J Mater Sci: Mater Med (2007) 18:2371–2376
DOI 10.1007/s10856-007-3153-z

In vivo evaluation of titanium implants coated with bioactive glass by pulsed laser deposition

Jacinto P. Borrajo · Julia Serra · Pío. González · Betty León · Fernando M. Muñoz · M. López

Received: 16 December 2005 / Accepted: 21 August 2006 / Published online: 14 June 2007

Abstract During the past years, different techniques, like chemical treatment, plasma spraying, sputtering, enamelling or sol–gel; and materials, like metals, hydroxylapatite, calcium phosphates, among others, have been applied in different combinations to improve the performance of prostheses. Among the techniques, Pulsed Laser Deposition (PLD) is very promising to produce coatings of bioactive glass on any metal alloy used as implant. In this work the biocompatibility of PLD coatings deposited on titanium substrates was examined by implantation in vivo. Different coating compositions were checked to find the most bioactive that was then applied on titanium and implanted into paravertebral muscle of rabbit.

Introduction

The main challenge of the implant technology is the development of load-bearing devices with adequate mechanical properties as well as improved fixation, resulting in the firm attachment of surrounding tissues, and avoiding the loosening of the implants and subsequent revision surgery. Titanium and its alloys have arisen as adequate materials that fulfil those requirements, i.e. good mechanical strength and bioinertness when implanted. Moreover, their surface can be modified to improve fixation by direct bonding with bone, thus, leading to cementless prosthesis. Chemical treatments have been applied to induce the formation of bioactive apatite nucleation points on the titanium surface [1–3], but the most extended method clinically used to enhance the adhesion of implants are the application of bioactive ceramic coatings on their surface [4–7]. These coatings provide the interface to which leaving tissues can attach avoiding the formation of a fibrous capsule that provokes the loosening of the implant. Several materials, e.g. bioactive glasses [8–10] and hydroxylapatite [5–7], and techniques, like sputtering [11, 12], enameling [13, 14], electrophoretic deposition [15, 16] or sol–gel [17, 18], have been used to produce bioactive coatings. Among them, Pulsed Laser Deposition (PLD) of bioactive glass has arisen as a very interesting alternative to coat metallic substrates for implantation [19–24] due to the high adhesion of the coatings to the metallic substrates and absence of contamination and porosity [21]. The bioactive behaviour of the coating is guaranteed due to the ability of this method to transfer the bulk glass composition to the coating [19–22, 24]. This fact makes the coating to behave as a bulk bioactive glass and reproduce, in physiological environments or fluids that mimic the human plasma composition, the same sequence of surface reactions that end with the formation of an apatite film on top of a SiO_2-rich layer. With enough time, the amorphous apatite layer (CaP) forms a crystalline hydroxylapatite to which living tissues can bond directly [25]. Moreover, the bioactive glass coatings produced by PLD do not elicit any cytotoxic reaction on cell cultures in vitro, as it was demonstrated in previous works [26–28]. The coatings (30 μm thick) were

J. P. Borrajo (✉) · J. Serra · Pío.González · B. León
Departamento de Física Aplicada, ETSE Industriais e Minas, Lagoas-Marcosende, Universidade de Vigo, Vigo 36200, Spain
e-mail: jpb@uvigo.es

F. M. Muñoz · M. López
Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, Lugo, Spain

deposited on Ti6Al4V alloy by PLD and evaluated following the norm ISO 10993 parts 5 (Test for in vitro cytotoxicity) and 12 (Sample preparation and reference materials). The cytotoxicity of MG-63 osteoblast-like cells in the presence of extracts obtained from the coated samples were compared with reference materials like Ti6Al4V alloy, bioactive glass and Thermanox (negative control) and Poly-vinyl chloride (positive control) following the MTT assay. The cellular activity of the PLD coated titanium alloy was at the same level of the Thermanox negative control, uncoated alloy and bioactive glass for different extract concentration, demonstrating the absence of any cytotoxic effect on MG-63 cells. In addition, the cells proliferated at a high rate on the coating surface forming a monolayer that totally covered it.

