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Cell behaviour on phospholipids-coated surfaces

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Abstract Effective integration of orthopedic biomaterials requires the rapid formation of the inorganic mineral phase during the first hours of implantation and the subsequent adhesion and proliferation of the osteoblasts. It has recently been demonstrated that phosphatidylserine-rich phospholipid coatings can induce a fast mineralisation of titanium implant surfaces on incubation in simulated body fluids. The aim of this work was to investigate the biocompatibility of these coatings in terms of cytotoxicity and ability to support osteoblast adhesion and activity. Cytotoxicity and cell adhesion to uncoated titanium, calcified phospholipid-coated titanium and HA-coated titanium was assessed using fibroblasts and osteoblast-like cells. The synthesis of type I collagen by osteoblast-like cells cultured on the calcified-phospholipid coatings was also comparable to that observed for osteoblast-like cells cultured on the titanium and HA-Ti surfaces.

The results suggest that the fast mineralization of the phospholipid matrix, obtained *in vitro* by its pre-treatment in a SBF, exposes the cells to an environment similar to that present in the bone during its natural formation that allow cells to adhere, proliferate and produce proteins fundamental for bone growth. The biocompatibility of these phospholipid-based coatings, in combination with their ability to initiate rapid mineralisation, provides a promising material that could *in vivo* create bone cell interactions and bone integration.

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Introduction

The key clinical objective in the orthopedic field is to develop implantable prosthetic materials which achieves rapid fixation with the surrounding bone [1]. Over the last two decades the development of biomaterials for orthopedic applications has been directed towards achieving complete integration of the prosthetic materials with bone tissue [2]. The most important characteristic of a new orthopedic material has to be the capability to promote osteointegration, which is defined as a "direct structural and functional connection between ordered living bone and the surface of a load-carrying implant" [3, 4]. Effective osteointegration seems to be the only way of ensuring good long-term prosthesis functionality [5]. Although some progress has been made in improving osteointegration the materials used as prosthetic alternatives to natural bone do not promote a satisfactory osteointegration in the required timeframe. In fact the available implants tend to provide an indirect bone-implant contact through the formation of a fibrous layer at the bone-implant interface which often contributes to the mobilization of the implant [3]. The development of new osteointegrative polymeric coatings, although proposed as a solution to the promotion of osteointegration, is still in embryonic development. In recent years scientists have focused on the development of bioactive osteoconductive materials which limit the host immune response [6], and promote the formation of new bone tissue through, for example, fabrication of polymeric scaffolds which can support bone in-growth [2, 3].

Phospholipids have previously been shown to have many potential applications in the biomedical field through their

ability to form liposomes for diagnostic imaging and drug delivery [7, 8]. More recently the use of phospholipids as biomaterial has generated considerable interest [9] in light of their ability to mimic the cell surface [10–12]. Extracellular membrane vesicles (matrix vesicles) contain phospholipids associated with mineralized material. These matrix vesicles seem to be involved in initial formation of calcium hydroxyapatite crystals via the interaction of calcium and phosphate ions with phosphatidylserine (PS), an acidic phospholipid found in high concentration in the membrane bilayer [13]. The matrix vesicles appear to be the foci for initial calcification with their acidic phospholipids providing sites for initial calcium binding and their interior offering an environment in which mineralisation can be facilitated through the increase of the ion concentrations to saturating levels [14]. Recent studies [8] suggest a specific role for the amino group of phosphatidylserine in interacting with calcium in the mineral phase thereby linking the vesicle membrane to the mineral phase. In particular, it has been postulated that phosphatidylserine binds to the calcium phosphate ion cluster providing a nucleating site for growth of the crystalline apatite molecule. The important role of these types of lipids in the mechanism of bio-mineralisation has been clearly demonstrated *in vitro* [15], and the crystal nucleation and growth processes which occur during the initial stages of mineral formation in the matrix vesicles has been clearly highlighted [16]. PS-based phospholipid coatings have been recently developed which induce a fast mineralisation when exposed to simulating body fluids whose salt composition is similar to that of the bone interstitial fluids [17–19]. These types of coatings aim to produce early phase mineralisation at the bone-implant interface by mimicking the role of matrix vesicles during the development of bone.

