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Bioactive glass coating: physicochemical aspects and biological findings

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Glass coating material was investigated before and after spraying to see whether it maintained the chemical and physical properties; *in vivo* and *in vitro* studies were done to evaluate the biological results. Following the spraying process, the Biovetro® coating on the $TiAl_6V_4$ plate – as evidenced from chemical and physical analysis – maintains the properties of the original glass unchanged as far as the amorphous structure and its behaviour in a hydrolytic environment are concerned. *In vitro* and *in vivo* studies underline the positive features of the coating obtained by the plasma spray process, confirming that it has the typical properties of bioactive glass patented under the trade mark, Biovetro®, i.e. biodegradability and osteoconductivity already confirmed by previous experimental protocols carried out by our group using powdered and fibre Biovetro.

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The development of coatings made of bioactive inorganic materials for orthopaedic implants has been made easier thanks to plasma spray techniques for which powdered granular materials are used. The process takes place by injecting a gas flow (plasma spray) into a chamber where, by means of an electric arc, the gas temperature is raised up to 10 000–30 000 K. Powders with a grain size below 80 μm are thrust into the chamber; they are heated up and, following accelerated flow through a nozzle, are forcefully sprayed on the surface of the metal for coating.

The speed of the particles may range between 100 and 350 $m\,s^{-1}$, and therefore the flight time may be only 10^{-3} of a second. When the particles hit the material, they yield their thermal and kinetic energy to the substrate, which undergoes deformation taking up a lenticular shape and solidifies in a matter of 10^{-6} seconds. By spraying the whole piece of material, the particles deposit one on top of the other making up the coating to the desired thickness, which usually is about 100 μm.

The advantages of the plasma spray technique are represented by the high deposition speed, decreased modification of the metal substrate and minimum size tolerance. Some disadvantages may arise because of the need to improve the coating deposition on the metal; to do so, the temperature of the beads going through the chamber is, for instance, increased. In the presence of thermodynamically unstable substances, a temperature increase may lead to structural and morphological changes such as:

(a) modification of the crystallinity and of the ratio between the atomically ordered crystal phase and the amorphous phase, i.e. the phase that does not display a long-term order in the atomic distribution;
(b) appearance of new phases following the formation of new crystal compounds;
(c) modification of the chemical composition due to evaporation of volatile components.

As regards the morphological features, the disadvantages may be due to the presence of excessive macro- and microporosity in the coating with negative effects on the adhesion at the interface between the coating and the metal, and on the mechanical properties along with modifications of the biological response.

Among osteoconductive coatings that are currently used in orthopaedic surgery, we find, for instance, hydroxyapatite, calcium phosphate and, more recently, bioactive glass. The use of such coating materials is meaningful in as much as they allow the prosthesis to adapt to the bone cavity, prevent the formation of fibrous tissue at the prosthesis–bone interface and favour a strong chemical bond between implant and bone tissue.

On the basis of these conditions, we decided to investigate the glass material before and after the spraying stage to see whether it maintained the chemical and physical properties; we also decided to carry out *in vivo* and *in vitro* studies to evaluate the biological results.

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MATERIALS AND METHODS

We used the material and the methods described below to obtain a Biovetro® coating on a titanium plate that was to undergo chemical and biological controls. Biovetro belongs to a group of compounds whose biocompatibility and osteoconductive properties have been previously assessed[1–3]. The glass had the following composition: Na_2O (7–24%), K_2O (2–8%), CaO (9–20%), MgO (0.1–2%), Al_2O_3 (0.1–2%), SiO_2 (46–53%) and P_2O_5 (4–8%). It was made at SEIPI laboratories using an electric furnace and melting at 1350° C a mixture of raw materials of analytical grade purity. After the melting process, the glass was cast, crushed and transformed into powder of grain size suitable to the spraying process.

