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Biodegradable microgrooved surfaces for skeletal muscle regeneration

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Introduction

During tissue formation, skeletal muscle precursor cells fuse to form multi-nucleated myotubes. The formation of such structures requires the orientation and alignment of myoblasts in a correct structure prior fusion (1). To understand the mechanisms regulating skeletal muscle development, *in vitro* systems promoting cells alignment need to be developed. One method to obtain micrometer-scale features on substrate surfaces is by means of photolithography (2). This technique can be used to control and affect cell size, shape, spatial organization, proliferation and gene regulation.

The aim of this work is to investigate how differently polymeric microgrooved surfaces, obtained by photolithography and polymer casting can affect, *in vitro*, myoblast alignment, fusion and successive myotubes formation.

Materials and methods

Microgrooved polymeric films were obtained by solvent casting on microgrooved silicon wafers with different grooves widths (5, 10, 25, 50, 100 μm) and depths (0.5, 1, 2.5, 5 μm) obtained by standard photolithographic techniques. A solution of biodegradable L-lactide/trimethylene carbonate copolymer (PLLATMC, Resomer LT 706, Boerhinger Ingelheim) in chloroform (5% w/v) was used. The surface morphology of all wafers and films was investigated by Dektakt Surface Profiler and SEM (Hitachi S3000), to verify the accordance of morphology of the obtained micropatterns to the master ones.

In vitro static cell tests were performed using C2C12 murine myoblasts, seeded with a density of $5*10^5$ cells/ml. Cell adhesion and proliferation were studied up to 7 days after confluence. Cell morphology was investigated by optical microscope and immuno-fluorescence assays. Expression of myosin was detected using an anti myosin primary antibody (MF20) and an Alexa 633 secondary antibody; actin was detected using FITC Phalloidin and nuclei were labeled with Hoechst.

Results and discussion

Good reproducibility of the polymeric replicas, obtained from the micropatterned silicon wafers was confirmed by SEM for all the different grooves dimensions; Figure 1 shows a similar morphology for the master (a) and polymeric replica (b) with a 2.5-50 μm microgrooves dimensions. Comparing profilometric data, no significant differences ($p>0.05$) in groove dimensions were found between wafers and PLLATMC films, and very regular profiles were detected for all polymeric samples.

In vitro analysis demonstrated different cell behaviour, depending on groove width and depth. At 24 hour from seeding for all depths an increased degree of alignment of the cells was observed on films with grooves width of 5, 10 and 25 μm. On films with grooves width of 50 and 100 μm cells were not aligned and no preferential orientation was observed; this likely due to the grooves dimensions being larger than a single cell.

Seven days after confluence, on all films the development of myotubes was observed from fluorescence images: multinucleated cells were detected all over the surfaces. On films with deeper grooves (2.5 and 5 μm) myotubes were more aligned. On 5 and 10 μm grooves width, myotubes were always aligned, but appeared to be packed with few nuclei, especially for 5 μm grooves depth. On 25 (Fig. 2a) and 50 μm width it seemed to be a good alignment and myotubes formation, while for 100 μm grooves width the alignment is less pronounced. Considering shallower films (0.5 and 1 μm) a less evident alignment was observed. Especially for films with 0.5 μm depth a certain degree of alignment was detected but only on 5 μm grooves width. On smooth PLLATMC films used as control, myotubes were randomly oriented as shown in Figure 2b.

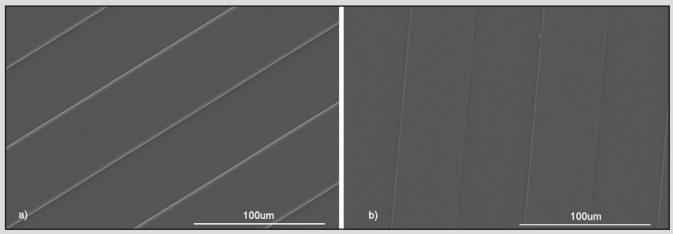

Figure 1 - SEM micrographs of a wafer (a) and the corresponding polymeric film (b) with 2.5 μm grooves depth and 50 μm grooves width.

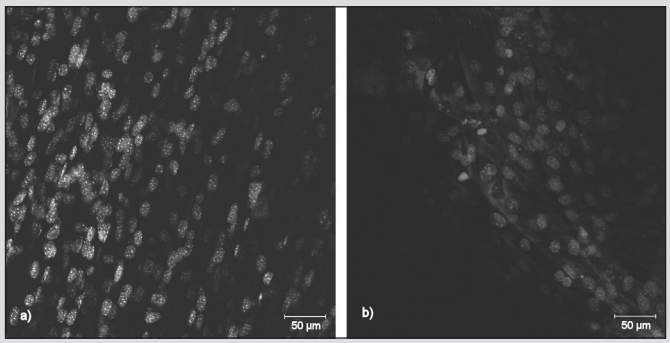

Figure 2 - Immunofluorescence images of C2C12 nuclei after 1 week of culture on 2,5-25 μm polymeric film (a) and smooth film (b).

Conclusions

Microgrooved surfaces seem to be a promising method to improve myotubes formation and alignment. Best alignment and myotube formation was observed for 2,5 and 1 μm depth especially for 50 and 25 μm width.

For films with more adequate microgrooves dimensions *in vitro* dynamic test will be performed. The possibility of using mesenchymal stem cells is also under investigation.

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Polyurethane foams and Ca-P composites for bone tissue engineering

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Introduction

Calcium-phosphates (hydroxyapatite, HA, and α-tricalciumphosphate, α-TCP) can be added to synthetic polymers to develop biomimetic composites for bone regeneration. In fact, a biomimetic inorganic coating of the porous structure can improve the early osteointegration process. We have recently set up a process to obtain cross-linked polyurethane foams (PUFs) with a controlled range of pore size, open porosity and slow degradation rates. Polymeric scaffolds with slow degradation rate, compared with biodegradable polymers, can offer the essential advantage of a slow release of non toxic degradation products, which do not evoke aspecific inflammatory response. These polyurethane foams (PUFs) and their composites with HA or α-TCP have been proposed as scaffolds for bone regeneration (1).

Materials and methods

The PUFs were synthesized with a one step bulk polymerization by reacting a polyether-polyol mixture with polymeric MDI, using Fe-acetylacetonate as catalyst (0.001% w/wp), and water as expanding agent (2% w/wp) (2). Two families of foams (EC and EF) were developed using two different polyether-polyol formulations. EF-based foams proved to be more hydrophilic and flexible than the EC-based ones. Composites were obtained by a co-expansion process of the matrices with hydroxyapatite (HA, A6021, Plasma Technik) or α-TCP (CNR-CSFM "G. Ciamician", Bologna, Italy) and/or by coating the PUFs with CaP, in order to improve the mechanical properties of the base foams and support osteointegration (3). Before coating, specimens did not undergo any surface treatment because, after surface modification, CaPs would be attracted mostly by the outer part of the specimen and therefore they would probably not penetrate deep into the foam. With the aim to mimic the *in vivo* conditions of the ECM environment, the coated samples were immersed into Simulated Body Fluid (SBF) up to 14 days, while the loaded and coated composites were treated in the same way up to 21 days. Before and after the SBF treatment, weight variations and SEM analysis were performed. XRD and EDS analyses were also carried out on PUFs coated with the CaPs.

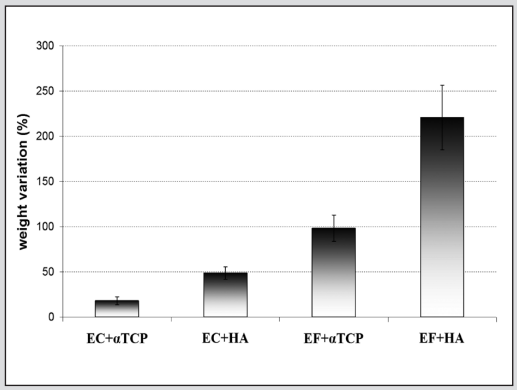
To evaluate *in vitro* growth and gene expression of human bone marrow stromal cells (MSCs) and primary osteoblasts from human donors (HOBs), MSCs and HOBs were seeded (10^5 cells/well) on loaded or unloaded PU foams, and cultured up to 5 weeks. Cell viability and ALP release were investigated; morphology was assessed by SEM analysis and ALP and osteocalcin mRNA expression were analyzed after 5 weeks of *in vitro* culture.

Results and discussion

Possibly due to its hydrophilicity, the EF-type foam showed a better ability to be coated with CaPs (Fig. 1). After the SBF treatment, a weight increase was evidenced by α-TCP coated foams, according to a phase change of CaPs, from α-TCP to HA. This result was also confirmed by XRD analysis.

The results of *in vitro* tests indicated that HOBs and MSCs showed higher viability after the fourth week of culture onto the composites. A higher number of MSCs than HOBs was observed on EC and EF foams, and MSCs also formed a thick layer of extracellular matrix. Moreover, by SEM, it was possible to evidence a deeper colonization of the EF-type foams, compared to the EC-type (Fig. 2). On both EF and EC matrices and composites, ALP mRNA expression was higher for MSCs than for HOBs. Osteocalcin mRNA expression was higher for MSCs than HOBs in EC matrices and EF α-TCP composites. Cells onto EF composites, and particularly onto EF α-TCP, expressed ALP and osteocalcin mRNA at a higher level than cells onto EF matrices.

Figure 1 - PUFs weight variations after coating with CaPs.



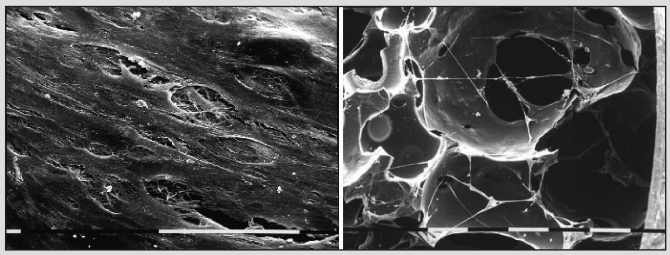


Figure 2 - MSCs on EC α-TCP composites (left) and HOBs on EF α-TCP (right) after 4 weeks of culture (bar = 100 μm)).

Conclusion

The deposition method allowed an uniform coating of PU foams with a high amount of CaPs; in particular, α-TCP demonstrated to be more strongly bound to the substrate, particularly on EF matrices. The loading of the matrices with HA and α-TCP particles improved the osteoconductivity of the scaffolds. MSCs grew quickly and differentiated to osteoblasts, providing a good source of active bone forming cells. In conclusion, the described PUF/CaP composites can be advantageously proposed as scaffolds for bone tissue engineering.

Note

The base reagents for PU foaming were provided by Elastogran (Italy), Group BASF, until 2004 (Projects financed by CNR and FIRB, Italy). Starting 2006 BASF does not supply its "plastics" for the manufacture of implants of any risk class.

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Structure and *in vitro* behaviour of alendronate modified hydroxyapatite nanocrystals

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Introduction

Bisphosphonates (BPs) were developed in the 19th century, but were first investigated in the 1960s for use in disorders of bone metabolism. Nowadays bisphosphonates are the most popular drugs for the treatment of osteoporosis, Paget's disease, bone metastasis (with or without hypercalcemia), multiple myeloma and other conditions that feature bone fragility (1). In particular alendronate is a first-line bisphosphonate, which displays a very high affinity for bone tissue being rapidly absorbed onto the bone surface (2). In the laboratory, the great affinity of alendronate for Calcium ions hinders the direct synthesis of hydroxyapatite (HA) crystals modified with BPs, and this is for sure one of the reasons why the structural interaction of alendronate with hydroxyapatite has not been completely investigated, yet. We have recently developed a modified method of hydroxyapatite synthesis for the preparation of composite nanocrystals containing alendronate. Herein we report the chemical-physical properties of this new material and its effects on cells behaviour.