The aim of this study was to evaluate *in vitro* the adhesion of osteoblasts and fibroblasts to porous titanium materials coated with both PS- and phosphatidyl inositol-based coatings to assess whether the phospholipid-rich microenvironment would affect the adhesion and proliferation of these cells. In addition the production of collagen by cells on these surfaces has been evaluated as an indication of whether these coating materials will modulate the ability to support the formation of new bone.

Materials and methods

Materials

Titanium-300 nm porous disks (12 mm diameter) have been used for cell adhesion and proliferation studies and 25-mm diameter disks for collagen synthesis analysis. An additional control used in the experiments was hydroxyapatite-coated titanium (HA-Ti). All the substrates were provided by SAMO SpA (Bologna, Italy). Three phospholipidic coatings were studied: (i) phosphatidylserine (PS); (ii) phosphatidylserine: phosphatidylcholine (PC): Cholesterol (C) (7:3:1 molar ratio) and (iii) phosphatidylcholine: phosphatidylinositol (PI): Cholesterol (7:2:1 molar ratio). All the phospholipids were of natural source and purchased from Sigma (Poole, UK).

Disk coatings

The three candidate phospholipidic coatings were prepared as previously described [17]. The phospholipids were dissolved in an appropriate volume of chloroform by sonication on ice to minimize solvent evaporation to give a final concentration of 222 mM. Complete disk coating of the rough surface of Titanium was obtained by adding 20 μl of the phospholipidic solution. After solvent evaporation (15 min at sterile conditions), coating hydration and mineralisation was induced by pre-conditioning the disks in Vogel's Simulating Body Fluid (SBF) for 1 h at 37°C [14]. This pre-treatment has been previously proven to induce substantial mineralisation of the phospholipid matrix [17]. After pre-equilibration in the cell growth medium, the disks were seeded with cells as described below.

Cell culture method

Cytocompatibility and cytotoxicity tests were performed using two different cell lines: human fibroblast MRC5 cell line derived from normal lung tissue (ATCCCCL 171) and osteoblast-like cells isolated enzymatically from trabecular fragments obtained at surgery from adult human bone [20]. Briefly, bone trabecular fragments after treatment with bacterial collagenase 3 mg/ml, 6.35 U/ml elastase, 18.22 mg/ml D-sorbitol and 6 mg/ml chondroitin sulfate (all from Sigma) were placed in 90 mm tissue culture dishes and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin, 15 μl streptomicin and 2 mM L-glutamine at 37°C in 95% air/5% CO_2. Outgrowth of cells from the bone fragments appeared within one week and formed a confluent monolayer after 3–4 weeks. The isolated bone cells were characterized by a number of parameters, including (i) osteoblast morphology, (ii) alkaline phosphatase expression and (iii) hormone responsiveness (PTH, 1,25$(OH)_2$D3).

Alkaline phosphatase activity

Alkaline phosphatase activity (APA), a marker of osteoblast-like cells differentiation, was determined by an assay based on the hydrolysis of p-nitrophenylphosphate to p-nitrophenol. Cultures were collected after 4 days incubation with the materials, rinsed three times with PBS and placed in 300 μl 0.05% Triton X100 in PBS and

sonicated on ice for 2 × 10 sec using a Branson 250 sonifier (Branson Ultrasonics, Danbury, USA). One hundred microliters of substrate (1 mM p-nitrophenilphosphate in 1 M diethanolamine + 1 mM $MgCl_2$ pH 9,8 all from Sigma) were added to 100 μl of cell extract. The mixture was incubated at 37°C until the color was comparable with the standardized series (a 20 mM p-nitrophenol solution from Sigma) in about 15–30 min. All sample, including the standardized series, were measured in duplicate on a Bio-Rad microplate spectrophotometer reader at 490 nm [21].