With these encouraging results, the next logical step was to perform an in vivo study on PLD coatings. Norm ISO 10993-6 about ''Biological Evaluation of Medical Devices. Tests for local effects after implantation'' indicate that muscle is an adequate tissue for short-term evaluation of biomedical materials. The aim of this study was the in vivo evaluation of the biocompatibility of titanium implants coated with bioactive glass produced by PLD. A previous step to select the most adequate coating for implantation, among different glass compositions, was carried out by in vitro test.

Materials and methods

Glass coatings were produced by laser ablation from bulk glasses of different compositions (Table 1). The glass targets were ablated with an ArF excimer laser (193 nm, 10 Hz, 4.2 Jcm^{-2}) and the substrates were kept at 200 °C. A detailed description of the experimental system can be found elsewhere [22].

Film bioactivity has been assessed by immersion of the samples in Simulated Body Fluid (SBF) [29] for 72 h at 37.0 ± 0.5 °C. The sample surface area to SBF volume ratio (SA/V) was kept at 0.5 cm^{-1}. The composition and morphology of the bioactive layers formed after the immersion was followed by Scanning Electron Microscopy (Philips XL30 and JEOL JSM6700F) and Energy Dispersive X-ray Spectroscopy (EDAX Dx4 and Oxford INCA Energy 300 SEM).

Concerning the in vivo study, the International Organization for Standardization (ISO) test 10993-6, 'Biological Evaluation of Medical Devices-Part 6: Tests for local effects after implantation', was followed in this test protocol. Coated Ti cylinders were implanted bilaterally into paravertebral muscles of 12 New Zealand white female adult rabbits. The animals were divided into three implantation periods (1, 4, and 12 weeks). A maximum of three samples were placed into the paravertebral muscles on each side of the spine of each rabbit. Three animals were used in the 1-week and 4-week study, and six in the 12-week study, providing 8 replicates at 1-week and 4-week, and 15 replicates at 12-week point. All implants were sterilized before implantation using gamma radiation. The placement of the implants was carried out according to the ISO 10993-6: 1994 guidelines. The rabbits were anaesthetized using intramuscular injections of ketamine and xylazyne. An area of skin was exposed by removal of hair from the dorsa-lumbar region of each rabbit. Implantation of the samples was achieved by intramuscular injection under aseptic conditions. Implants for the right paravertebral muscles were coated with bioactive glass and implants for the left side were uncoated Ti cylinders to serve as controls. The rabbits were housed individually in standard rabbit cages with an ambient temperature of 24–26 °C. They had 12-h light/12-h dark cycles, and they were fed standard rabbit chow and water ad libitum. The animals were euthanized after 1, 4, and 12 weeks.

For histological examination, the implants were removed together with the surrounding muscle and fixated in 10% neutral buffered formalin. The specimens were dehydrated in a graded series of alcohol, infiltrated and embedded with Technovit 7200 VLC (Heraeus Kultzer, Dormagen, Germany) and processed by the cutting–grinding method [30]. Final sections were ground down to the approximate thickness of 30 μm and stained with toluidine blue staining technique. Thickness of the fibrous tissue covering implants was calculated using a digital image analysis system (Olympus DP12 and Microimage 6.0, Media Cybernetics, Maryland USA).

Table 1 Composition of the bioactive glasses (wt%)

Glass	SiO_2	Na_2O	K_2O	CaO	MgO	P_2O_5	B_2O_3
BG42	42	20	10	20	5	3	–
BG50	50	15	15	15	2	–	3
BG55	55	21	9	8	2	4	1
BG59	59	10	5	15	5	3	3

Results and discussion

Figure 1 shows the bioactive products of the surface reactions that occurred on a bioactive glass coating after immersion in SBF. The formation of both SiO_2-rich and a CaP layers was observed, which is a clear indication of the bioactivity of the coating [25, 29]. A detailed EDS analysis revealed three layers with different composition. On the top, appeared a layer with a globular structure composed mainly of calcium and phosphorous that was identified as the CaP layer. A new layer type was found immediately under the CaP film composed mainly of a mixture of calcium, phosphorous, silicon and oxygen, the so-called CaP–SiO_2 mixed layer. Finally, the SiO_2-rich layer was located between the CaP–SiO_2 mixed layer and the substrate surface. The presence of the bioactive layers on the glassy coatings indicates identical evolution of the surface reactions as in bulk bioactive glasses.