The coating process was achieved by using plasma spray equipment operating at a controlled atmosphere in the presence of an electric arc of power 25–30 kW, and a modified torch with parameters that had been studied carefully for its use with the inorganic bioactive material under investigation. The metal support was made up of a titanium alloy, $TiAl_6V_4$, similar to that used for a prosthesis, either in the form of a plate of ($6 \times 10 \times 1.5\,mm^3$) or in the form of a dish of 15 or 19 mm diameter and 3.3 mm thick, depending on the needs of the experiment. The thickness of the Biovetro coating was kept constant at about 80 μm.

The inorganic bioactive material was investigated before and after the spraying stage with the following analyses.

Chemical and physical features

1. Chemical analysis using classic methods assisted by a Hilger Polyvac E 981 emission spectrometer and a Perkin Elmer 5000 atomic absorption spectrometer.
2. Analysis of the specific ion release with a hydrolytic test performed on 19 mm diameter dish-shaped metal samples coated with sprayed Biovetro. Three dishes were placed in 50 cm^3 of Tris solution buffered with hydrochloric acid at a pH of 7.2 and kept at 37° C in a thermostated bath for 1, 6, 24, 48, 72 and 100 h. Following extraction of the samples, the ion species released by the coating were determined in the remaining solutions by means of colorimetry for SiO_2, ion chromatography for P_2O_5 and atomic absorption spectrometry for Na_2O, K_2O and CaO. Results were assessed on the basis of the mean of three values obtained from three samples and expressed in terms of milligrams of oxide per square centimetre of surface exposed.
3. Microstructural analysis using X-ray diffractometry performed on PW 1720 Philips equipment, on a 600 mg sample.
4. Analysis of thermal evolution using the Netzsch differential thermal analysis between 20° C and 1200° C on a 450 mg sample.
5. Morphological analysis made with a scanning electron microscope equipped with a wavelength dispersion microprobe using a CAMEBAX 50 L. equipment.
6. Light microscopy with a Zeiss light microscope.

Biological analyses

For *in vivo* and *in vitro* studies titanium alloy samples were coated with sprayed biomedical Biovetro and titanium alloy samples without coating were used as controls. In this case round samples with a 15 mm diameter for cell culture tests and plates ($6 \times 10 \times 1.5\,mm^3$) were preferred for *in vivo* tests.

For *in vitro* testing, we used cell line L929 (Mouse Connective Tissue: BSCL56 from the Istituto Zooprofilattico di Brescia, Italy). This cell line was maintained in complete growth medium D-MEM containing antibiotics (penicillin (100 IU cm^{-3})) and streptomycin (0.1 mg cm^{-3}) supplemented with 10% fetal calf serum (Gibco Europe Ltd). Cell cultures were passed twice in 1 wk on Petri dishes (Costar, Italy) and incubated at 37° C in 5% CO_2 and over 90% humidity. At the time of the experiment, cell cultures in exponential growth were obtained by repeated washing with phosphate-buffered saline (PBS) (pH 7.4), and incubated for several minutes in 0.1% trypsin – 0.02% ethylenediaminetetraacetic acid in PBS. The cell suspension was then centrifuged at 1500 rev min^{-1} for 10 min and the pellet thus obtained was resuspended in growth medium. Various dilutions of the cell suspension were obtained according to the method described above and then seeded at a density of $1 \times 105\,cm^{-2}$ on both treated and control samples. The cells were seeded in the same growth medium with 10% fetal calf serum added on different samples, incubated for different time intervals at the same temperature and CO_2 values. After 24, 72, 96 and 120 h, without changing medium, each sample in triplicate was treated for cell count. The cells grown on the different surfaces were washed with PBS and detached by adding 100 μl of 0.1% trypsin – 0.02% ethylenediaminetetraacetic acid for 5 min at 37° C. The cell suspension was gathered after two further washing with PBS. The cells were counted in the resulting volume using a Coulter counter. Protein synthesis velocity was also determined as an index of cell activity, by measuring the uptake of radioactive L-leucine ({^{3}H}-leucine: 2.5 Ci mol^{-1}) during 30 min incubation. A monolayer of cells was incubated in complete growth medium, to which was added 0.8 mM of unmarked aminoacid plus 2 μCi cm^{-3} radioactive leucine. After incubation, the cell monolayer was washed three times with cold Earl's solution containing 0.1% glucose. Cells were then precipitated by adding 10% col trichloroacetic acid (TCA) (w/v) for 10 min, washing twice in TCA and solubilizing with 0.2 N NaOH; radioactivity was measured on known volumes of the acid-insoluble fraction by a liquid-phase radioactivity counter (Packard 2425). Total protein content was determined by colorimetry (Biorad) using bovine serum albumin as standard.