Cytotoxicity evaluation

Cytotoxicity was evaluated by lactate dehydrogenase (LDH) concentration test [22]. The experiments have been performed both on MRC5 and on osteoblasts-like cells. Observations have been performed on 5×10^3 cells/cm^2 for fibroblasts and on 1×10^4 cells/cm^2 for osteoblast-like cells. Two additional cultures have been used in both experiments: untreated cells (negative control) and cells treated with Triton X100 (positive control). LDH activity, corresponding to the oxidation of NADH to NAD+ in presence of LDH and pyruvate, has been measured after 12, 24, 48 and 72 h by measuring the color developed at 340 nm by BECKMAN DU®-68 spectrophotometer.

Cell adhesion and proliferation

The adhesion and proliferation of both fibroblasts and on osteoblast-like cells were assessed by initially seeding with 2×10^4 cells/cm^2 and quantifying the number of adherent cells at 6 h, 24 h, 48 h and 72 h. Cells were also cultured on polystyrene as a control. The experiments were repeated three times for each kind of material. After each incubation period, the medium was removed and cells were detached by treatment with trypsin and counted using a Kova's slide (Boheringer Mannheim Italia, Milano) on an inverted microscope.

The morphology of the adherent osteoblast-like cells was assessed by scanning electron microscopy (SEM). After the experiment the sample were fixed in 2.5% (w/v) glutaraldehyde, stepwise dehydrated in increasing concentrations of ethanol and freeze-dried overnight. The samples were sputter-coated with palladium and analyzed by SEM at 5 keV.

Collagen type I production

The potential of the tested materials to favor the formation of a collagen-based extracellular matrix on the surfaces has been evaluated by testing the production of type I collagen by osteoblast-like cells.

Osteoblast-like cells were incubated for 4 days following initial seeding at 1×10^5 cells/cm^2 as described above, with the addition of 50 μg/ml L-ascorbic acid to the culture media. After incubation, the culture media were collected separately and dialysed against distilled water at 4°C for 24 h, freeze-dried and resuspended in 100 μl of distilled water. Ten microliters of resuspended culture media were added with 10 μl SDS-PAGE sample buffer (4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 40% (w/v) glycerol and 0.02% (w/v) bromophenol blue in 62.5 mM Tris-HCl, pH 6.8 Sigma) and loaded onto 10% polyacrylamide gel.

Adherent cells were washed with PBS and sonicated on ice in 100 μl of a mixture composed by water (50%), SDS 10% (25%) and Tris-HCl pH 6.8 (25%). Twenty microliters of the cell lysate were mixed with the SDS-PAGE sample buffer (2-mercaptoethanol:glycerol 1:10 and 0.02% w/v bromophenol blue) and loaded onto the gel. Total protein was also determined using a total protein assay kit BCA (Pierce). Electrophoresis was carried out at 100 V by a Bio-Rad mini Protein II electrophoresis system and stained by a BioRad Silver Stain Plus kit [23] or blotted onto nitrocellulose membrane (Amersham) in a Bio-Rad Mini Trans-Blot electrophoresis Transfer Cell [24]. The membranes were then treated with 0.3% w/v bovine serum albumin (Sigma) in PBS for 1 h at room temperature and then, after washings with PBS/Tween-20 0.1%, incubated with a mouse anti-human collagen type I (5 mg/ml in PBS) overnight at 4°C. After washing, the membranes were incubated with 1:2000 diluted anti-mouse antibody conjugated with horseradish peroxidase (Amersham, Milan, Italy) for 1 h at room temperature. ECL™ Western Blotting detection reagents (Amersham) were used for immunodetection of the proteins. Collagen produced from the osteoblast-like cells cultured onto the tested materials was quantified by a picrosirius dye staining method [25, 26]. Standards were prepared by dissolving type I collagen (Sigma) at concentrations of 4–0.5 μg/50 μl of culture medium. Samples (50 μl of culture medium) and collagen standards, were added into a microtiter plate and incubated at 37°C for 16 h (humidified) and then 24 h at 37°C (dry). Wells were washed in distilled water, filled with 100 μl of 0.1% Sirius Red F3BA (Fluka) in saturated picric acid (Sigma) and stained for 1 h at room temperature. The plates were then washed five times with 200 μl of 10 mM HCl (10 sec per wash); the collagen bound stain was washed with 200 μl of 0.1 M NaOH for 5 min and the eluted stain was read at 540 nm in a model 450 microplate reader (Bio-Rad).