As shown in Fig. 2, the thickness of the bioactive products, i.e. SiO_2-rich and CaP layers is highly dependent on the content of network modifiers, i.e. alkali and alkali-earth ions, that the original glass has in its composition [25, 31]. For coatings produced from glasses ranging from 42 to 50% (wt%) of silica content (BG42), an excellent bioactive behavior was found. The thickest SiO_2-rich and CaP layers were obtained and no traces of the mixed layer were observed. When the composition of the silica content ranges from 50 to 55% (wt%) (BG50) the bioactive behavior is still found. However, the thickness of the CaP layer decreases dramatically and the mixed layer appears between CaP and SiO_2-rich layers. For higher amounts of silica (BG55 coating), the bioactive process is not completed and only the mixed layer and the SiO_2-rich layer are obtained, being the thickness of the last lower than for smaller contents of silica in the glass composition. If the amount of silica increases to higher levels (BG59, Table 1) the coating looses its reactivity and only limited dissolution of the surface is found. It can be concluded that the higher the concentration of modifier ions on the glass compositions (lower silica content) the thickness of the bioactive layers increases [31].

Once BG42 coating was selected as the most bioactive coating, the main objective of this work was to study its biocompatibility in vivo. The SEM-EDS evaluation of the

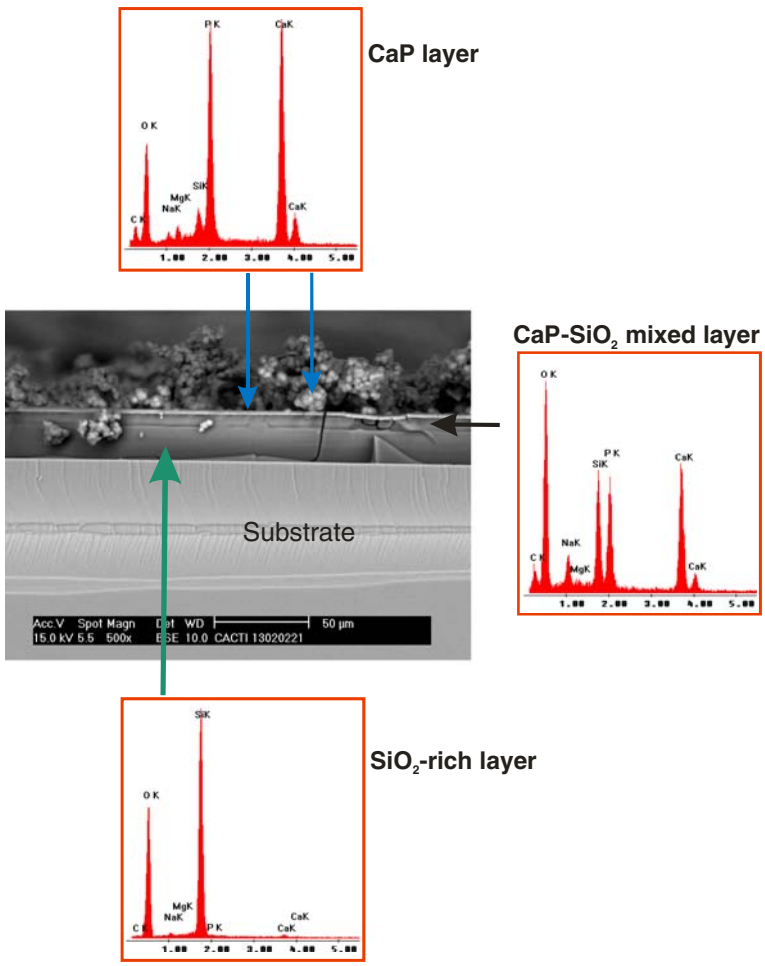

Fig. 1 Typical cross-section micrograph of the BG50 glass coating after immersion in SBF for 72 h at 37.0 ± 0.5 °C