For *in vivo* testing, the experimental protocol involved the use of a group of rabbits with assessments made after 30, 90 and 180 d. In every animal, at the level of the metaphysis of one tibia, a sample coated with Biovetro was introduced, whereas in the contralateral tibia we introduced an uncoated sample, *Figure 1*. On the day of the intervention, 7 and 14 d thereafter, and again 21, 14 and 7 d before killing the animal, tetracycline, xylenol orange and calcein were sequen-

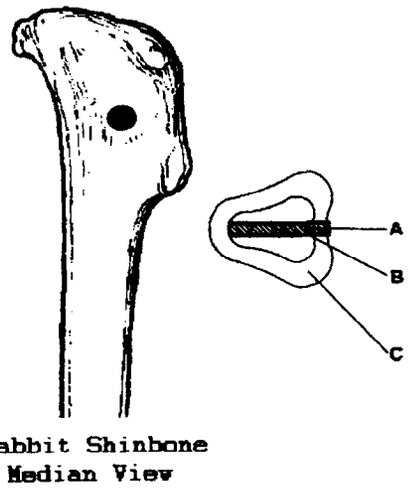

Figure 1 *In vivo* implantation and positioning of the $TiAl_6V_4$ plate: **A**, metal; **B**, Biovetro®; **C**, bone.

Figure 2 Surface of the Biovetro® coating sprayed on a $TiAl_6V_4$ plate as observed under the scanning electron microscope (original magnification, ×500).

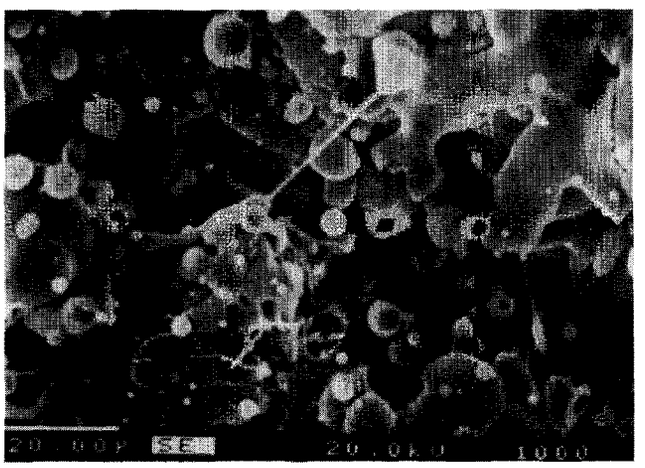

Figure 3 Magnified detail of the Biovetro® surface appearing in *Figure 2* (original magnification, ×1000).

tially injected as labelling substances used to assess the degree of new osteogenesis. The bone segments containing the samples under investigation were treated with suitable histological techniques to preserve not only the biological tissue but also the implant and its relations with the bone itself. By means of diamond blades, sections 50 μm thick were cut to be studied under the polarized light microscope, ultraviolet light microscope and under the scanning electron microscope equipped with a wavelength dispersion electronic microprobe.

RESULTS AND DISCUSSION

Following the plasma spraying process, the structure of the coating on the metal samples sprayed with Biovetro were examined. The behaviour in a simulated solution (hydrolytic test) and in a biological environment was then analysed. The results are reported below.