Statistical analysis

Statistical analysis of the data was carried out using a Dell computer equipped with SPSS for Windows software. The Bonferroni test was performed and data were considered significantly different at $p < 0.05$.

Results

Osteoblast-like cells differentiation (APA)

90% of the cells obtained by enzymatic isolation from human trabecular bone had a typical osteoblast phenotype, with respect to cell morphology and alkaline phosphatase activity. From the incubation of these cells on the different materials APA levels were found to be significantly lower than those obtained on the control. There was no significant difference between the APA levels for the cells seeded on different substrates (Fig. 1).

Cytotoxicity

The LDH activity of all the tested materials was found to be statistically lower than that observed for the positive control (Triton X100-treated cells), but not significantly different from the negative control. No time dependent increase in LDH activity was observed when either fibroblasts or osteoblast-like cells were cultured on the test materials (Fig. 2).

Cell adhesion and proliferation

The adhesion of fibroblasts on all the materials tested appeared to be similar but titanium and PS coatings evidenced statistically lower fibroblast cell number when compared to the polystyrene control. Fibroblasts adhesion to the HA coated Ti-300, Ti-PS:PC:C and Ti-PC:PI:C appeared to be statistically enhanced compared to uncoated titanium and PS coated Ti-300 (Fig. 3A). It appears an increase in the number of cells after 72 hours on all the materials; if comparing the PS-coated material to uncoated titanium and to HA coated titanium no statistical differences were seen for fibroblast number at 72 hours incubation time (Fig. 3B).

The adhesion of osteoblast-like cells to all the tested materials showed (Fig. 4A), a statistically reduced adhesion at 6 hours to uncoated titanium and to the HA coated material. No statistical differences were seen in cell adhesion to the three phospholipid-based coatings, which were comparable to the control. After 72 hours osteoblast-like cell cultures evidenced low cell proliferation respect to cell number obtained at 6 hours adhesion time; on the tested materials osteoblast-like cell number evidenced no statistical differences between the three phospholipids formulation (Fig. 4B). SEM of the osteoblast-like cells adhering on the surface of the phospholipids showed a good cell spreading and the typical morphology of the osteoblasts (Fig. 5).

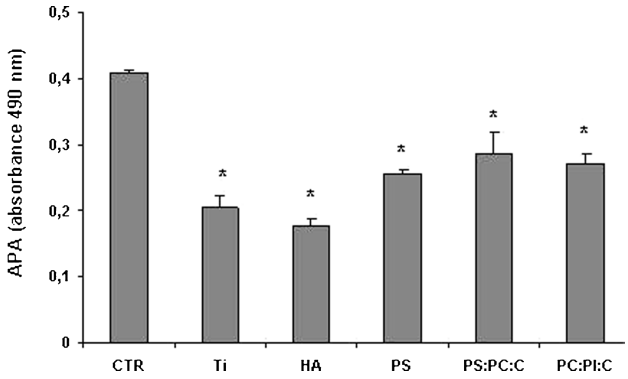

Fig. 1 The total amount of alkaline phosphatase activity after 4 days of incubation of human osteoblast-like cells onto the materials tested. Data expressed as absorbance at 490 nm. Mean ± SD ($n = 3$) CTR, untreated cells cultured in proliferation on polystyrene wells; Ti, cells proliferated on Titanium; HA, cells proliferated on Titanium coated with hydroxyapatite; PS, cells proliferated on Titanium coated with phosphatidylserine; PS:PC:C, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcoline:cholesterol; PC:PI:C, cells proliferated on Titanium coated with phosphatidylcoline:phosphatidylinositole: cholesterol. ($^*p < 0.05$ respect to control untreated cells)