Materials and methods

The synthesis of hydroxyapatite was carried out in N2 atmosphere by dropwise addition of a $(NH_4)_2HPO_4$ solution to a $Ca(NO_3)_2 \cdot 4\ H_2O$ solution, pH 10, 90 °C. We verified that the direct approach to synthesize a HA-alendronate composite material yielded to a mixture of amorphous material and crystalline HA (3). So, a modification was made to the above procedure by adding alendronate dropwise (concentration range 7–28 mM), after completing the phosphate solution addition. Powder X-ray diffraction, TEM, BET, spectrophotometric, thermal and chemical analyses were used to characterize the structure, morphology and composition of the products. Alendronate release tests were performed in Phosphate Buffer Solutions pH 7.4 at 37 °C. Disk shaped samples of HA-alendronate composite materials were prepared by pressing the powders into cylindrical moulds. Gamma rays sterilized disks, and HA as reference, were cultured with human osteoblasts and osteoclasts up to 21 days. In order to assess the effect of alendronate on osteoblasts proliferation and activity, WST-1, alkaline phosphatase activity, osteocalcin, type I collagen, matrix metalloproteinase-1 (MMP1) and matrix metalloproteinase-13 (MMP13) were tested, whereas TRAP staining and pit assay were performed on osteoclasts.

Results and discussion

With the new method of synthesis we obtained hydroxyapatite as unique crystalline phase in the whole range of alendronate concentration. Thermogravimetric analysis provides evidence that the products are composite nanocrystals. Alendronate is incorporated into HA crystals up to about 7%wt, without significantly affecting the values of the lattice constants of HA and the Ca/P molar ratio. The results of the structural refinements indicate no significant variation of the atomic positions, occupancy factors, and thermal parameters of hydroxyapatite. The diphosphonate anion most likely interacts with the calcium ions on the HA surface through a bidentate chelation of deprotonated oxygen atoms. On the other hand, alendronate affects the morphology of the composite crystals, which exhibit significantly smaller dimensions (about 20×100 nm) than HA crystals (usually 40×200 nm) and increased surface area.

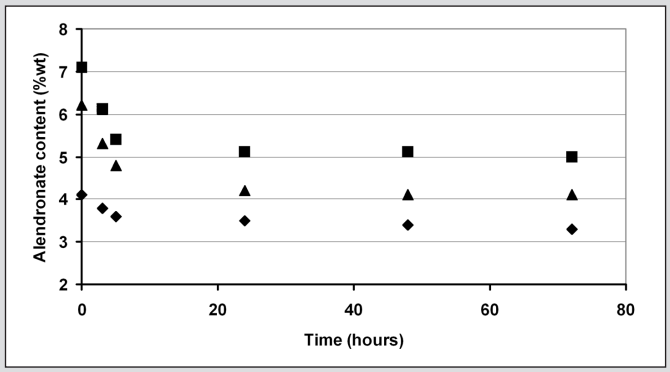

Figure 1 - Release of Alendronate from composites stored in Phosphate Buffer Solutions pH 7.4 at 37°C. Tested samples were prepared at alendronate concentration of (◆) 7 mM, (▲) 14 mM and (■) 28 mM.

Kinetics of alendronate release do not appear to be affected by initial bisphosphonate load and release is maximum in 24 hours of soaking in PBS (Fig. 1). Osteoblast-like MG-63 cells cultured on composites display good proliferation and enhanced activity indicating that alendronate promotes extra-cellular matrix mineralization processes, in agreement with the increased values of alkaline phosphatase activity, collagen type I and osteocalcin production. Furthermore, the production of MMP1 and MMP13 measured at 14 days significantly decreases on increasing alendronate content, indicating that alendronate does not provoke any biological processes involving collagen degradation. By contrast, the data indicate that osteoclasts activity decreases as a function of alendronate content of the composites.

Acknowledgements

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Osteoblast adhesion on biomaterials: focal adhesion pattern relating to SEM morphology

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Introduction
Understanding the cellular basis of osteoblast cell-biomaterial interaction is crucial to the analysis of osseointegration, a requirement for long-term endosseous implants stability (1). Cell adhesion is one of the initial events to subsequent proliferation and differentation of bone cells before bone tissue formation (2, 3). The initial cell adhesion is a major determinant of surface cytocompatibility *in vitro* and is suggested as a possible determinant of biocompatibility in the *in vivo* situation (4). This study is focused on the following question: how do the cell-substrate interactions detected by immunofluorescence adhesion assay appear morphologically?

Materials and methods
We study the relationship between cell-substrate interactions and cell morphology by means of immunofluorescence and Scanning Electron Microscopy analysis. Two titanium surface treatments (chemically treated T1, electrochemically treated, T2) were used as substrate for cell adhesion. Human mandibular osteoblast (5) were seeded on different surfaces at the density of 1×10^4 cells/cm^2 in D-MEM supplemented with 10% foetal calf serum (FCS). The attachment, spreading and morphology of osteoblast cells were evaluated after 6, 24, 72 hours of culture on different materials by Scanning Electron Microscopy (SEM). To study the relationship between focal adhesion sites and cytoskeletal morphology, after 6 and 24 hours of culture a triple immunofluorescene (IF) labelling for focal adhesion (F-actin red, vinculin-green, DAPI-blue) was performed.

Results and discussion
Obtained results from SEM and IF analysis were compared and the morphological features were detailed. At 6 hours, cells were spreading on all titanium surfaces and showed a roundish spread out morphology with long fine cytoplasmatic extension in multiple direction. Cells cultured on the electrochemically treated titanium demonstrated greater spreading with large lamellopodes (Fig. 1). The IF study showed that after 6 hours, the cells on the titanium surfaces were not completely spread yet and the vinculine positive focal contacts begun to be present on the surfaces round the nuclei (Fig. 2).
At 24 hours intercellular connections can frequently be observed by SEM. At this time, cells were spread and displayed fewer cytoplasmatic extension and presented bi-polar or tri-polar spindle-like morphology. On the electrochemically treated titanium large spread lamellopodes supported the cell-material interactions, while on chemically treated titanium short filopodia were still present (Fig. 1).
The IF analysis showed that the vinculin-positive focal contacts were numerous, homogeneous in size and morphology and distributed at the cell periphery where the cells were more flat. The immunolabelled focal adhesion had become more organised as the cell spreading and contact area increased (Fig. 2).
On both surfaces, after 72 hours of culture the trend to forming a cell monolayer was evident (Fig. 1).

Conclusion
After 6 hours of culture the cell adhesion onto the surfaces was not stabilised by any focal contact at the cell peripheral edge, indicating an attachment phase, while, after 24 hours, numerous, homogeneous "dot" contact were present on both surfaces, indicating that a stable cell adhesion had occurred. SEM analysis combined with immunofluorescence for focal adhesion and cytoskeletal organisation is a useful method to study cell to biomaterials interaction by a morphological point of view.

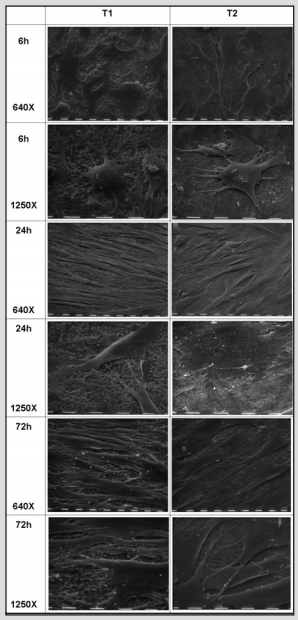

Figure 1 - Morphology (SEM: ×640, ×1250) of osteoblast cells grown on Titanium surfaces T1 and T2 after 6, 24 and 72 hours of culture.

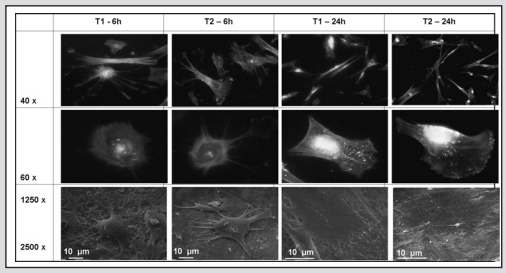

Figure 2 - Immunofluorescence labelling relating to high magnification SEM picture (1250×, 2500×) of osteoblast cells cultured on Titanium surfaces T1 and T2 after 6 and 24 hours.

Acknowledgements
The authors would like to acknowledge Mr. Davide Dallatana for SEM technical support.

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Growth and osteogenic differentiation of hASCs (human Adipose-derived Stem Cells) on scaffolds

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Introduction

Adipose tissue can be harvested in large amount with minimal morbidity. It contains numerous cells types, including cells (hASCs, human adipose-derived stem cells) that have the ability to differentiate into several lineages, such as fat, bone, cartilage, muscle, endothelium, hepatocytes and neuronal cells (1).
The aim of our study, after characterization of the hASCs (2), has been the analysis of their osteogenic differentiation potential. The interaction between undifferentiated or differentiated cells and several scaffolds have been particularly examined, in order to support the use of autologous hASCs in regenerative medicine.

Materials and methods

Cells were enzymatically isolated from fat derived by liposuction from adult donors under informed consensus, as previously described (3), and then purified and expanded *in vitro*. hASCs CD expression pattern was analyzed by cytofluorimetry (4).
Expanded hASCs have then been differentiated into osteogenic lineage in monolayer condition using an induction medium (DMEM/10% FBS supplemented with 10 mM glicerol-2-phosphate, 10 nM dexamethasone, 150 µM L-ascorbic acid-2-phosphate and 10nM cholecalciferol) for 14 and 21 days.
Here we just show data from hASCs osteogenic differentiation on fragments of human bone and on two different types of hydroxyapatite, with a variable grade of porosity (35% and 60%) (5), for different culture time.
10^5 undifferentiated hASCs were let adhere to each scaffold and then the constructs were cultured in static condition at 37°C and 5% CO_2 , either in cDMEM (non inductive medium, CTRL) or in osteogenic medium (OM).
The osteogenic differentiation of hASCs both in monolayer and on scaffolds was monitored evaluating changes in their proliferation rate and in calcium depots, quantifying the formation of calcified matrix stained with Alizarin Red S, by cetylpyridinium chloride (CPC) extraction process (6). Furthermore, cell-scaffold constructs were observed by scanning electronic microscopy (SEM).

Results and discussion

hASCs show a very similar surface expression pattern of BMSCs (Bone Marrow Stromal Cells); they were CD13+, CD105+, CD90+, CD29+, CD44+, CD49d+, CD14-, CD45-, CD71- , confirming the specific MSC features.
Both osteogenic pre-differentiated hASCs and cells directly differentiated on scaffolds show good adhesion to the tested scaffolds, as demonstrated by the SEM images (Figs. 1, 2). Pores are filled by cells, in particular in the high porosity HAP (60%) which seems to better promote the inside cell migration.
Undifferentiated hASCs cultured on bone fragments for 14 days in CTRL and OM show good adhesion capacity (Fig. 1A). Calcium deposition of hASCs cultured in monolayer in absence of scaffolds in CTRL e OM for 14 days were compared with calcium level produced by cells cultured on bone fragments in the same media for the same time. Both hASCs cultured in OM in monolayer and seeded on bone, produced significantly higher levels of calcified matrix in comparison to cells in CTRL (Fig. 1B, §). In both media, cells loaded on bone fragments are able to produce more calcium than cells grown in monolayer (Fig. 1B,*). In the graph the basal level of calcium deposition produced by bone fragments maintained in culture without hASCs is also shown.
hASCs loaded on the two types of HAP cultured in OM filled pores (Fig. 2A) and produced a more abundant amount of calcium depots than the same number of cells cultured for the same period in monolayer (Fig. 2B and submitted manuscript). No significative difference of calcified matrix has been revealed between the two types of HAP. Calcium deposition reach similar level

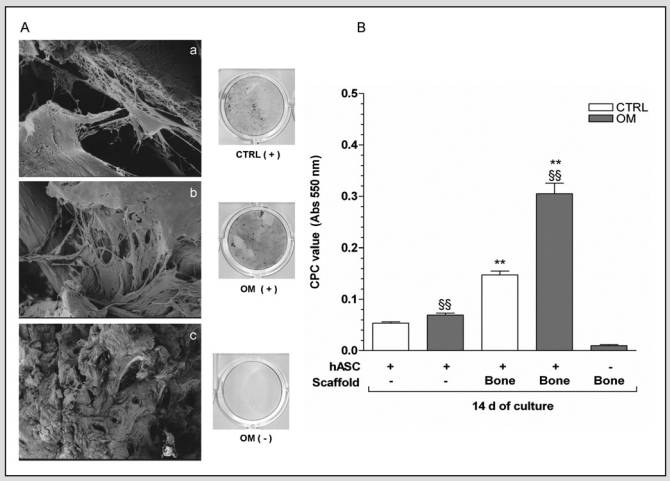

Figure 1 - hASCs osteogenic differentiation on human bone fragments.
A. (Left) Scanning electron microscopy (SEM) images of human bone fragments loaded with hASC cultured for 14 days in non-inductive medium [CTRL] (a), osteogenic medium [OM](b) and unloaded human bone in OM(c) (50X magnification). (Right) Alizarin red S staining of calcified extracellular matrix of the corresponding culture wells.
*B. Quantification of calcified extracellular matrix formation by cetylpyridinium chloride (CPC) extraction of alizarin red S staining (fig. 1A a-b-c). Values are represented as mean ± SD (n=3) (§§:OM vs CTRL; **: scaffold vs monolayer p<0.01).*