Morphological aspects and structure of the surface

The morphology of the surface of the coating can be seen in *Figures 2* and *3*, which relate to the study of a portion of the sprayed surface under the scanning electron microscope. The morphological aspects and the structure of the surface are quite similar, showing a wide superficial area of microcavities with round grains more or less deformed and joined together. Adhesion to the metal is not homogeneous and there appear to be discontinuities. These data suggest that the plasma spraying process does not provide a continuous structurally compact coating. Therefore, it could be assumed that as the bioactive material moves in a high-temperature environment during the spraying process, it undergoes modifications which can affect the composition and properties found in the original samples. This doubt concerning Biovetro was dispelled by X-ray studies and by the results of the chemical analysis performed before and after the spraying process. X-ray diffraction (*Figure 4*) confirms that the material has an amorphous nature, and shows the lack of a crystal phase in the sprayed Biovetro coating. The fact that an amorphous structure remains is therefore due to heat treatment whereby the temperature of the glass grains greatly exceeds 1000° C. This statement is justified by the fact that following dilatometric and differential thermal analysis, non-sprayed Biovetro samples showed an α dilation coefficient of 130×10^{-7}, a glass transition temperature, T_g, of 505° C, a glass ceramic transformation due to the exothermic effect recorded by differential thermal analysis between 720 and 780° C, with its maximum at 760° C, and associated with calcium phosphosilicate crystallization, and the softening and melting of glass due to the endothermic effect recorded by differential thermal analysis at 1000° C, i.e. the critical temperature range associated with a glass–ceramic formation effect.

Examination of the chemical composition of Biovetro before and after the spraying process proves that due to the heat treatment, the Biovetro coating loses a moderate quantity of volatile components such as Na_2O and P_2O_5, with variations which, however, do

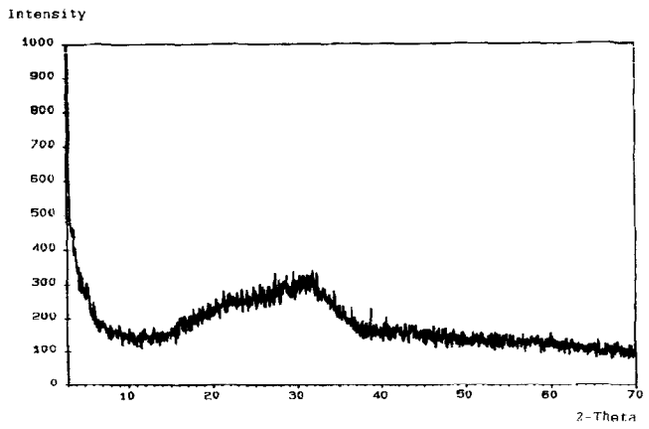

Figure 4 X-ray diffraction of a Biovetro® amorphous coating.

not affect the chemical and physical features of the glass under investigation.

Behaviour in a simulating solution: hydrolytic test

The hydrolysis test described above showed the effect of contact of Biovetro with biological solutions; in particular, the metal dishes coated with an 80 μm layer of sprayed Biovetro were placed in a Tris solution. At the end of every time-period, following extraction of the sprayed metal samples from the fluid in the remaining solutions, the concentration of ions released by the material under investigation was measured with particular attention to SiO_2, P_2O_5, Na_2O, K_2O and CaO calculated in milligrams of oxide per square centimetre of the surface of exposed sprayed Biovetro. The ion release results are reported in *Table 1*.

It can be noted that the Biovetro coating produces microreleases of Na_2O, K_2O and SiO_2 which increase with time, reaching a constant release after 100 h.

Different behaviour is noted in relation to specific migrations of P_2O_5 and CaO. The migrations of released ions appear to be quite considerable during the first hours of the hydrolytic test, with a maximum concentration being observed after 24 h from the beginning of the test. Thereafter, there is a stage of slow decrease. Many authors state that such a pattern is due to the development of two opposite processes at the Biovetro–fluid interface. In particular, the first feeds the solution whereas the second favours the decrease of phosphorus and calcium concentration in the fluid as a result of the hydrothermally induced formation of a single layer of new apatite crystals, which grow on the surface of the components of the sprayed coating[4,5].