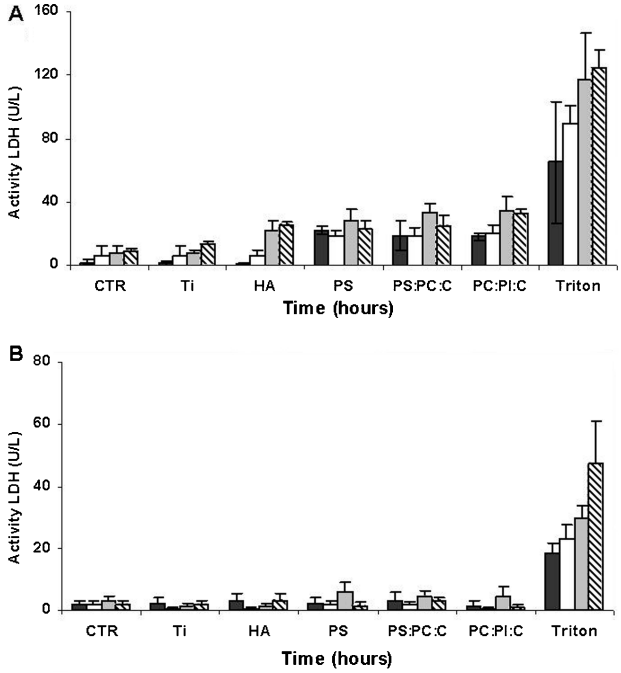

Fig. 2 LDH activity at long-term incubation MRC5 fibroblasts (Fig. 2A) and human osteoblast-like cells (Fig. 2B) cultured onto the tested materials. Data expressed as LDH activity (U/l) respect to the different incubation times (12, 24, 48 and 72 h). Mean ± SD ($n = 3$). CTR, untreated cells cultured in proliferation on polystyrene wells; Ti, cells proliferated on Titanium; HA, cells proliferated on Titanium coated with hydroxyapatite; PS, cells proliferated on Titanium coated with phosphatidylserine; PS:PC:C, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcholine:cholesterol; PC:PI:C, cells proliferated on Titanium coated with phosphatidylcoline:phosphatidylinositol:cholesterol; TRITON, cells cultured in proliferation on polystyrene wells and treated with Triton X100 (positive control)

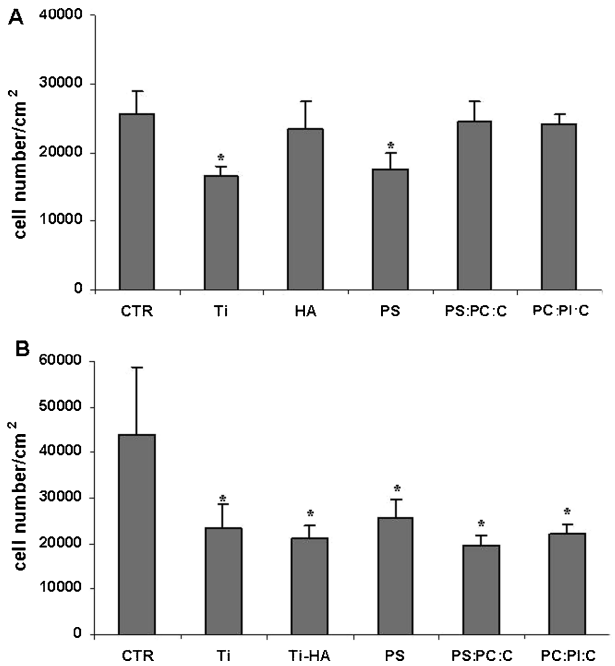

Fig. 3 Histogram showing the fibroblasts adhesion (A) and proliferation (B) by direct cell counting. Data expressed as cell number/cm^2. Mean ± SD ($n = 3$); $^*p < 0.05$ respect to CTR. CTR, untreated cells cultured in proliferation on polystyrene wells; Ti, cells proliferated on Titanium; HA, cells proliferated on Titanium coated with hydroxyapatite; PS, cells proliferated on Titanium coated with phosphatidylserine; PS:PC:C, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcholine:cholesterol; PC:PI:C, cells proliferated on Titanium coated with phosphatidylcoline:phosphatidylinositol:cholesterol