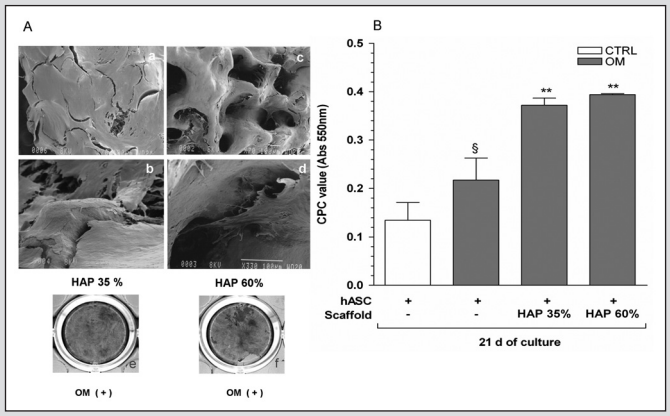

Figure 2 - hASCs osteogenic differentiation on two different HAP.
A. Scanning electron microscopy images (70X and 330X magnification respectively) of HAP 35% porosity seeded with hASC and cultured for 21d in OM (a-b).; Scanning electron microscopy images (70X and 330X magnification respectively) of HAP 60% porosity seeded with hASC an cultured for 21d in OM (c-d). Alizarin red S staining of calcified extracellular matrix of the corresponding culture wells HAP 35% porosity (e) and HAP 60% porosity (f).
*B. Quantification of alizarin red S staining of previously described culture wells (fig. 2A e-f) by cetylpyridinium chloride (CPC) extraction compared with hASCs grown in monolayer in OM and non-inductive medium. Values are represented as mean ± SD (n=3) (§:OM vs CTRL media p<0.05; **: scaffold vs monolayer p<0.01).*

in the presence of HAP and of bone fragments, but bone seems to be a faster osteogenic inducer since the calcium level of hASCs grown on bone are referred to two weeks of culture.

Conclusions

Adipose tissue can be considered a very useful source of MSCs (7). When seeded on bone fragments and HAP granules, hASCs are able to populate interconnected pores of both scaffolds and to further increase their differentiation capacity respect to the monolayer condition. We also believe that, due to their abundance and their differentiation capability, hASCs may be used soon for screening of new scaffolds by checking the biocompatibility and cell adhesion, both in static and dynamic conditions, before testing them in selected models *in vivo*.

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Bioreactor coating of titanium fiber-mesh with human osteoblasts and bone matrix

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Introduction

In the present work of bone tissue engineering we show a "biomimetic strategy" that consists in the surface modification of titanium fiber-mesh with proliferated bone cells and their extracellular matrix produced *in loco*: during the culture period we have applied an electromagnetic wave because the osteoblastic cell function can be electromagnetically modulated in terms of proliferation and differentiation (1).

Materials and methods

Titanium fiber-mesh sheets were harvested from Harris-Galante Porous acetabular components (Zimmer). The mesh was composed of sintered non-woven titanium fibers (fiber diameter, 440 ± 10 μm; scaffold density, 2.7 ± 0.1 g/cm^3; scaffold porosity, 40% ± 3%). Cell culture scaffolds (diameter, 12 mm; height, 0.8 mm) were cut from the mesh with a die.

The human osteosarcoma cell line SAOS-2 was cultured in McCoy's 5A medium, supplemented with 15% fetal bovine serum. A cell suspension of 4×10^5 cells in 100 μl was added onto the top of each scaffold. An electromagnetic stimulus (frequency, 75 Hz; magnetic field, 2 mT) was applied to the seeded scaffolds for 22 days.

Cultured scaffolds were processed for SEM analysis, DNA extraction, and bone matrix extraction.

Results and discussion

The SEM images revealed that, due to the physical stimulation, the cells proliferated over the available titanium surface (Fig. 1).

This observation was confirmed by the measure of the DNA content after 22 days of culture: in the static culture the cell number per scaffold grew to $8.5\times10^6 \pm 5.1\times10^4$, whereas in the electromagnetic culture to $16.4\times10^6 \pm 3.2\times10^4$ ($p<0.05$).

To evaluate the amount of bone matrix over the scaffold surface, an ELISA of the extracted matrix was performed: at the end of the culture period, in comparison with the static culture, the physical stimulus greatly increased the coating with type-I collagen ($p<0.05$) (Tab. I).

The aim of this study was the surface modification of titanium fiber-mesh with extracellular matrix and osteoblasts to make the biomaterial more biocompatible for the bone repair *in vivo*.

The physical stimulus increased the cell proliferation around 2-fold and

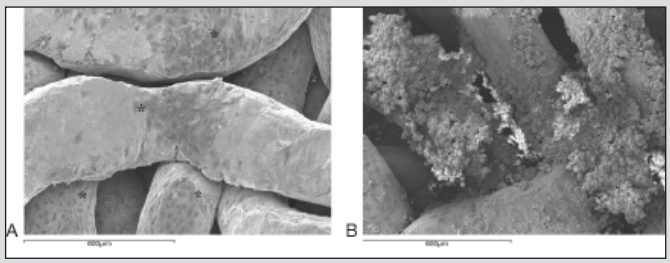

Figure 1 - SEM images of the static (A) and electromagnetic (B) cultures, 100X, bar=600 μm.

TABLE I - TYPE-I COLLAGEN COATING IN fg/(cell × scaffold)

Static	Electromagnetic
917.40 ± 150.25	8971.94 ± 380.12

the coating with type-I collagen was enhanced around 9.8-fold. The use of a cell line showed the potential of the physical stimulus; nevertheless, a better result could be obtained with autologous bone marrow stromal cells instead of SAOS-2 osteoblasts for total immunocompatibility with the patient.

Conclusion

In conclusion, we could theorize that the cultured biomaterial could be used fresh, that is, rich in autologous cells and matrix, or after sterilization with ethylene oxide, that is, rich only in autologous matrix in order to handle a simpler storable tissue-engineering product for bone repair.

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Physically enhanced coating of titanium plasma-spray with human osteoblasts and bone matrix

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Introduction
The events leading to the integration of an implant into the bony tissue take place at the interface between tissue and implant: in order to obtain a stable primary stabilization of the implant and the following osteointegration, rough implant surfaces have been developed (1).
Aiming at an accelerated and enhanced *in vivo* osteointegration of a rough titanium surface during the early postimplantation period, we show a particular "biomimetic strategy" that consists in the surface modification of titanium plasma-spray with proliferated bone cells and their extracellular matrix produced *in loco*. In addition, during the culture period we have applied an electromagnetic or an ultrasonic wave because the bone cell function can be physically modulated in terms of mitosis and differentiation (2).

Materials and methods
Disks (diameter, 12 mm; height, 4 mm) were cut from the titanium alloy Ti6Al4V. The disks were plasma-sprayed with titanium powder of the same alloy by a Vacuum Plasma-Spray (VPS) procedure (Lima). The resulting rough surface had the following characteristics: mean roughness depth Rz of 30 μm and thickness of plasma-sprayed layer equal to 175 ± 75 μm.
The human osteosarcoma cell line SAOS-2 was cultured in McCoy's 5A medium, supplemented with 15% fetal bovine serum. A cell suspension of 4×10^5 cells in 100 μl was added onto the top of each disk.
An ultrasound (frequency, 1.5 MHz; power, 149 mW) or an electromagnetic (frequency, 75 Hz; magnetic field, 2 mT) stimulus was applied to the seeded disks for 22 days.
Cultured disks were processed for SEM analysis, DNA extraction, and bone matrix extraction.

Results and discussion
The SEM images revealed that, due to the physical stimulations, the cells proliferated over the available titanium surface (Fig. 1).
This observation was confirmed by the measure of the DNA content after 22 days of culture: in the static culture the cell number per disk grew to $2.1\times10^6 \pm 4.1\times10^4$, in the ultrasonic culture to $5.2\times10^6 \pm 3.6\times10^4$, and in the electromagnetic culture to $5.6\times10^6 \pm 3.2\times10^4$ ($p<0.05$ in the comparisons "static vs. ultrasonic" and "static vs. electromagnetic", $p>0.05$ in the comparison "ultrasonic vs. electromagnetic").
To evaluate the amount of bone matrix over the disk surface, an ELISA of the extracted matrix was performed: at the end of the culture period, in comparison with the static culture, the physical stimuli greatly increased the coating with type-I collagen ($p<0.05$ in the comparisons "static vs. ultrasonic" and "static vs. electromagnetic", $p>0.05$ in the comparison "ultrasonic vs. electromagnetic") (Tab. I).
The aim of this study was the surface modification of titanium plasma-spray with extracellular matrix and osteoblasts to make the biomaterial more biocompatible for the bone repair *in vivo*.
The physical stimuli increased the cell proliferation around 2.5-fold and the coating with type-I collagen was enhanced around 9÷11-fold. The use of a cell line showed the potential of the physical stimuli; nevertheless, a better result could be obtained with autologous bone marrow stromal cells instead of SAOS-2 osteoblasts for total immunocompatibility with the patient.

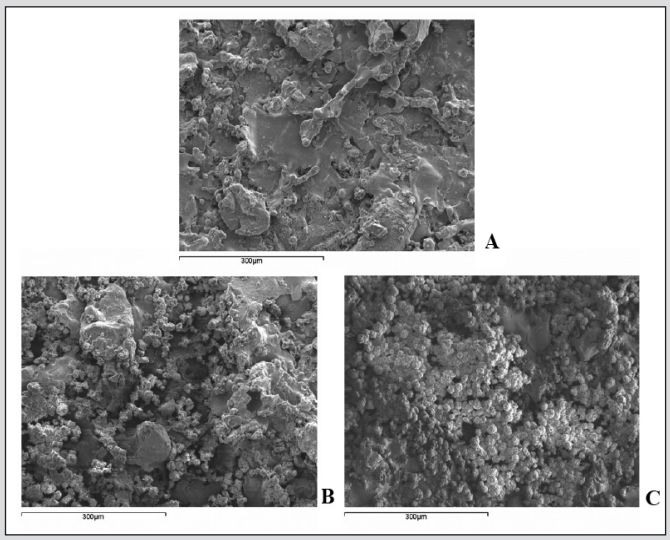

Figure 1 - SEM images of the static (A), ultrasonic (B), and electromagnetic (C) cultures, 200×, bar=300 μm.

TABLE I - TYPE-I COLLAGEN COATING IN fg/(cell×disk)

Static	Ultrasonic	Electromagnetic
769.25 ± 31.56	7177.24 ± 150.23	8453.44 ± 122.61

Conclusion
In conclusion, cultured biomaterial could be used fresh, rich in autologous cells and matrix, or after sterilization with ethylene oxide, rich in autologous matrix in order to handle a simpler storable tissue-engineering product for bone repair.

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DNA delivery through specifically designed thermosensitive cationic nanospheres

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Introduction
RDNA synthetic vectors based on polymeric systems are very promising since they are safe and able to protect DNA from enzymatic degradation, thus improving its *in vivo* bioavailability. In addition, they are easy to prepare on large scale and to store. Within this frame, we prepared a novel class of thermosensitive polymeric cationic core-shell nanospheres, specifically designed to reversibly bind nucleic acids on their functional and hydrophilic expanded shell.

Materials and methods
Functional core-shell nanospheres were obtained by emulsion polymerization of methylmethacrylate in the presence of (N-isopropylacrylamide) and (2-(dimethyloctyl)ammonium ethyl methacrylate bromine) which should stabilize the latex, once copolymerized, through an electrosteric mechanism (1). The polymerization reaction was performed in a 1 litre five-neck reactor at 80±1.0 °C for 2 hours under constant stirring. At the end of the reaction, the product was purified by repeated dialysis against water to remove the residual monomers. After this procedure, the polymeric nanoparticles were dried under vacuum at room temperature. Particle size and size distribution were measured by scanning electron microscope (SEM) and photon correlation spectroscopy (PCS). The amount of quaternary ammonium groups per gram of nanoparticles was determined by potentiometric titration of the bromine ions deriving from the electrostatic stabilizer and obtained after complete ionic exchange. Z-potential values were measured by a Zetasizer 3000 HS system (Malvern, UK) using a 10 mV He–Ne laser. DNA binding ability was investigated on selected samples of nanospheres in cell-free experiments run in PBS buffer at room temperature for 2 hours.