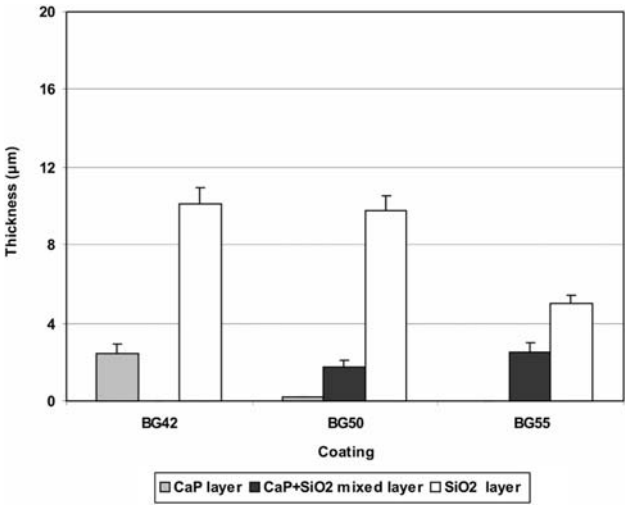

Fig. 2 Thickness of the bioactive products obtained after immersion of the glassy coatings in SBF for 72 h

coated titanium-living tissue interface revealed that when the coatings are implanted in vivo they develop the same reaction pattern as in vitro (Fig. 3). The bioactive glassy coating evolves towards the formation of the SiO_2-rich layer and a mixed CaP–SiO_2 film appears between the SiO_2-rich layer and the living tissue. The mixed layer increases its content on calcium and phosphorous elements when we move from the SiO_2-rich layer to the living tissue interface, which is an indication of the direct contact of an apatite-like layer generated by the coating and the living tissue.

Histologically there was no evidence of an adverse tissue reaction in any of the sections according to the standard UNE-EN ISO 10993-6: 1994. No significant inflammatory response, necrosis, granuloma formation or soft tissue calcification around implants could be observed in both groups, demonstrating the biocompatibility of the glass coating surface. Around all implanted materials, light microscopy highlighted a reactive and fibrous capsule formation that could be caused by surgical insertion of materials and handling of the muscular tissue. The capsule was composed of layers of cells, mainly fibroblasts, and collagen fibers (Fig. 4). At 1 week after implantation, histomorphometric measurements revealed a statistically significant stronger response, in terms of capsule thickness, surrounding Ti implants than bioglass coated implants. In both, coated and control implants, a progressive decrease in capsule thickness was seen in rabbits sacrificed 4 and 12 weeks after implantation. For the 4-week group the capsule thickness decreased sharply, but from 4 to 12 weeks, the thickness decreased slightly (Fig. 5). Comparisons of the groups didn't reveal statistically significant differences among coated and control implants at these longer implantation times.

Histological and morphologic in vivo examinations are well-accepted methods to assess biocompatibility. The accepted international standard for biocompatibility testing is ISO 10993, which is the standard used in this study with