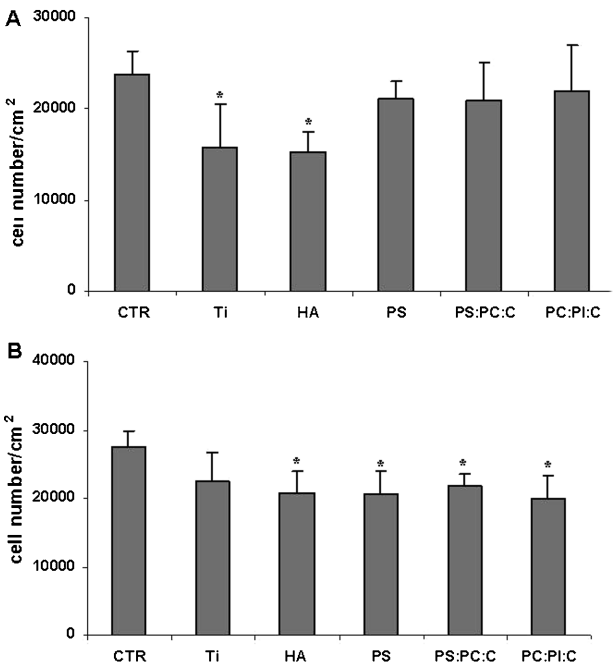

Fig. 4 Histogram showing the osteoblast-like cells adhesion (A) and proliferation (B) by direct cell counting. Data expressed as cell number/cm^2. Mean ± SD ($n = 3$); $^*p < 0.05$ respect to CTR. CTR, untreated cells cultured in proliferation on polystyrene wells; Ti, cells proliferated on Titanium; HA, cells proliferated on Titanium coated with hydroxyapatite; PS, cells proliferated on Titanium coated with phosphatidylserine; PS:PC:C, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcholine:cholesterol; PC:PI:C, cells proliferated on Titanium coated with phosphatidylcoline:phosphatidylinositol:cholesterol

Collagen Type I production

Silver Staining of the SDS-gels for the supernatant obtained from the osteoblast-like cells (Fig. 6) showed a similar pattern of protein production for all the materials tested. Cells incubated on the test materials seemed to produce higher levels of a protein at approximately 80 kDa compared to that secreted into the tissue culture supernatant in the control sample (Fig. 6, lane 1). Some of the low molecular weight proteins expressed by cells cultured on titanium and HA-Ti appeared to be expressed to a lesser extent by the osteoblast-like cells cultured on the phospholipid-coated materials (lanes 4–6). Western Blot of osteoblast-like cells culture media (Fig. 7) showed that type I collagen was present in both culture media and the cell lysate from all the materials tested, (Figs. 7a and b). Sirius Red staining showed that the production of collagen released from osteoblast-like cells into the culture medium (Fig. 8) was similar regardless of the material on which the cells were cultured.

Discussion and conclusion

The functionality of orthopedic prosthetic implants is primarily determined by the osteointegrative potential of the biomaterial which serves to ensure the integration of the implant surface to the surrounding bone and promotion of new bone tissue formation. A key aspect of new generation orthopedic biomaterials is the ability to catalyze the formation of new inorganic mineral phase during the first hours of implantation and allow the subsequent adhesion and proliferation of the osteoblasts. In recent years *in vitro* studies have suggested the use of lipid vesicles as models for the study of the biological calcification process [14, 15]. These investigations have demonstrated that PS-based formulation can promote calcification *in vitro* [16]. Recently, it has been shown that, when used as coating materials, PS-rich phospholipid coatings can induce a fast mineralisation of the implant surface during incubation in SBF [17–19]. The aim of this work was to investigate the biocompatibility of these coatings in terms of cytotoxicity and ability to support osteoblast adhesion and activity.