Results and discussion
Several samples of cationic nanospheres were prepared by emulsion polymerization (1) and characterized (Tab. I).
Their core is mainly constituted by poly(methylmethacrylate) whereas an highly

TABLE I - PHYSICO-CHEMICAL PROPERTIES OF CORE-SHELL NANOSPHERES

Sample	SEM diameter (nm)	PCS diameter (nm)	Surface charge (µmol/g)	Z-potential (mV)
ZM2-1	130.0 ± 7.0	189.7	280	+ 24.0 ± 3.6
ZM2-2	137.0 ± 5.0	153.5	202	+ 40.1 ± 8.7
ZM2-3	125.1 ± 10	180.1	217	+ 31.4 ± 5.7
ZM2-4	152.7 ± 10	163.3	219	+ 47.5 ± 6.6

hydrophilic shell, constituted by hydrosoluble arms is obtained, bearing positively charged functional groups, able to reversibly bind DNA, and poly(N-isopropylacrylamide) (NIPAM) chain segments, able to improve the biocompatibility and contributing to the colloidal stability of the latex (Fig. 1A). In contrast to many liposomes and polycation formulations, these nanospheres can be obtained in large scale reactors with highly homogeneous size (Fig. 1B). The nanosphere aqueous suspensions are stable at room temperature and can be lyophilized and stored for several months.
Preliminary experiments run with sample ZM2-2 show that DNA adsorption occurs easily and with high efficiency, with final loading values up to 15% wt/wt, whereas desorption is nearly quantitative in the presence of high ionic strength buffers. The presence of poly(NIPAM) chains in the shell is able to induce thermosensitive behavior. Figure 2 shows the variation of the hydrodynamic diameter with temperature for both free nanospheres (panel A) and nanospheres with adsorbed DNA (panel B). In both cases, above 37 °C a marked decrease in size occurs as a consequence of the temperature induced conformational changes of PNIPAM arms (2-4). It is interesting to note that the presence of

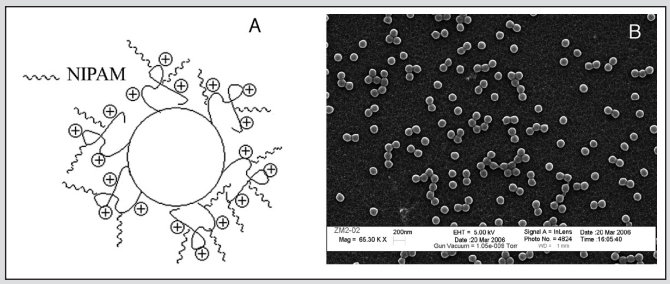

Figure 1 - Core-shell nanosphere structure: schematic representation (A) and SEM micrograph (B) of a representative sample (ZM2-2).

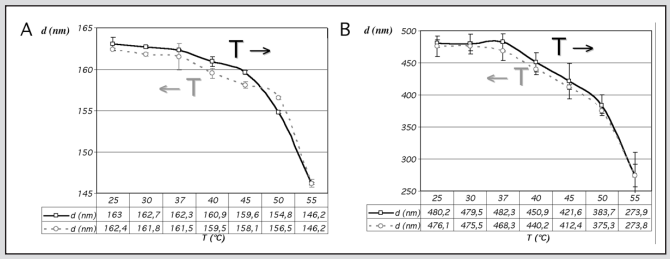

Figure 2 - Thermosensitive behavior of core shell nanospheres without (A) and with (B) adsorbed DNA.

ionic comonomers in the PNIPAM based shell does not change its thermosensitive propensity. In addition DNA adsorption does not affect shell thermosensitivity as the same trend was observed with free nanospheres as well as with DNA/nanospheres complexes (Fig. 2).

Conclusion
Functional PMMA core-shell nanospheres with a cationic thermosensitive shell were obtained by emulsion polymerization on a large scale. As these cationic nanospheres are able to reversibly bind plasmid DNA, they represent a promising delivery system for nucleic acids towards both therapeutic and vaccine approaches.

Acknowledgements
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Rationale for the use of a hyaluronan-based scaffold to treat cartilage lesions in patient with osteoarthritis

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Introduction
The use of autologous chondrocyte transplantation, performed either by cells in suspension or carried by a biomaterial, is limited to the repair of cartilage damaged after traumatic lesions or in patients with osteochondritis dissecans (1, 2). In osteoarthritis (OA) the degenerative lesions are usually treated with or without drugs (3). Many of these drugs, however, are beset with serious side effects. In OA several factors, including mechanical, biochemical and genetic ones are involved leading to an increased synthesis and release of catabolic enzymes, prostaglandins and oxygen radicals (4). The rationale of this study was to look at the behaviour of the molecules involved in cartilage catabolic pathways once human chondrocytes are seeded in a hyaluronan-based scaffold with the aim of evaluating their use to treat early degenerative lesions in these patients.

Materials and methods
The biomaterial used in this study was a hyaluronan-based three-dimensional scaffold (Hyaff®-11, Fidia Advanced Biopolymers). Human articular cartilage was obtained from the knees of 5 patients with a history of trauma. Informed consent was obtained from all the patients who entered into the study, and the work was approved by the Ethics Committee of Istituti Ortopedici Rizzoli.
Chondrocytes were isolated, grown in culture for up to three passages and seeded onto scaffolds. Constructs and surnatants were collected and analyzed at 1, 3, 7, 14 and 21 days from the seeding to evaluate: metalloproteinase-1 (MMP-1) and metalloproteinase-13 (MMP-13) secretion by specific ELISAs, nitric oxide (NO) production by Griess method, mRNAs for collagens I and II, aggrecan, Sox-9, MMP-1 and MMP-13 by Real-Time PCR (RT-PCR), apoptosis by Tunel assay, caspase-3 by immunohistochemical analysis.

Results and discussion
From the data obtained in our study, we clearly demonstrated that chondrocytes grown onto a hyaluronic acid-based scaffold are able not only to re-differentiate and express their original phenotype but also reduce the production and expression of many factors involved in cartilage degradation (Fig. 1). It is well known, that NO is highly produced by chondrocytes from OA-derived chondrocytes and it is one of the molecules that triggers cartilage apoptosis and breakdown via activating matrix metalloproteinases activity (5). Chondrocytes grown onto hyaluronic acid-based scaffold reduce the secretion of nitrites and the expression and secretion of MMP-13 and caspase, resulting in a lower percentage of apoptotic cells (Fig. 2). It is possible to hypothesize that synthetic matrices based on hyaluronic acid or derivatives of this molecules, such as Hyaff®, mimic the embryonic hyluronic acid-rich environment and when used as a delivery vehicle for cell-based therapies in adult organisms, promote a recapitulation of these embryonic events. This results in the activation of anabolic factors, which induce the differentiation of the cells with the final aim of facilitating tissue repair.

Conclusion
The hyaluronan-based biomaterial used in this study acts on chondrocyte metabolism downregulating catabolic pathways. The ability to reduce the expression of molecules involved in cartilage degenerative processes by chondrocytes indicates its use also in the transplantation therapeutical strategy to treat early lesions in patients with osteoarthritis.

Acknowledgements
We thank Patrizia Rappini and Graziella Salmi for their assistance in the preparation of the manuscript and Luciano Pizzi for his technical assistance.

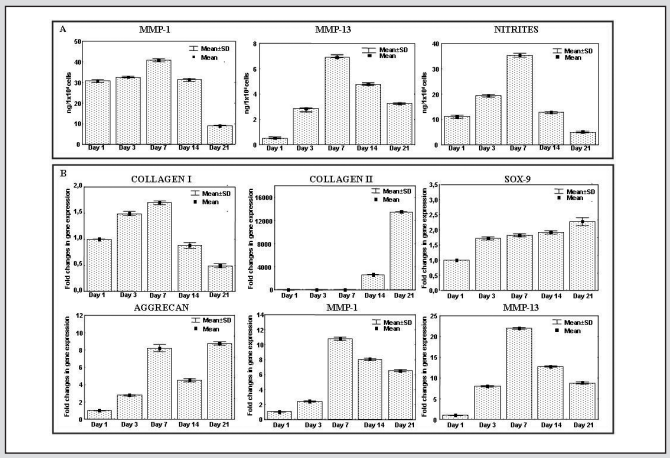

Figure 1 - A. MMP-1 (A), MMP-13 (B) and nitrite levels in the supernatants of chondrocyte grown onto Hyaff®-11 scaffold at different experimental times. Values are expressed as mean ± standard deviation (SD). B. Kinetics for type I collagen, Type II collagen, aggrecan, Sox-9 MMP-1 and MMP-13 messenger RNA expression in chondrocytes grown onto Hyaff®-11 scaffolds at different experimental times. Results are reported as fold changes in gene expression and reported as mean ± standard deviations (SD).

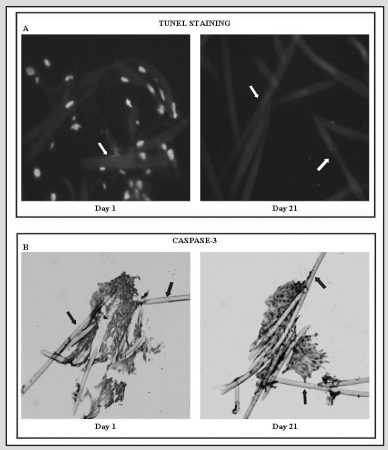

Figure 2 - A. Tunnel immunostaining in dedifferentiated cells at Day 1 and cells cultured on Hyaff®-11 at day 21. Dedifferentiated cells showed a marked positivity, while only a small number of cells showed apoptotic nucleus at day 21. The arrows indicate the Hyaff®-11 fibers. B. Immunohistochemistry for caspase-3 in the cells grown on Hyaff®-11. The cells were all positive at day 1 and completely negative at day 21. The arrows indicate the Hyaff®-11 fibers.

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Poly ϵ-caprolactone temporary substrates via salt leaching technique for bone repair

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Introduction

In the field of bone tissue engineering, the choice of adequate materials and processing techniques is crucial to produce a scaffold able to realize temporary substrates for bone repair. Indeed, materials and processing techniques determine, to a great extent, the main characteristics of the scaffold (1). In this way, bioerodible and non-cytotoxic polymer as poly ϵ-caprolactone (PCL) is able to maintain physical properties and mechanical response for at least 6 months before degradation (2). Phase inversion and particulate leaching technique allows to modulate scaffold porosity through the careful employment of soluble porogen agents in order to carry out a three dimensional polymer matrix with pore shape and size able to stimulate new bone growth (2).
In this work, three-dimensional porous poly ϵ-caprolactone-based composite scaffolds were fabricated via solvent casting/salt leaching process using sodium chloride (NaCl) crystals as porogen agent.

Materials and methods

Poly ϵ-caprolactone pellets (Sigma Aldrich MW 65 kDa) were dissolved in a 20 wt % N-N-dimethylacedammide (J.T. Baker 06/2007) solution (5 g polymer in 20 ml solvent) by stirring for about 3 hours at 58 °C. NaCl particles, sieved into specific size ranges (212-300 μm), was added to form an homogeneous mix, and it was placed into Petri dishes which act as mould. After mixture compression previously realized by 0.127 N/mm^2 pressure, ethanol (CH_3OH) was used for 24 hours to extract used solvent and bidistilled water for 7 days to leach out salt and any other contaminants. Morphology investigation and an estimation of porosity have been performed by scanning electron microscopy (Leica 420) and mercury intrusion porosimetry (Thermo Electron Pascal 140-240 ultra macro kit). Furthermore, compressive properties were measured at room temperature on dynamometric machine (Instron 4204) according to ASTM D695 standard.