The specific migrations of Na_2O, K_2O and SiO_2 due to Biovetro degradation lead to pH variations in the test environment as shown in *Table 1*. Recent studies confirmed that a pH increase in the environment is necessary for the growth of apatite crystals in the hydrogel layer, which develops at the interface between the Biovetro and the biological tissue[6]. Moreover, such ion releases may be considered to be relevant for a biological process, and particularly so for osteogenesis[7,8].

In vitro and *in vivo* studies

Given the modifications of the chemical and physical parameters of the coatings due to the variables used during the plasma spraying process, we resorted to cell culture growth studies and *in vivo* studies on rabbits to assess the biological behaviour of titanium alloy surfaces coated with Biovetro layers. The results of such tests are reported and discussed below.

In vitro *studies*

Using *in vitro* tests, we wanted to verify the behaviour of cell line L929 on the material analysed, given its morphologic characteristics and the results of the hydrolytic test, in order to correlate the results with known data. This particular cell line has already been used by our research group. The data concerning the growth curve (*Figure 5*) show analogous proliferation compared to Petri dishes (Costar) and titanium dishes ($TiAl_6V_4$). A decrease in cell proliferation is noted, however, on titanium dishes coated with Biovetro, which shows a rough, uneven surface as demonstrated in scanning electron microscopy images. Cells which were seeded on titanium, especially on titanium coated with Biovetro, showed an increase in protein synthesis (*Figure 6*) compared with controls on plastic, indicating greater cell activity. These results can be interpreted in the light of data obtained from previous studies by our group[9–11] and of data cited in the references[8,12]. The decrease in cell proliferation seems to be associated with a particular ionic release, as shown by the results obtained from the hydrolytic test detailed in *Table 1*, and with the consequent change in the medium pH. Matsuda *et al.*[8], in an analogous experiment, showed a variation in culture medium pH towards alkalinity in relation to ionic release by Biovetro which was

Table 1 Results of oxide release expressed in milligrams per square centimetre of sprayed Biovetro® surface as a function of the time (h) used for the hydrolytic test

Component (mg cm^{-2})	Time (h)					
	1	6	24	48	72	100
SiO_2	0.050	0.266	0.499	0.852	0.878	0.854
P_2O_5	0.031	0.118	0.184	0.143	0.113	0.097
Na_2O	0.150	0.488	0.855	1.043	1.113	1.089
K_2O	0.043	0.136	0.230	0.276	0.287	0.270
CaO	0.114	0.388	0.772	0.736	0.717	0.715
pH	7.30	7.42	7.73	7.73	8.10	8.00

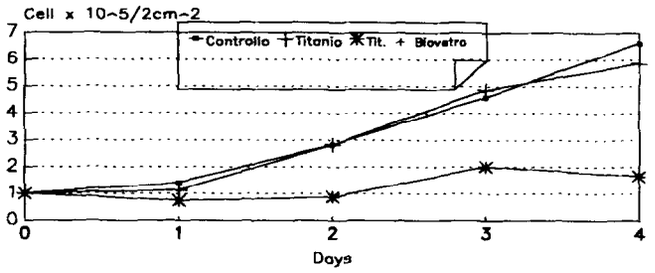

Figure 5 Proliferation rate for fibroblasts (L929).