The data showed no cytotoxicity, good cell vitality and osteoblast collagen type I synthesis for all the phospholipid coatings, comparable to those of the titanium and HA-Ti surfaces.

These results suggest that the fast mineralisation of the phospholipid matrix, obtained *in vitro* by its pre-treatment

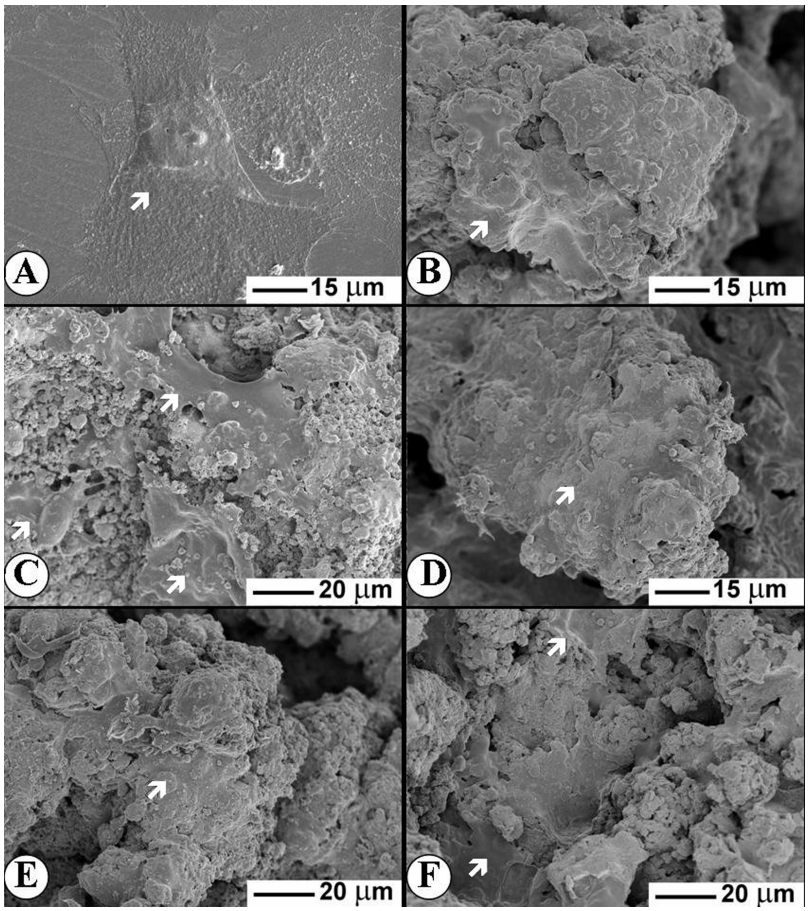

Fig. 5 SEM Micrographs showing osteoblasts on (a) CTR, cells on tissue culture plastic (×1200) (b) Ti, Uncoated Ti-300 (×1200) (c) HA, coated Ti-300 (×1000) (d) PC:PS:C coated Ti-300 (×1200) (e) PS coated Ti-300 (×1000) and (f) PC:PI:C coated Ti-300 (×1000)