Results and discussion

Morphological analysis performed by SEM on surface of scaffolds with 18/82 (v/v) polymer/salt volume ratio verifies the presence of highly interconnected porosity and of an homogeneous distribution of pores ranged from 100 to 200 μm (Fig. 1) according to features ideal to correct osteoblast attachment and growth reported in literature (3).
By mercury intrusion porosimetry measurements of PCL scaffold with 18/82 (v/v) PCL/NaCl volume ratio (Fig. 2), porosity degree results equal to about 84% of scaffold total volume equal to expected value calculated by theoretical NaCl volume fraction (82%). The achieved macroporosity with radius ranged to 1 and 150 μm shows typical pore features required to regenerating bone temporary scaffold as reported in many studies on biomaterials (4).
Finally, compressive response of PCL scaffold is well described by typical σ-ϵ curve with a toe region at low deformations followed by a steeper region. Toe region elastic modulus referred to pore compression E* is equal to about 109 ± 0.032 kPa and bulk modulus E referred to skeleton material response is equal to 2.84 ± 0.078 MPa. Such values evaluated along with toe region are suitable for sustain temporarily physiological loads.

Conclusions

In this work, degradable composite scaffolds were prepared by solvent casting/salt leaching methods. An interconnected macroporous structure was noticed with pore size feasible to promote cell seeding and proliferation. Sufficient mechanical properties able to sustain loads were evaluated *in vitro* conditions allowing to maintain the spaces required for cell ingrowth until the complete degradation of polymeric network. Such features allows to consider PCL scaffold obtained by salt leaching technique as an excellent candidate for bone tissue engineering.

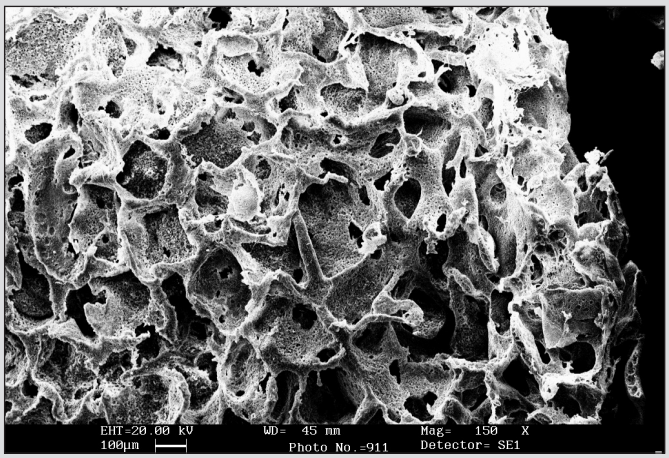

Figure 1 - SEM micrographs of degradable poly ε-caprolactone-based scaffolds with 18/82 PCL/NaCl volume ratio by solvent casting and particulate leaching.

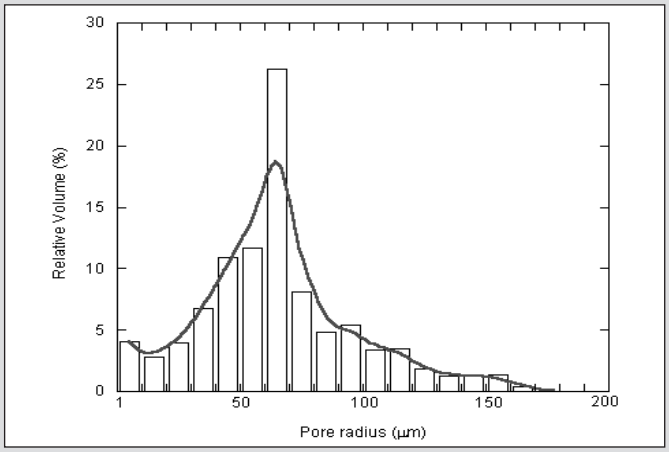

Figure 2 - Mercury intrusion porosimetry degradable poly ε-caprolactone-based scaffolds – pore size distribution of scaffold with 18/82 PCL/NaCl volume ratio.

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Bioactive and antibacterial glasses and glass-ceramics

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Introduction

One of the most serious problem connected with orthopaedic surgery is the development of infections, that can cause several problems to patients, implant failure and high medical costs (1); the realization of surfaces both antibacterial and biocompatible/bioactive represents a challenge. Silver, a well known antibacterial agent with a broad spectrum activity, could be introduced onto different materials to obtain an antibacterial behaviour. In this research silver ions have been introduced onto glasses and glass ceramics surfaces, with different degrees of bioactivity, through ion-exchange process, a technique that modifies only the external surface layer of the materials without altering bulk characteristics (2, 3).

Materials and method

Glass and glass-ceramics with different composition have been studied both in bulk form, in order to optimize the process, and as coatings applied via Plasma Spray (Eurocoating S.p.a) on metallic substrates, to mimic possible applications. The ion-exchange was performed using a silver aqueous solution, process parameters (temperature, time, Ag concentration) were varied in function of material composition and surface nature, in order to obtain a controlled diffusion profile and concentration of Ag. All samples have been analyzed by means of XRD, SEM and EDS before and after the treatment to investigate their structure, morphology and composition and to evaluate the influence of ion-exchange process. Coatings have also been analyzed in order to verify their roughness, porosity and adhesion resistance. *In vitro* reactivity was carried out soaking samples in SBF solution and then by analysing the samples with the above listed techniques. The amount and rate of silver released was investigated by GFAAS; moreover biocompatibility tests were performed by means of cells adhesion and proliferation evaluation. The effective antibacterial behaviour of Ag-doped samples has been investigated through two antibacterial tests (in accordance to NCCLS standards) on *S. Aureus:* the count of colonies forming units (CFU), by means of the broth dilution method, and the measurement of inhibition zones.

Results and discussion

Characterization analyses show that ion-exchange process does not induce any structural or morphological change on Ag-doped samples; moreover the introduced silver does not influence the material reactivity and so the bioactivity process, as confirmed by SEM and EDS analyses. GFAAS analysis demonstrates that silver is gradually released in solution, in particular during the first days of immersion, the most dangerous for infections development, and the amount is correlated with the process parameters. As well the coating adhesion resistance is higher then the limit provided by ISO standard for HAp coatings. Cellular tests demonstrate that biocompatibility is generally maintained after treatment but it is closely connected to silver amount released from samples. Microbiological tests confirm the antibacterial behaviour of silver-doped samples: CFU count test show a bacterial reduction of 2-3 magnitude order both for adhered bacteria and proliferated ones, and all sample are able to produce an inhibition zone of about 3 mm (Fig. 1). In particular, the broth dilution test has permitted to verify the bacteriostatic behaviour of silver introduced onto materials: Ag ions are able to limit the bacterial colonies proliferation and adhesion, in-depth studies will investigate the possible bactericidal effect.

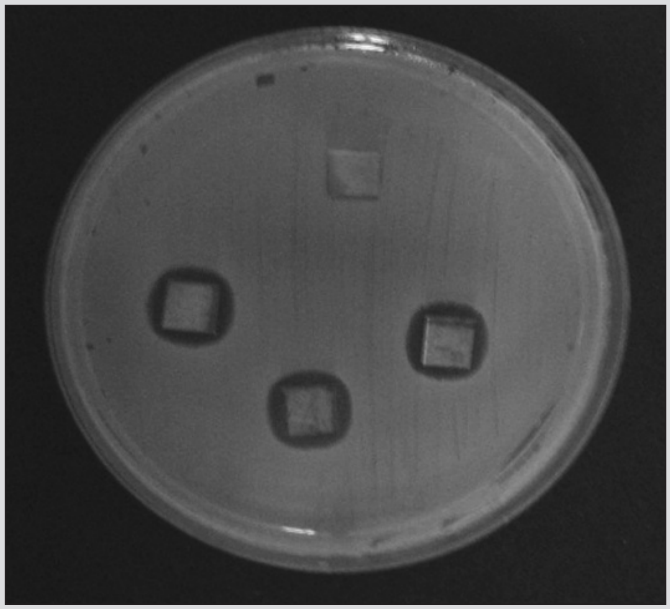

Figure 1 - Inhibition zone on Mueller Hinton agar plate containing three Ag-doped samples and one untreated as a control.

Conclusion

Ion-exchange technique permits the introduction of controlled silver amount in different glass-ceramic materials without modifying their structural and morphological properties; it is possible to modulate the amount of Ag ions introduced and released in glass ceramic materials varying the process conditions. The process is reproducible, is easily transferable to different material and so to medical devices partially or totally glass realized. Comparing cellular and microbiological tests it is possible to design the silver amount intended to be loaded in the materials surface to confer, at the same time, antibacterial properties and a not cytotoxic behaviour.

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Biomimetic scaffolds for bone and cardiac tissue engineering

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Introduction

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The major components of the ECM include cell adhesive or anti-adhesive molecules, such as fibronectin, vitronectin, laminin and tenascin; structural components, such as collagen and elastin; and proteoglycans, a complex array of proteins with glycosaminoglycans side-chains. Moreover, it contains bioactive molecules, such as cytokines and growth factors. ECM molecules interact with each other and with their specific receptors on the cell surface. Such interactions now are believed to play an important role in the regulation of several cellular processes, such as adhesion, migration, proliferation and differentiation (1, 2).

For this reason, the development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials, that mimic the native ECM and its natural interactions with cells.

The aim of this work was the preparation and characterization of scaffolds with a composition similar to that of the natural extracellular matrix, for bone and cardiac tissue engineering.

Materials and methods

Sponges based on collagen (CLG) and hydroxyapatite (HA), with a 20:80 weight ratio, were prepared for bone tissue engineering. 1% w/v CLG solution was added to transglutaminase (TGase) enzyme (0.05 U/mg of protein) and then mixed with HA. The mixture was incubated at 37 °C for 30 minutes, for the enzymatic cross-linking and finally freeze-dried.

Sponges based on alginate (ALG) and CLG, with a 20:80 weight ratio, were prepared for cardiac tissue engineering. 0.5% w/v solutions of the two biopolymers were mixed together and freeze dried. Then, they were exposed to glutaraldehyde (GTA) vapours, for collagen cross-linking and immersed in $CaCl_2$ solution, for alginate cross-linking. Consequently, the samples were immersed in a coagulation bath in acetic acid, to promote the ionic interactions among the two biopolymers and finally freeze-dried. Both systems underwent a morphological, physicochemical, functional and biological characterization.

Results and discussion

The morphological analysis, carried out by scanning electron microscope (SEM), showed for the ALG/CLG sponges an highly porous structure with well interconnected pores. The SEM images of CLG/HA sponges displayed the presence of interconnected pores, within a compact and inhomogeneous structure.

For both systems, the physicochemical characterization, carried out by infrared spectroscopy, thermogravimetric analysis and differential scanning calorimetry, pointed out the presence of interactions among the chemical groups typical of the two components. Moreover, a good chemical homogeneity, both on the surface and in the thickness of the samples, was proved by FT-IR Chemical Imaging.

High values of water uptake were obtained during the swelling tests. Hydrolytic degradation tests showed a suitable stability of CLG/HA and ALG/CLG sponges in aqueous environment. Enzymatic degradation tests were also performed and higher values of weight loss were obtained, showing that the cross-linking process did not modify the biodegradability of these materials.

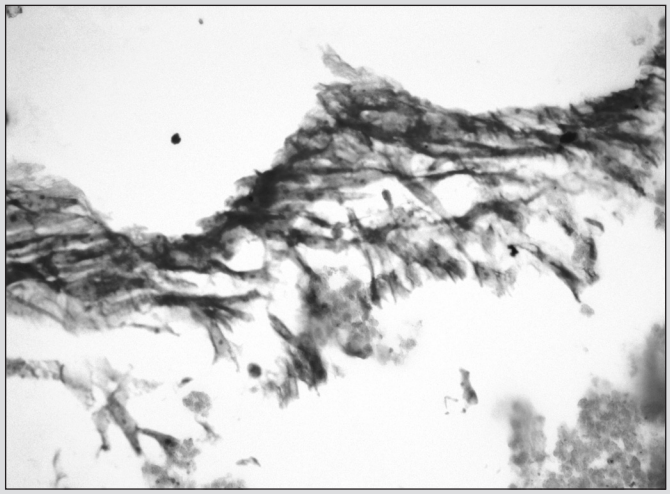

Figure 1 - Adhesion and proliferation of mesenchymal stem cell from umbilical cord on CLG/HA scaffold.

The biological characterization of CLG/HA tests was carried out using mesenchymal stem cells from umbilical cord. As shown in Figure 1, cell adhesion and proliferation on the support was very good.

Proliferation and differentiation tests were performed on ALG/CLG sponges using C2C12 line myoblasts. The number of cells proliferated on the sample were very close to that on control. Moreover, ALG/CLG sponges were able to promote stem cell differentiation, with the comparison of multinucleated myotubes, as shown in Figure 2.