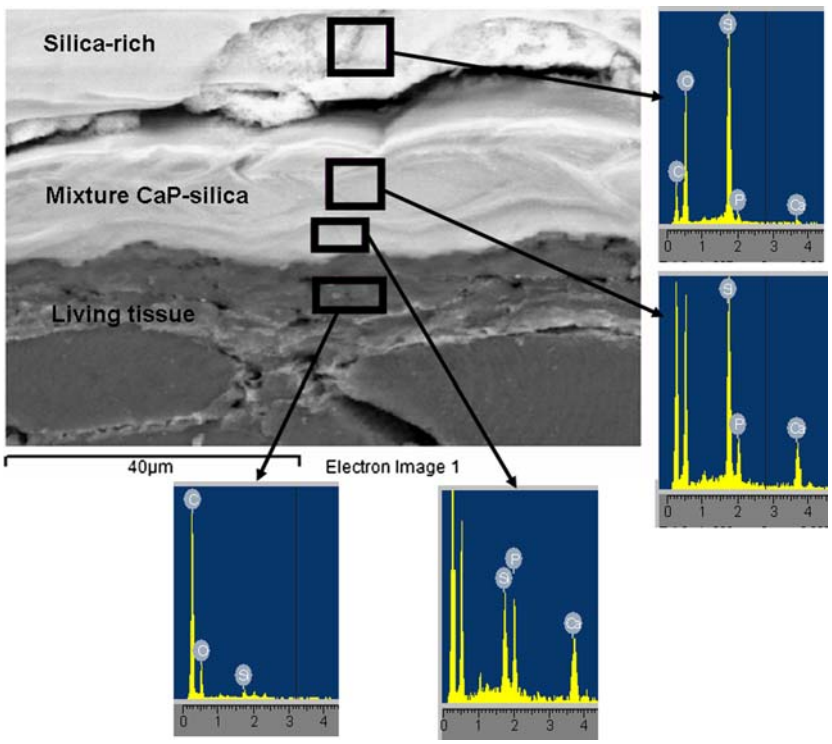

Fig. 3 SEM micrographs and EDS spectra obtained for a titanium cylinder coated with BG42 glass after 12 weeks of implantation in paravertebral muscle of rabbits

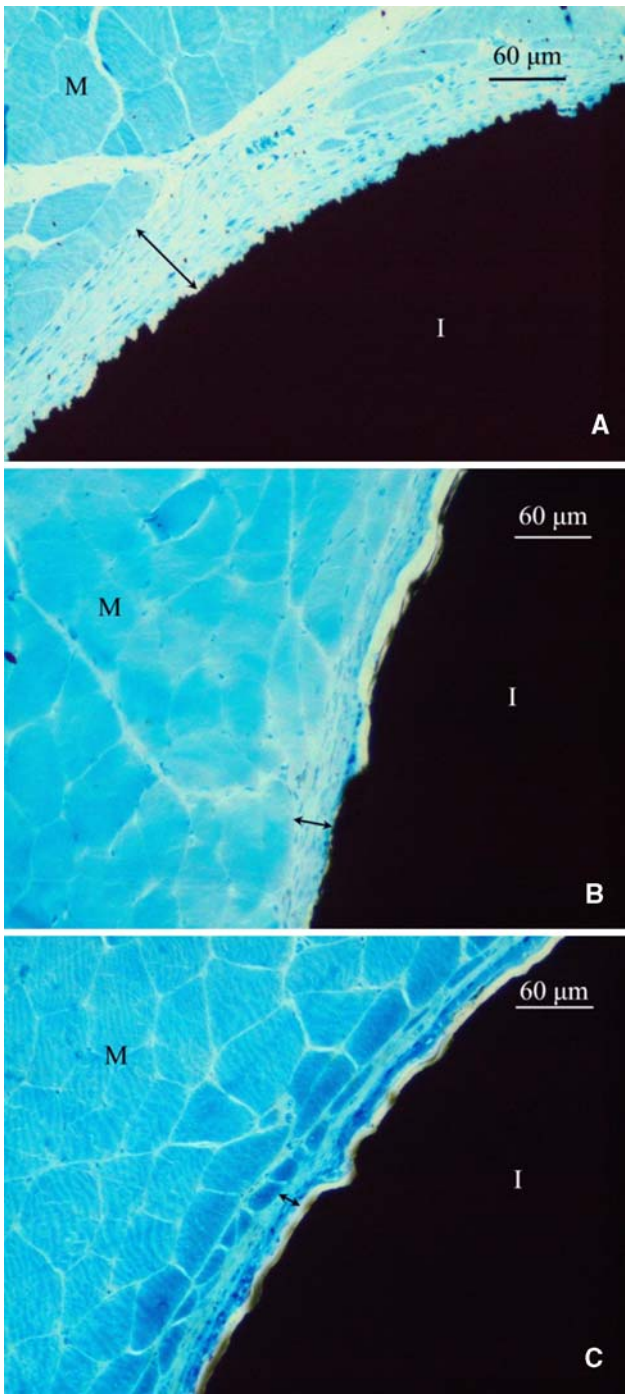

Fig. 4 Histological images obtained from bioactive glass coated titanium implant for implantation times of 1 week (**A**), 4 weeks (**B**) and 12 weeks (**C**). Arrows show the thickness of the fibrous tissue. Absence of an adverse tissue reaction and a progressive decrease of capsule thickness can be observed. M: muscle. I: implant