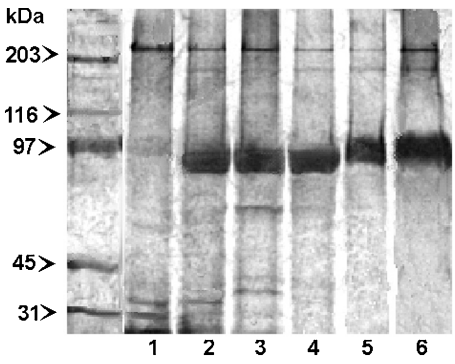

Fig. 6 Silver Staining of protein synthesized by the human osteoblast-like cells during 4 days-proliferation onto the tested materials. Lane 1, basal production of unstimulated cells; Lane 2, cells proliferated on Titanium; Lane 3, cells proliferated on Titanium coated with Hydroxyapatite; Lane 4, cells proliferated on Titanium coated with phosphatidylserine; Lane 5, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcholine:cholesterol; Lane 6, cells proliferated on Titanium coated with phosphatidylcholine:phosphatidylinositol:cholesterol. The first lane without number corresponds to the standard molecular weights

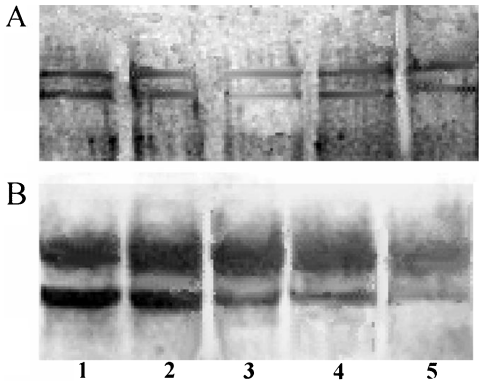

Fig. 7 Western Blot analysis of Type I Collagen expressed by osteoblast-like cells cultured 4 days onto the tested materials. Figure 7A shows Type I Collagen secreted by the cells into the culture medium; Fig. 7B shows Type I Collagen associated with cell lysate. Lane 1, cells proliferated on Titanium; Lane 2, cells proliferated on Titanium coated with Hydroxyapatite; Lane 3, cells proliferated on Titanium coated with phosphatidylserine; Lane 4, cells proliferated on Titanium coated with phosphatidylserine:phosphatidylcholine:cholesterol; Lane 5, cells proliferated on Titanium coated with phosphatidylcholine:phosphatidylinositole:cholesterol

in a SBF [17], exposes the cells to an environment similar to that present in the bone during its natural formation that allow cells to adhere, proliferate and produce proteins fundamental for bone growth. The biocompatibility of these phospholipid-based coatings, in combination with their ability to initiate rapid mineralisation [17], provides a promising

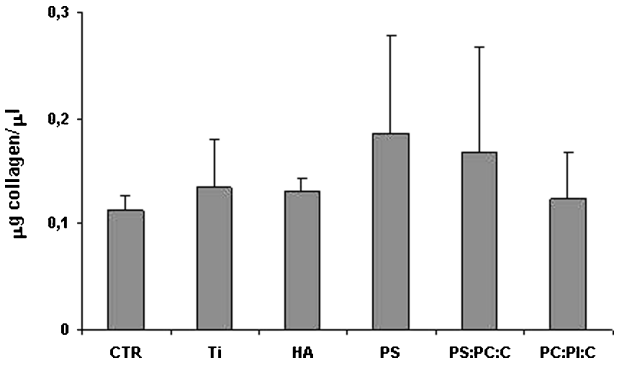

Fig. 8 Sirius Red staining of osteoblast-like cells total Collagen production after 4 days incubation in contact with the tested materials. Data obtained reading absorbance of Sirius red at 540 nm verses μg/μl of calf skin Type I Collagen standard curve and expressed as μg collagen/μl. Mean of triplicate determinations. CTR, untreated cells cultured in proliferation on polystyrene wells; Ti-300, cells proliferated on Titanium; HA, cells proliferated on Titanium coated with hydroxyapatite; PS, cells proliferated on Titanium coated with phosphatidylserine; PS:PC:C, cells proliferated on Titanium coated with phosphatidylserine: phosphatidylcoline: cholesterol; PC:PI:C, cells proliferated on Titanium coated with phosphatidylcholine: phosphatidylinositole: cholesterol

material that could *in vivo* create bone cell interactions and bone integration.

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