Conclusion

Porous three dimensional scaffolds with a composition similar to that of natural bone and cardiac tissues were prepared in this work. The results obtained in the characterization suggest their potential use for bone and cardiac tissue engineering. Next step toward the development of biomimetic systems will be the functionalization with peptide sequences of other extracellular matrix proteins present in the same tissues and the loading with controlled release system of growth factors and cytokines.

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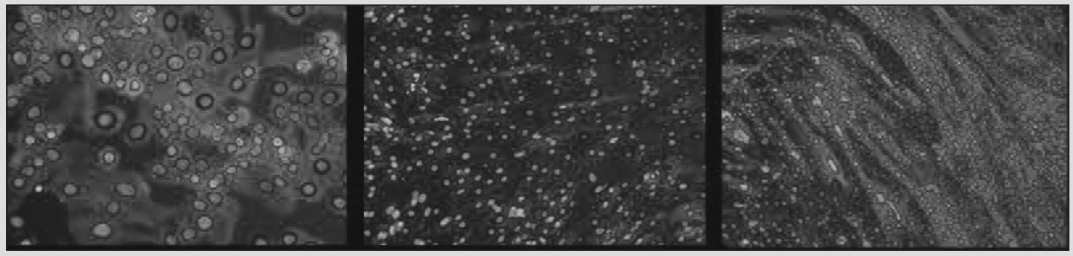

Figure 2 - Proliferation and differentiation of C2C12 myoblasts on ALG/CLG scaffold, at days 2, 5 and 8 after seeding (from left to right).

Ligament repair: a molecular and immunohistological characterization

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Introduction

The anterior cruciate ligament (ACL) is an important static stabilizer of the knee, its function is to limit rotation and forward motion of the tibia. The most commonly surgical treatment for ACL reconstruction is an autologous transplant with the patellar tendon (PT) or with the semitendinosus and gracilis tendon (STGR), which provide good results in limiting anterior tibial displacement under anterior loads and similar outcomes in the medium term (1, 2).

The aim of our study was to quantify the gene expression of some matrix molecules (collagen I, II, X, SOX-9, tenascin-C, aggrecan) and of some molecules involved in the extracellular matrix remodelling in human ACL, PT, STGR and reconstructed ACL with patellar tendon (rACL-PT) and with semitendinosus and gracilis tendon (rACL-ST). An immunohistochemical evaluation of all the matrix markers previously reported was also performed in the ACL, rACL-PT, rACL-ST samples.

Methods and materials

Human ACL, PT, STGR and rACL specimens, obtained from the knees of five male patients during elective reconstruction of ACL, were minced into very small pieces and plated onto cell culture flasks for a week. At confluence, cells were harvested and pellets processed for Real-time RT-PCR. Real-Time RT-PCR analysis was performed to evaluate the expression of types I, II and X collagen, aggrecan, Sox-9, tenascin-C, cathepsin B, MMP-1 and MMP-13 mRNAs. Immunohistochemical analysis for matrix proteins and remodelling proteins was carried out on paraffin embedded sections in the samples by using specific monoclonal antibodies.

Results and discussion

RT-PCR results were reported in Figure 1. We found that all the tissues analyzed expressed collagen I and tenascin C, two characteristic markers of fibrous tissue. These results were confirmed by immunohistological evaluations (Fig. 2). We also demonstrated the presence of collagen II, aggrecan and Sox-9. Collagen X mRNA was detected only in the STGR samples and, at very low levels in the rACL-ST; the protein was slightly expressed in the extracellular matrix of all the samples evaluated. This molecule, originally found to be a product of hypertrophic chondrocytes of growth plate, has already been demonstrated to be also present, at the ligament/tendon-bone interface. rACLs present high levels of MMPs and cathepsin B, suggesting that during ligamentization active remodelling processes are needed. Our results showed only slight differences among the tissues analyzed; the more interesting being the higher expression of collagen type II in the cells from rACL-STGR compared to rACL-PT. This difference is probably due to the biomechanical characteristics of the two tendons.

Conclusion

Despite the low number of patients evaluated we believe that our study could give a reasonable identification of genetic and protein markers specific to tendon/ligament tissues and be helpful in testing tissue engineering approaches for ACL reconstruction.

Acknowledgements

We thank Patrizia Rappini and Graziella Salmi for their assistance in the preparation of the manuscript and Luciano Pizzi for his technical assistance.

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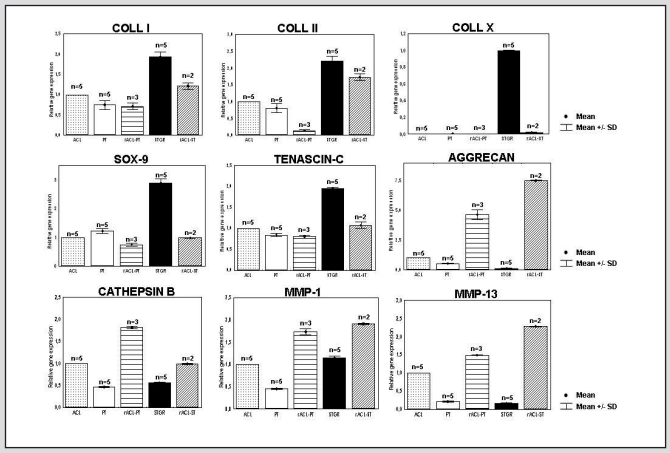

Figure 1 - PCR analysis of differential gene expression of collagen I, II, X, Sox-9, Tenascin-C, aggrecan, cathepsina B, MMP-1 and MMP-13 in anterior cruciate ligament (ACL), patellar tendon (PT), ACL reconstructed with PT (rACL-PT), double loop semitendinosus and gracilis tendon (STGR), and ACL reconstructed with STGR (rACL-ST). Values are expressed as mean ± standard deviation (SD). n= number of samples evaluated.

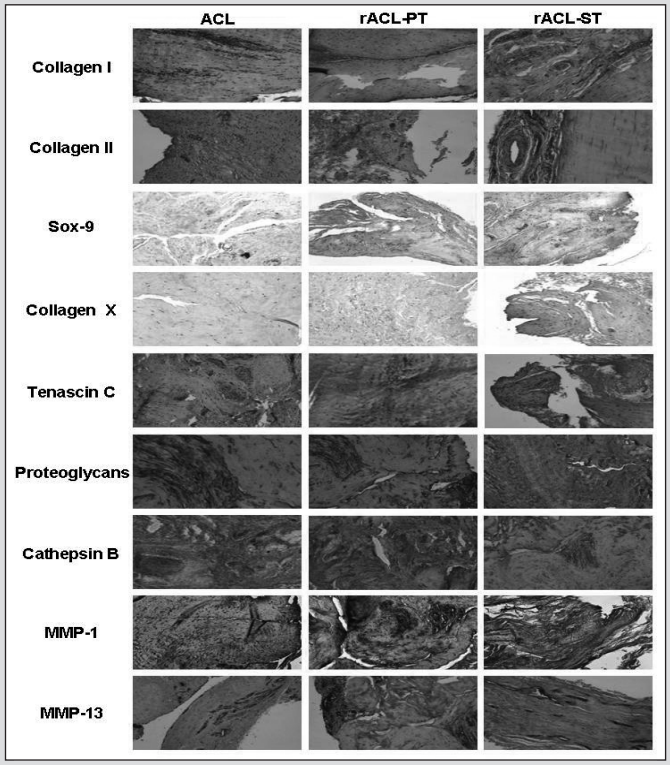

Figure 2 - Immunohistochemical observations of collagen type I, II, X, Sox-9, tenascin-C proteoglycans, cathepsin B, MMP-1 and MMP-13 in the different tissues from a representative patient. ACL= anterior cruciate ligament, rACL-PT= ACL reconstructed with patellar tendon, rACL-ST=ACL reconstructed with semitendinosus and gracilis tendon. Proteins were developed using Fast Red.

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Wood structures with organized morphology for bone substitutes

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Introduction

The painful disability of bone related diseases is a major problem of ageing populations in modern societies and its social impact is enormous. With such a demand, there is a recognized societal and economic need of developing bone scaffolds able to regenerate the natural bone tissue with appropriate mechanical and biofunctional performance, in order to attain a rapid patient recovery. This is possible if the bone scaffold expresses a high level of biomimetism either from the chemical and from the morpho-structural point of view (1).

This study reports the preliminary results of the ceramisation process from wood to innovative ceramic scaffolds for bone substitution. These final structures will be highly organized from the molecular to macro-scales, with extremely functional architectures able to constantly adapt to ever changing mechanical and biofunctional needs.

Starting from suitable vegetal raw materials pyrolysed to produce carbon templates characterized by complex pore structure, then infiltrated by vapour phase calcium to produce calcium carbide and finally transformed into porous ceramic of calcium phosphate-based materials by multi-step thermic and hydrothermal treatment in controlled environment.

Materials and methods

Some wood templates were used as starting tests for ceramisation process and transformed in carbon template at 1000 °C. The carbon piece was exposed to the vapour of calcium in argon flow, for transformation in CaC2, in temperatures higher than boiling point of calcium. The carbonation reactions with CO_2 were carried out into cylinder reactors in controlled conditions.

Different analytical techniques were used to check the phase composition and morphology of raw, intermediate and final materials.

Results and discussions

The pyrolysis process was performed on wood both on native form and after chemical treatment suitable to eliminate the resins. Pyrolysis treatment permit to maintaining the highly organized microstructure of the native wood (Fig. 1).

The following step was the chemical reaction between carbon templates and vapour phase calcium to achieve calcium carbide ceramics. Figure 2 shows the calcium carbide template obtained after the thermal treatment, it appear with original structure preserved. XRD and SEM-EDS confirmed the extensive formation of calcium carbide phase in the wood structure. Vapour phase reaction is particularly effective because involve all the struts between pores.

The transformation of calcium carbide into calcium carbonate is important because this compound is a good precursor to synthesize biomimetic materials. In fact phosphatation processes of calcium carbonate constituting natural source to form hydroxyapatite were successfully studied (2, 3).

Best result of carbonation was the following synthesis:

Reaction 1) $CaC_2 + H_2O + Q \rightarrow CaO + C_2H_2$

Reaction 2) $CaO + CO_2 \rightarrow CaCO_3$

Total reaction $CaC_2 + H_2O + CO_2 \rightarrow CaCO_3 + C_2H_2$

Reactions 1 and 2 were performed in two different step: the formation of calcium oxide was obtained from calcium carbide in air at 1000 °C, then the calcium carbonation starting from calcium oxide, was run in pCO_2 10 bar at 350 °C.

The carbonation processes of calcium carbide had a yield of about 100% in term of calcium carbonate formation (determined by XRD analysis).

Conclusions

On the way of the multi-step process required to achieve calcium phosphate-based scaffolds with highly organized morphology, a ceramisation process able to transform natural woods in calcium carbonate ceramics is set up. Pyrolysis, infiltration, and chemical transformation processes have been developed to succeed in maintaining the initial morphology of the wood. The development of material synthesis able to mimic biological principles and hierarchic morphology, may pave the way for realizing prosthetic devices closer to the extraordinary performance of human tissues. This study is addressed also to solve the problem of the long bone regeneration or under stresses.

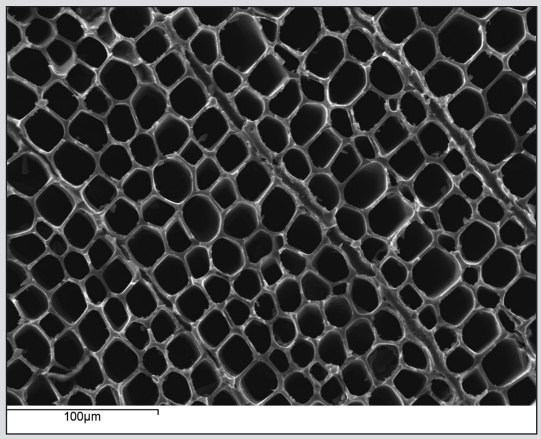

Figure 1 - Pyrolysed pine-wood.

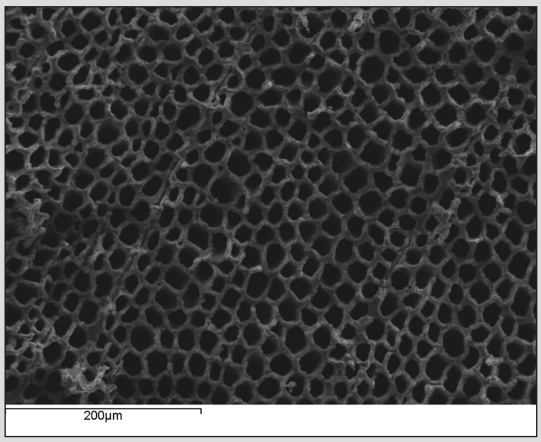

Figure 2 - Calcium carbide template.

