of our knowledge, PLL has been mainly investigated as a Ti implant surface coating in copolymers or composite materials,^{8,11,12} but in vitro and in vivo studies on the effect of an electrostatically adsorbed PLL coating on Ti are rarely reported, although of great interest to optimize surface properties of dental and orthopedic implants.

We obtained a PLL surface coating, indeed XPS high resolution spectra both of C1s, N1s and O1s confirmed the presence of PLL amino-acid: N1s revealed a single peak close to 400 eV, typical of organic nitrogen species.^{30,31} In addition, similar observations were made through the O1s high



FIGURES 2 AND 3. FIGURE 2. Alizarin Red assay (a), performed at 28 days of cell culture, revealed how TiPLL disks induced a significantly higher amount of calcified secreted matrix ($P < .05$, Student's t test), not only in comparison to Ti disks. Pro-inflammatory IL-6 production (b). IL-6 expression showed a reduced trend in osteoblasts seeded on TiPLL disks than on Ti, also under LPS stimulation, even if without a statistical significance. **FIGURE 3.** Vicker Microhardness evaluation (after 12 weeks from implantation): trabecular and cortical refer to peri-implant bone, while native bone refers to bone around natural tooth. A higher mean value of cortical bone around implants coated with PLL (TiPLL) can be found, as compared to uncoated implants (Ti; $P < .05$; Student's t test).

resolution spectra with higher contributions of the characteristic bands C – O and C = O, at 531.8 eV and 533.4 eV, respectively. Moreover, XPS also detected the presence of Ti, revealing that PLL coating was not sufficiently homogeneous or thick to fully cover the Ti plate.

However, the presence of PLL was able to provide higher hydrophilicity and roughness to the titanium disk surface, as suggested by contact angle and AFM measurement. Cytocompatibility, osteodifferentiation, enhanced calcium deposition, and a decreased proinflammatory IL-6 production, were found in the osteoblasts seeded onto TiPLL, in comparison to uncoated Ti. These results suggest good biocompatibility of TiPLL implants. In particular, ALP activity was high at 7 days and reduced at 14 days. This result is consistent with the ALP property of being a strong early marker of osteogenic differentiation, which can no longer be found at later stages. Alizarin Red showed superior matrix calcification, a late osteogenic marker, in presence of PLL coating. A general reduced trend of IL-6 production can suggest a reduced bone remodeling process. These observations are related to the presence of a polyelectrolyte (PLL), which increases cell adhesion,^{8,10,13} and hydrophilicity, promoting the interaction between cells and substrate.

Our data are consistent with those reported by Park et al, who found that selected polyelectrolytes, including PLL, enhanced surface wettability of Ti implants.²

In our work, in vivo experiments in sheep showed how cortical bone micro-hardness, 3 months after implantation, significantly improved in the presence of PLL coating, although no significant difference was found referring to peri-implant trabecular bone. In this model system, in fact, the cortical portion is the most represented part of the bone, while trabecular bone is only a minimal section, unable to contribute to peri-implant bone microhardness.

On the other hand, no significant difference was found between coated and uncoated Ti in relation to BIC. These data are in accordance with the enhanced calcium deposition found with the above mentioned in vitro studies. Probably, PLL coating affects 3 months' deposited bone hardness, without modifying the bone-implant interface.

Enhanced osteoblast calcium deposition, together with a lower early bone remodeling, in an unloaded implant model, may be promising to accelerate healing time in the initial phase of implant integration, and to improve early implant stability.

CONCLUSION

The present study suggests that the PLL coating of Ti implants safely promotes osteoblast calcium deposition and peri-implant cortical bone hardness in animals.

Further in vivo studies to specifically investigate the electrostatic interaction between PLL and Ti and, in vivo, the bone fill and interfacial shear strength will be useful to further elucidate the role of PLL. The final aim is to optimize surface properties for dental implants in order to reduce healing time and thus enhance osseointegration.

ABBREVIATIONS

AFM: atomic force microscope
 ALP: alkaline phosphatase
 BIC: bone-to-implant contact
 HV: Vickers unit or Vickers hardness degrees
 LPS: lipopolysaccharide
 PLL: polyelectrolyte poly-L-lysine
 pNPP: 4-nitrophenylphosphate disodium salt hexahydrate
 TiPLL: Ti disks coated with PLL

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