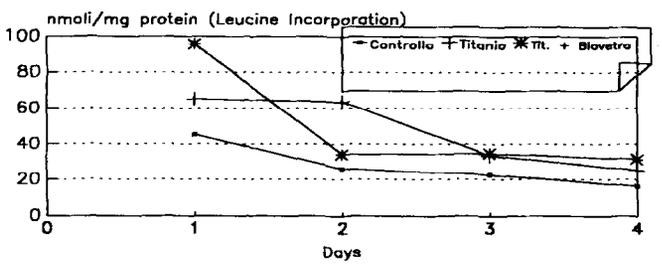

Figure 6 Protein synthesis of fibroblasts (L929).

associated with a reduction of cell proliferation. Another factor that could affect cell growth is the unevenness of the surface. Scanning electron microscopy images, taken during earlier work by our group[11] and by others[8], show that on rough surfaces most cells assume a lengthened shape with long pseudopods (polygonal form) that anchor themselves to the spiny projections of these same cells. Cells placed on a smooth surface, such as culture dishes, assume a spherical shape. According to Ohnishi[16], L-cells take on a spherical shape during mitosis and a polygonal shape with pseudopods during synthesis. Our results, along with those cited in the references, allow us to confirm that the increase in protein synthesis in those cells seeded on titanium, and particularly on titanium coated with Biovetro, represents the adaptation of the cells to the particular surface and environment. On the rough surface obtained by spraying the titanium dishes with Biovetro, increased protein synthesis is accompanied by a marked decrease in the proliferation curve. Therefore, most of the cells can be considered to be in the synthesis phase and not in the mitotic phase, which would explain the increase in protein synthesis. This increase in protein synthesis, associated with a decrease in cell proliferation, has already been observed by our group in other analogous experiments on materials lacking completely smooth surfaces or materials which show ionic release.

It can be deduced that this material acts as an inhibiting factor to this cell line. This is certainly a positive result: in fact, such characteristics offer the possibility that this material could favour direct anchorage of an eventual prosthesis to bone tissue without the interposition of fibrous connective tissue, as shown by data obtained by various authors[8,12–15].

In vivo *studies*

Samples of the short-term experimental protocol (30 d) were examined by light microscopy; the occlusion of the gap between the cortex and the implant by the formation of new bone tissue directly in contact with the Biovetro coating without interposition of fibrous tissue was noted. At the level of the medullary canal there is formation of a shell of new bone tissue around the Biovetro-coated implant, as already described in other studies[9].

The preparations obtained from samples of the experimental protocol after 3 and 6 month were studied under light microscopy and with X–ray microanalysis. Such studies confirmed the presence of new osteogenesis with bone remodelling activities at the level of both the cortex and the medullary canal (*Figure 7*) where the thickness of newly deposited bone is greater than was observed after 1 month. In particular, the X-ray microanalysis (*Figure 8*) shows the presence of silicon as a residue of the Biovetro coating after 3 month, confirming the observation made with the light microscope, i.e. the presence of a glass layer between the bone and the metal.

Observations made at 6 month show the bone tissue directly in contact with the metal support without interposition of any other material. At the level of the medullary canal (*Figure 9*) the growth of new bone tissue was so extensive as to fill in the whole cavity and to establish good contact with the metal. The light microscope results were confirmed by studies made with the electronic microprobe. *Figure 10* shows the disappearance of Si, indicating the presence of coating glass material, from between the bone tissue and the metal, and the reappearance of Ca and P, components of the new bone formation.

CONCLUSIONS

Following the spraying process, a Biovetro coating on a plate of $TiAl_6V_4$ (as evidenced by chemical and physical analyses) maintains the properties of the original glass unchanged as far as the amorphous structure and its behaviour in a hydrolytic environment are concerned.

In vitro and *in vivo* studies underline the positive features of the coating obtained by the plasma spray process, conforming that it has the typical properties of Biovetro, i.e. biodegradability and osteoconductiv-

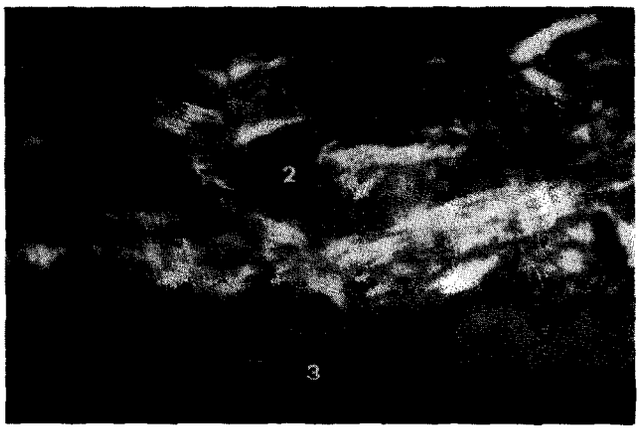

Figure 7 Light micrograph of implanted region after 3 month *in vivo*: **2**, medullary canal: the thickness of newly deposited bone is greater than that observed after 1 month; **3**, metal with coating (original magnification, ×40).