Acknowledgements

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New morphosynthetic processes for biomaterials development

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Introduction

New methodologies of synthesis allowing a direct morphological control of the final product represent an extremely stimulating and polyvalent strategy today. Organic templates with complex and organized structures can be used to control the crystals morphology or the spatial distribution of the inorganic phases to get final products with hierarchically organized structures or with specific ability linked to the very high specific surface (1, 2). The potentialities of these new synthetic techniques can be applied to control the specific surface, the porosity, the 3-D organization and therefore to produce materials that associates morphological gradients to gradients of functionality.

Materials and methods

Morphosynthetic process was exploited for:

- the realization of composite materials based on hydroxyapatite (HA) and alginate (Alg) exploiting the high affinity of this natural polymer for Ca^{2+} ions, present in HA structure. The mineral phase which is in form of HA nanoparticles, precipitates on the natural polymer thanks to an acid-base neutralization process between $Ca(OH)_2$ and H_3PO_4 under ideal conditions. The so obtained HA/Alg hydrogel dripped in a solution containing bivalent ions like Ca^{2+} and Sr^{2+}, brings to the formation of spheres. The spheres separated by the solution, can be subsequently freeze dried to obtain hollow spheres.
- the precipitation of porous particles of micrometric dimensions and complex morphology with elevated specific surface. In this process the joined action of the reagent urea and EDTA as template was exploited. The formation of the mineral phase and the generation of the complex morphology are stimulated under opportune and controlled pH and temperature conditions. In function of the relative concentrations of PO_4^{3-} and Ca^{2+} ions the morphology and final chemical composition of the mineral phase can be changed.

Results and discussion

The studied morphosynthetic processes, allows to realize composite materials with complex porous structures, formed by nanocrystalline HA as mineral phase, interactive with complex molecules of organic nature that are the main responsible of the particular obtained morphologies. The polymeric chains of alginate in the first case and the molecules of EDTA in the second one, thanks to their affinity with Ca^{2+} ions, manifest their templating action growing of the inorganic component and stimulating the formation of stable and morphologically complex composites materials. The obtained HA/Alg hydro-gel submitted to a freeze dried process gave a beehive morphology analogous to the egg-box structure proposed for the Ca^{2+}-Alg interaction (3, 4) very useful as bone scaffold. The same hydrogel gave the possibility to produce spherical samples that can be modulated in composition (HA/Alg ratio), dimension and morphology (hollow or not depending from the drying process) (Fig. 1) which can be designed and developed as drug delivery system. Likewise the second morphosynthetic process gave the possibility to obtain final products with complex 3-D organizations modifiable in composition and dimension in function of the synthesis conditions (Fig. 2) which comprises both the above discussed functions.

Conclusion

The studied morphosynthetic processes, exploiting the ability of organic molecules like alginate and EDTA to organize themselves in complex structures, allowed the realization of systems with morphologies that make them proper for applications as bone substitute and transport/release of bioactive molecules. In particular the mineral phase constituted by HA nanoparticles, results bioactive and bioresorbable and with high affinity to bind natural bone. Secondly the natural organic phase, allowing the realization of products with organized multifunctional structures, is very important to favour cell adhesion and more in general osteoconductive behavior of the composites.

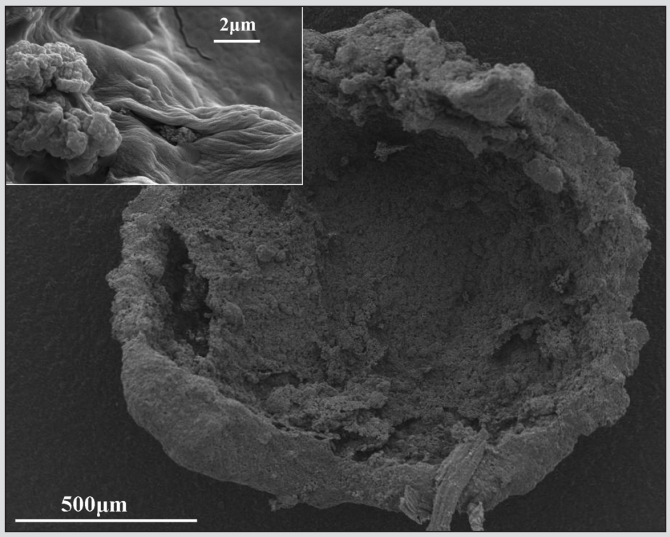

Figure 1 - HA/Alg hollow sphere; in detail, HA particles merge in the polymeric structure of alginate.

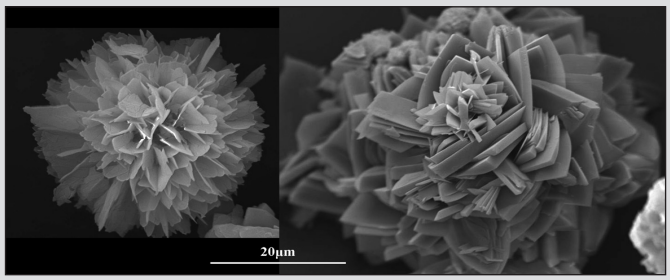

Figure 2 - HA/template hierarchically structured porous spheres.

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Combined drug release from biodegradable bi-layer coating for endovascular stents

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Introduction

Restenosis post-stenting is the most common problem related to stent implantations and it is caused by mechanical injury to the intima during stent implantation and deployment, stretching of the arterial wall and changes in the blood flux.

The restenosis post-stenting is also called in-stent restenosis and it is due to the smooth muscle proliferation and migration, followed by extracellular matrix and collagen synthesis that leads to the neointimal hyperplasia. In order to limit the in-stent restenosis, stents have been covered by polymeric coatings containing pharmacological agents that inhibit smooth muscle cell proliferation and migration. The setup and control of drug delivery from polymer coating is clearly an important challenge in the designing of advanced drug elution coatings for stent (1, 2).

In the present work, the characterization of a composite biodegradable system used for the controlled and combined drug delivery is proposed. For this aim, two different active principles were selected and used, in order to keep down the post-stenting restenosis problem after the device implantation. In particular, the main goal was the study and the control of the simultaneous drug delivery kinetic, regulated from diffusive processes and from the biodegradation of the selected polymers.

Materials and methods

In order to achieve the aim of the present study, biodegradable materials such as poly(lactic-co-glycolic) acid (PLGA), polyhydroxy(butyrate-co-valerate) (PHBV), were selected and tested under chemo-physical (SEM, DSC, TGA, FT-IR) and functional (*in vitro* drug release tests, evaluation of interaction between polymers and stent materials by adhesion tests, anti-restenosis drugs loading tests) aspects. Drugs used were tacrolimus (immunosuppressant) and paclitaxel (antiproliferative and antitumor), two active principles used for restenosis treatment.

Samples containing drugs were obtained in different morphologies, for example single and multi-layer. Adhesive properties were tested upon surfaces of interest, that were metallic (AISI 316L) and carbo-coated with turbostratic carbon. Adhesion tests were carried out immersing polymeric samples in a standard solution (pH = 7.4 containing 0.5% of sodium dodecyl sulphate) for a period of 30 days. Samples were monitored every 24 hours, paying attention that polymeric coatings were always stuck on to the surface.

Drug delivery tests were carried out *in vitro* immersing samples in the same solution used for the adhesion tests. At established times, samples of the delivery solution were taken and analyzed using a chromatographic method (HPLC) for evaluating the exact amount of drug released from the matrix.

Results and discussion

A great adhesion capability onto the surfaces of interest was shown for PLGA biodegradable polymer. Then, interaction between polymer and drug and the homogeneity of obtained coatings were identified using chemical and physical characterization.

Bi-layer systems obtained and tested, showed a great potential in controlled drug delivery. In particular, the capability of obtaining a simultaneous and synergic tacrolimus and paclitaxel release (without any hindrance between them) from a biodegradable matrix were confirmed. In Figure 1 paclitaxel delivery from a PLGA layer covered with a PHBV layer is compared with paclitaxel delivery from PLGA covered with a PHBV layer loaded with tacrolimus. In Figure 2 tacrolimus delivery from a PHBV layer shed upon a PLGA layer is compared with tacrolimus delivery from PHBV shed upon a PLGA layer loaded with paclitaxel. No relevant differences were detected, and an improvement in the cumulative elution of both drugs could be attributed to the interaction between active compounds.

Drug release kinetic that appears from tests had a sigmoid trend; it means that a release controlled by diffusive processes was present during the early period of *in vitro* tests. Then, after about 20 days, a matrix erosion occurred, and drug release was controlled by a mix mechanism, involving diffusive and degradation controls (degradation was monitored by GPC: polymer films showed a progressive decrease of the molecular weight).

This second phase seemed to guarantee the achievement of the initial purpose of the present work: the development of a specific biodegradable matrix that was able to deliver at medium-late time a drug amount higher than commercial systems now available, composed by biostable polymer matrix.

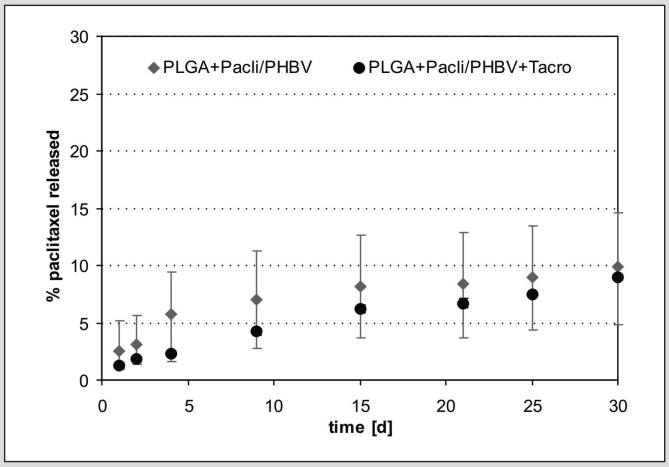

Figure 1 - Cumulative percentage Paclitaxel release from bi-layer PLGA/PHBV coatings; (●) PHBV top-layer containing tacrolimus, (◆) PHBV top-layer without tacrolimus.

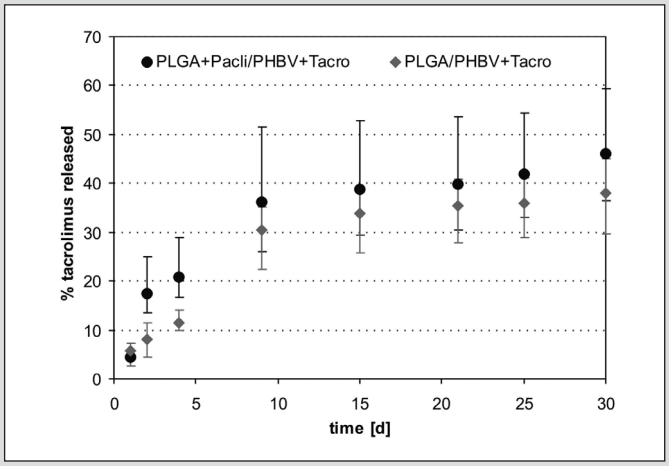

Figure 2 - Cumulative percentage Tacrolimus release from bi-layer PLGA/PHBV coatings; (●) PLGA bottom-layer containing paclitaxel, (◆) PLGA bottom-layer without paclitaxel.

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In vitro culture of human osteoblasts on sandblasted titanium modified with plastic deformation

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Introduction
The early osteointegration of a macro-rough or a micro-rough metallic surface depends mainly on the ingrowth of the bone tissue onto the material. Titanium surfaces with micro-roughness have been studied to substitute machined titanium, with the focus on enhancing the bone apposition onto the implant; in particular they promoted the proliferation and the differentiation of MG63 osteoblasts (1).
Aiming at an accelerated and enhanced *in vivo* osteointegration of a micro-rough titanium surface during the early postimplantation period, we show a particular strategy that consists in the surface modification of the sandblasted titanium with plastic deformation and, afterwards, with proliferated bone cells and their extracellular matrix produced *in loco*.
The first mechanical modification (the plastic deformation of the biomaterial surface) is proposed to enhance the second biomimetic procedure (the culture of bone cells on the plastically deformed surface); the last in vitro biological strategy is proposed in order to benefit the *in vivo* bone formation.