respect to the type of animal used, study period, implant site, surgical procedure and histologic evaluation. Measuring the capsule membrane thickness around the implant is a basic and important tool for estimating biocompatibility. In

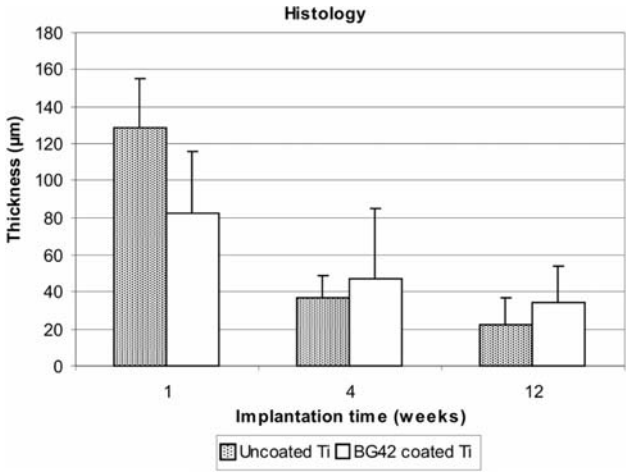

Fig. 5 Thickness of the fibrous tissue for titanium (dark bars) and glass coated titanium (white bars) samples

our study, for short implantation time (1 week), beneficial effects of the bioactive glass coating was pointed out by the statistically significant reduction of the fibrous tissue thickness. For a longer implantation times no statistically significant differences were detected.

Conclusion

The bioactivity of different glassy coatings produced by PLD was evaluated by immersion in simulated body fluid. The most bioactive coating was used to produce films on titanium metal and the resulting material was implanted in paravertebral muscle of rabbit.

The most bioactive coatings are produced from glasses with the highest amount of modifier ions. Moreover, their biocompatibility in muscle tissue is corroborated because they do not elicit any inflammatory response of surrounding tissues, but to ensure the fact of total compatibility with other tissues, new works with different tissues need to be performed.

References

1. H.-M. KIM, F. MIYAJI, T. KOKUBO and T. NAKAMURA, *J. Biomed. Mater. Res.* **32** (1996) 409
2. S. NISHIGUCHI, H. KATO, M. NEO, M. OKA, H.-M. KIM and T. KOKUBO, *J. Biomed. Mater. Res.* **54** (2001) 198
3. H. B. WEN, J. R. de WIJN, F. Z. CUI and K. de GROOT, *Biomaterials* **19** (1998) 215
4. J. DUMBLETON and M. T. MANLEY, *J. Bone Joint Surg. Am.* **86** (2004) 2526
5. R. G. T. GEESINK, *Clin. Orthop. Relat. Res.* **395** (2002) 53
6. Y. C. TSUI, C. DOYLE and T. W. CLYNE, *Biomaterials* **19** (1998) 2015