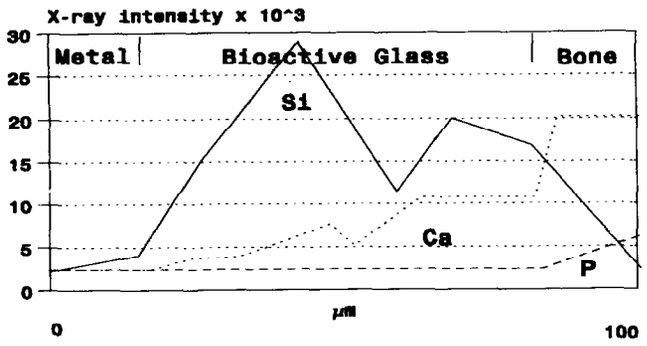

Figure 8 Mapping of X-ray scanning results for Si, Ca and P obtained by the electronic microprobe (after 3 month).

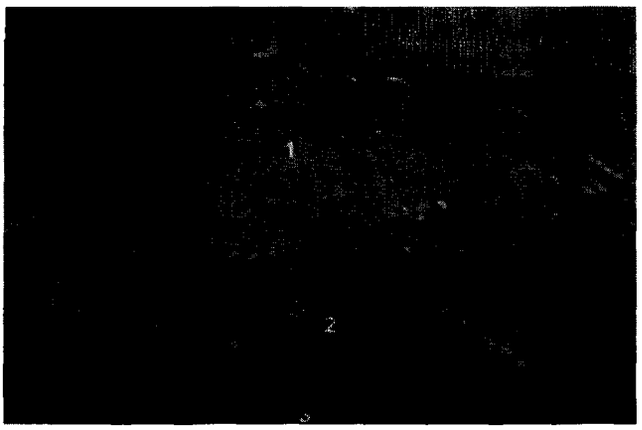

Figure 9 Light micrograph of implanted region after 6 month *in vivo*: **1,** cortical tissue; **2,** medullary canal: new bone tissue is directly in contact with the metal support; **3,** metal. (Original magnification, ×25.)

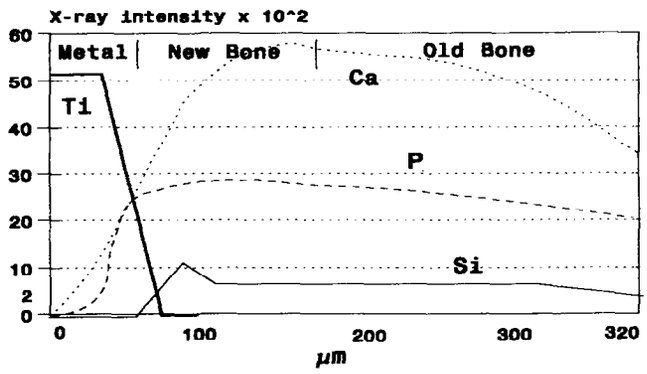

Figure 10 Mapping of X-ray scanning results for Si, Ca and P obtained by the electronic microprobe (after 6 month).

ity, already confirmed by previous experimental protocols carried out by our group using powdered and fibre Biovetro. The properties of such material lead, with time, to the reabsorption of the Biovetro layer, which is replaced by newly formed bone thus preventing fibrous tissue from filling the gap between the implant and the bone tissue which is present at the time of implantation.

This confirms that the material we used, Biovetro, is bioresorbable and osteoconductive.

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