Materials and methods
Control disks (diameter, 12 mm; height, 4 mm) were cut from the titanium alloy Ti6Al4V. The disks were sandblasted by Al_2O_3 powder (granulometry, 16 mesh) (Lima). The resulting rough surface had a mean roughness depth Rz of 26 μm. The surface of the preceding disks was then plastically deformed with a punching process (Lima). The resulting craters had the following characteristics: upper diameter of 500 μm, lower diameter of 300 μm, and depth of 170 μm. The sandblasted titanium surface was maintained between the punched craters.
The human osteosarcoma cell line SAOS-2 was cultured in McCoy's 5A medium, supplemented with 15% fetal bovine serum. A cell suspension of 4×10^5 cells in 100 μl was added onto the top of each disk.
Cultured disks were processed for SEM analysis, DNA extraction, and bone matrix extraction.

Results and discussion
The SEM images revealed that, due to the plastic deformation, the cells proliferated over the available titanium surface (Fig. 1). After 22 days of culture, in the control culture the cell number per disk grew to $1.5\times10^6 \pm 1.1\times10^4$, whereas on the punched titanium to $3.1\times10^6 \pm 1.3\times10^4$ with $p<0.05$.
An ELISA of the extracted matrix was performed: at the end of the culture period, in comparison with the control culture, the plastic deformation of the sandblasted titanium increased significantly the protein coating of the surface with $p<0.05$ (Tab. I).

TABLE I - BONE MATRIX COATING IN fg/(cell×disk)

	Sandblasted Ti	Punched Ti
Decorin	387 ± 83	911 ± 81
Osteopontin	313 ± 20	525 ± 90
Type-I collagen	2136 ± 333	2836 ± 334

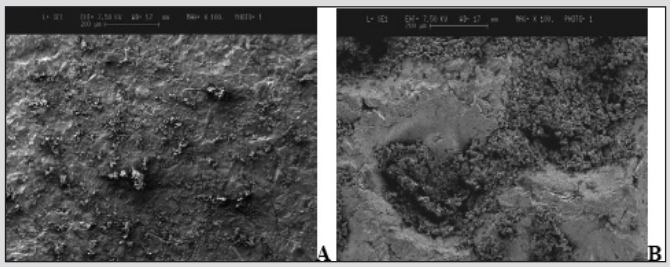

Figure 1 - SEM images of the cultured sandblasted (A) and punched (B) titanium surfaces, 100×, bar=200 μm.

Aim of this study was the plastic and biomimetic modification of a sandblasted titanium surface with punched craters, osteoblasts, and bone matrix to make the biomaterial more biocompatible for the bone repair *in vivo*.
The plastic deformation of the biomaterial surface was the "physical method" to obtain the biomimetic modification of the material: the plastic deformation increased the cell proliferation around 2-fold.
Furthermore, the synthesis of type-I collagen, decorin, and osteopontin was significantly enhanced. The effects of the plastic deformation were similar to those of the electromagnetic wave in terms of proliferation and bone matrix synthesis (2).

Conclusion
It is possible to conclude that the cultured biomaterial could be used fresh or after sterilization with ethylene oxide in order to obtain a storable tissue-engineering product for bone repair applications.

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Biocompatible 3D polymeric networks for cardiac tissue engineering applications

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Introduction

Tissue engineering is a multidisciplinary field that encompasses medicine, engineering, chemistry and biology for the purpose of fabricating replacement tissues able to restore damaged tissues. As far as cardiac diseases are concerned, the challenge is to identify the suitable combination between the best cell source for cardiac repair and the design of the optimal scaffold as a template for tissue replacement (1). Insights into the stem cells field lead to the identification of the suitable scaffold features that enhance the *ex vivo* proliferation and differentiation of stem cells. Scaffolds composed of natural and/or synthetic polymers can organize stem cells into complex architectures that mimic native tissues. A proper design of the chemical, mechanical, and morphological characteristics of the scaffold at different length scales is needed to reproduce the tissue complexity at the cell-scaffold interface. Hierarchical porosities are needed in a single construct, at the millimetre scale to help nutrition and vascularization, at the micrometer scale to accommodate cells, and at the nanometre scale to favour the expression of extra-cellular matrix components (2). Among the many available techniques for scaffold fabrication, electrospinning is a suitable technology to fabricate tuneable hierarchically porous matrices that mimic aspects of the cell native surroundings (3).

Materials and methods

A 10 wt.% poly(ε-caprolactone) (PCL) in a solution of chloroform-methanol (7:1) was electrospun between two electrodes at 20 kV, namely a positively charged syringe needle and a counter patterned electrode electrically grounded where electrospun fibers were collected. The syringe shaft was actuated by a programmable syringe pump that supplies the solution to the needle and fuels the process. The patterned electrodes consisted of two pairs of Cu bar electrodes placed at 90° from each other. Each bar was 50 mm long, with a square cross section 5 mm wide. They were placed 50 mm apart at a distance of about 170 mm from the syringe needle. The morphological properties of the scaffold were investigated by scanning electron microscopy (SEM). Scaffolds biocompatibility and bioactivity were investigated by implanting adult stem cells, namely bone marrow-derived mesenchymal stem cells (MSC) and resident cardiac stem cells (CSC). Cell adhesion and proliferation were assessed by immunofluorescence (IF).

Results and discussion

Figure 1 shows the morphology of the electrospun mat obtained with this experimental set-up.

The scaffold, made of fibers 2-4 μm in diameter, appears as quasi-woven network made with two groups of fibres oriented along orthogonal directions, exhibiting a macroscopic texture with a short-range ordered plot with periodic features orthogonal to each other. Our aim was the fabrication of a scaffold that could mimic the architecture of the natural extracellular matrix (ECM). Natural ECM consists of proteoglycans matrix embedding fibrous collagens organized in a three-dimensional porous network with fibres arranged in hierarchical structures from nanometer length scale fibrils to macroscopic tissue architecture. Similarly, electrospun scaffolds are inherently multiscale structures with nano- or micro fibres with macro scale interconnected pores. In general, if a simple collecting plate (e.g. an aluminium foil) is used, the electrospun fibres deposit randomly, forming a spaghetti-like stack. Our experiment shows how to obtain fibre alignment through the usage of patterned electrodes. Amongst other reasons, this is relevant for tailoring mechanical properties and large porosity. This approach for electrospinning appears to be a viable technique to modulate the in-plane elasticity of the scaffold. In fact, in a previous work (4) it has been demonstrated that cardiac progenitor cells differentiate into cardiac phenotype and align along the direction where the elastic properties of the scaffold matched the elastic properties of the cardiac cells. The biological validation of the produced scaffolds demonstrated that stem cells were able to colonize the pores created and to adhere functionally to the scaffolds (Fig. 2). In addition, no evidence of cell death was detected. The results showed not only the cytocompatibility of the materials used, but also significant adhesion and proliferation of seeded cells onto the constructs.

Figure 1 - SEM micrographs of quasi-woven textured PCL scaffold.

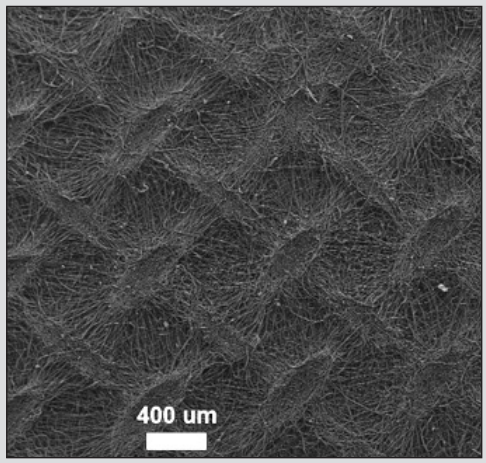
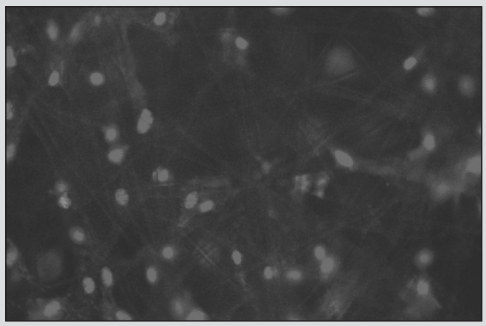


Figure 2 - IF images of the PCL scaffold.



Conclusions

We demonstrated that it is possible to switch from a totally random macrotexture to an ordered arrangement via designed electrodes. In summary, the fabrication of tuneable scaffolds with hierarchical porosities, made of biodegradable polymers, was demonstrated for cardiac tissue engineering.

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Surface functionalization of biomaterials through alkaline phosphatase anchoring

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Introduction

Rapid bone integration and fast healing of bad quality bones are important goals in prosthetic surgery. An interesting solution in this way is the realization of biomimetic surfaces that are able to pass signals directly to cells in order to promote healing and regeneration of damaged bone. The aim of this research work is the functionalization of biosurfaces by anchoring on them biomolecules involved in the process of bone integration. Alkaline phosphatase (ALP) was used as model protein, because it is involved in the mineralization processes (1, 2). The devices of interest for the research are dental or orthopaedic implants and substitutes of small bones. Treated materials will be bioactive both from a physico-chemical point of view (osteoconduction and hydroxyapatite precipitation) than from a biological one.

Materials and methods

In this research work two different kinds of biomaterials have been considered: bioactive glasses, with different compositions and degrees of bioactivity, and titanium alloys.

These surfaces, after suitable treatments are able to expose hydroxyls that could be employed in biomolecular anchoring. The firs step of this research work is the development of a reproducible technique to expose these groups on material surface. As for glasses it includes different washings in order to remove any contaminants and then expose reactive hydroxyls, while for titanium it include both an acid attack and a chemical treatment. The second step is surface activation in order to promote and stabilize biomolecule bonding. As for glasses silanization with an aminosilane has been chosen in order to expose amino group useful to graft enzyme, while for titanium tresyl-chloride activation has been performed.

The last step is enzyme grafting that has been realized for both materials by incubation of activated samples in a solution of ALP in PBS. For control bonding experiments have also been performed on materials with only hydroxyls exposed. Hydroxyls exposition has been verified by means of contact angle measurements and XPS analysis. Samples have been also analyzed by means of XPS in order to detect the presence of molecules (silane, tresyl-chloride, ALP) on the surface. In order to verify the activity of ALP anchored on surface enzymatic test has been performed by UV-vis analysis after the reaction with an appropriate substrate (p-nitrophenilphosphate). Samples have also been studied after different washings in order to evaluate the stability of bonding between silane and surface and enzyme and surface.

Results

Contact angles measurements on glasses and XPS spectra of oxygen region for titanium confirm the exposition of a significant amount of hydroxyls on sample surfaces after the first step of functionalization process. XPS analysis after the second step verifies the presence of silane on glasses and of tresyl-chloride on titanium. XPS spectra verify enzyme presence on material surfaces after functionalization trough an enrichment in carbon and nitrogen and a reduction in materials characteristic constituents. The detailed study of carbon region confirms that on functionalized samples the higher component of carbon peak is relative to C-O and C-N (at about 286eV) bonding typical of biomolecule (Fig. 1 – peak B- example for a bioactive glass). This result is similar both for bioactive glasses and for titanium.

Enzymatic test shows that ALP maintains its activity after anchoring on all tested materials. After washing the amount of enzyme active on the surface is reduced but maintained.

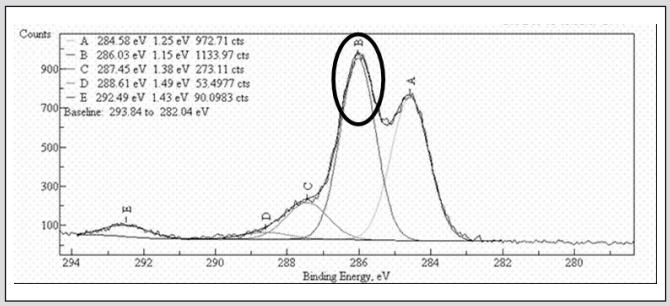

Figure 1 - XPS spectrum – detail of carbon region for a functionalized bioactive glass.

Conclusions

It is possible to successfully bond alkaline phosphatase to the surface of different biomaterials. The enzyme maintains its activity after anchoring and the bond is quite stable to washings. Cellular tests on functionalized samples are in progress.

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