7. W. SUCHANEK and M. YOSHIMURA, *J. Mater. Res.* **13** (1998) 94
8. L. L. Hench and Ö. Andersson, An Introduction to Bioceramics, edited by L. L. HENCH and J. WILSON (World Scientific: Singapore, 1993) p. 239
9. C. GABBI, A. CACCHIOLI, B. LOCARDI and E. GUADAGNINO, *Biomaterials* **16** (1995) 515
10. E. VERNÉ, M. FERRARIS, A. VENTRELLA, L. PARACCHINI and A. KRAJEWSKI, *J. Eur. Ceram. Soc.* **18** (1998) 363
11. C. X. WANG, Z. Q. CHEN and M. WANG, *J. Mater. Sci. Mater. Med.* **13** (2002) 247
12. Y. YANG, K.-H. KIM and J. L. ONG, *Biomaterials* **26** (2005) 327
13. P. TORRICELLI, E. VERNE, C. VITALE BROVARONE, P. APPENDINO, F. RUSTICHELLI, A. KRAJEWSKI, A. RAVAGLIOLI, G. PIERINI, M. FINI, G. GIAVARESI and R. GIARDINO, *Biomaterials* **22** (2001) 2535
14. T. KASUGA, T. MIZUNO, M. WATANABE, M. NOGAMI and M. NIIMONI, *Biomaterials* **22** (2001) 577
15. Y. HAN, K. XU and J. LU, *J. Mater. Sci. Mater. Med.* **10** (1999) 243
16. L. A. de SENA, M. C. de ANDRADE, A. M. ROSSI and G. de ALMEIDA SOARES, *J. Biomed. Mater. Res.* **60** (2002) 1
17. M. HAMADOUCHE, A. MEUNIER, D. C. GREENSPAN, C. BLANCHAT, J. P. ZHONG, G. P. LA TORRE and L. SEDEL, *J. Biomed. Mater. Res.* **52** (2000) 422
18. J. BALLARRE, J. C. ORELLANO, C. BORDENAVE, P. GALLIANO and S. CERÉ, *J. Non-Cryst. Solids* **304** (2002) 378
19. L. D'ALESSIO, R. TEGHIL, M. ZACCAGNINO, I. ZACCARDO, D. FERRO and V. MAROTTA, *Appl. Surf. Sci.* **138–139** (1999) 527
20. L. D'ALESSIO, D. FERRO, V. MAROTTA, A. SANTAGATA, R. TEGHIL and M. ZACCAGNINO, *Appl. Surf. Sci* **183** (2001) 10
21. R. TEGHIL, L. D'ALESSIO, D. FERRO and S. M. BARINOV, *J. Mater. Sci. Lett.* **21** (2002) 379
22. J. SERRA, P. GONZÁLEZ, S. CHIUSSI, B. LEÓN and M. PÉREZ-AMOR, *Key Eng. Mater.* **192–195** (2001) 635
23. P. GONZÁLEZ, J. SERRA, S. LISTE, S. CHIUSSI, B. LEÓN and M. PÉREZ-AMOR, *Vacuum* **67** (2002) 647
24. S. LISTE, J. SERRA, P. GONZALEZ, J. P. BORRAJO, S. CHIUSSI, B. LEON and M. PEREZ-AMOR, *Thin Solid Films* **453–454** (2004) 219
25. L. L. Hench and Ö. Andersson, An Introduction to Bioceramics edited by L. L. HENCH and J. WILSON (World Scientific: Singapore, 1993) p. 41
26. A. de CARLOS, J. P. BORRAJO, J. SERRA, P. GONZÁLEZ and B. LEÓN, *J. Mater. Sci. Mater. Med.* **17** (2006) 523
27. J. P. BORRAJO, P. GONZÁLEZ, J. SERRA, S. LISTE, S. CHIUSSI, B. LEÓN, A. de CARLOS, F. M. VARELA-FERIA, J. MARTÍNEZ-FERNÁNDEZ and A. R. de ARELLANO-LÓPEZ, *Mater. Sci. Forum* **514–516** (2006) 970
28. S. Liste. Recubrimientos basados en vidrios bioactivos producidos por ablación láser. Thesis dissertation. University of Vigo (2004)
29. T. KOKUBO, H.-M. KIM and M. KAWASHITA, *Biomaterials* **24** (2003) 2161
30. K. DONATH and G. BREUNER, *J. Oral Pathol.* **11** (1982) 318
31. J. SERRA, P. GONZÁLEZ, S. LISTE, S. CHIUSSI, B. LEÓN, M. PÉREZ-AMOR, H. O. YLÄNEN and M. HUPA, *J. Mater. Sci. Mater. Med.* **13** (2002) 1221