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In vitro mechanical and biological assessment of hydroxyapatite-reinforced polyethylene composite

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In vitro performance of hydroxyapatite (HA)-reinforced polyethylene (PE) composite (HAPEX™) has been characterized from both mechanical and biological aspects. The mechanical properties of HAPEX™, such as tensile strength and Young's modulus, showed little change after immersion in a physiological solution at 37 and 70 °C for various periods. In addition, the biological response of primary human osteoblast-like (HOB) cells *in vitro* on HAPEX™ was assessed by measuring DNA synthesis and osteoblast phenotype expression. Cell proliferation rate on HAPEX™ was demonstrated by an increase in DNA content with time. A high tritiated thymidine ([^{3}H]-TdR) incorporation/DNA rate was observed on day 1 for HAPEX™, indicating a stimulatory effect on cell proliferation. The alkaline phosphatase (ALP) activity was expressed earlier on HAPEX™ than on unfilled PE and increased with time, indicating that HOB cells had commenced differentiation. Furthermore, it was found that the HA particles in the composite provided favourable sites for cell attachment. It appears that the presence of HA particles in HAPEX™ may have the advantage of acting as microanchors for bone bonding *in vivo*.

1. Introduction

Bioactive ceramics with their bone-bonding ability have revolutionized the concept of biomaterials. However, their relatively poor mechanical properties have restricted potential clinical applications [1]. A composite material, incorporating bioactive hydroxyapatite (HA) ceramic particles into high-density polyethylene (HDPE) matrix, HAPEX™, has been developed with optimal stiffness, toughness and bioactivity [2, 3]. This type of composite is able to fulfil the mechanical and biological requirements for an implant material, which make it a successful orbital floor implant [4, 5].

The effects of various HA fillers and PE matrices and different processing routes on the properties of the composite have been investigated [6–10]. As an implant material, the composite will contact physiological fluids in service. Thus the changes in the composite in a physiological solution at 37 °C need to be evaluated. An *in vitro* simulation of mechanical properties of the composite has been established. As water absorption is a major deteriorating factor on the properties of a composite in solution [11–13], accelerated ageing tests have been performed at 70 °C for various periods to predict the long-term stability of the composite.

HAPEX™ has been shown to encourage bone formation *in vivo* [14], but the complexities associated with *in vivo* studies make it difficult to examine the specific interactions of various cells with the implant. *In vitro* cell culture models, allowing the biological assessment of materials at a cellular level, are helpful in evaluating specific cell response. Several models using animal or human osteoblast-like cells or stromal cells have been developed for studying the *in vitro* response of the cells to various biomaterials [15–19]. These cell culture models, with their limited variables, allow more direct observation of the cell–biomaterial interaction. In this work, a primary human osteoblast-like (HOB) cell model, a representative of the cell type that the material surface will contact *in vivo*, has been used to study the cell response to HAPEX™. The knowledge obtained will further aid in designing second generation implant materials to promote bone bonding.

2. Materials and methods

HAPEX™ was produced by incorporation of HA (Plasma Biotal, P88) particles into HDPE (BP Rigidex) through twin-screw extrusion and compression moulding [10]. The microstructure has been

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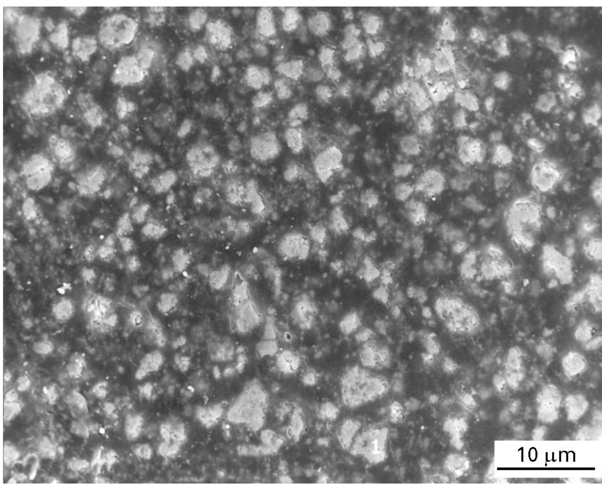


Figure 1 The uniform dispersion of HA particles (white) in the PE matrix (dark) on the polished surface of HAPEX™.

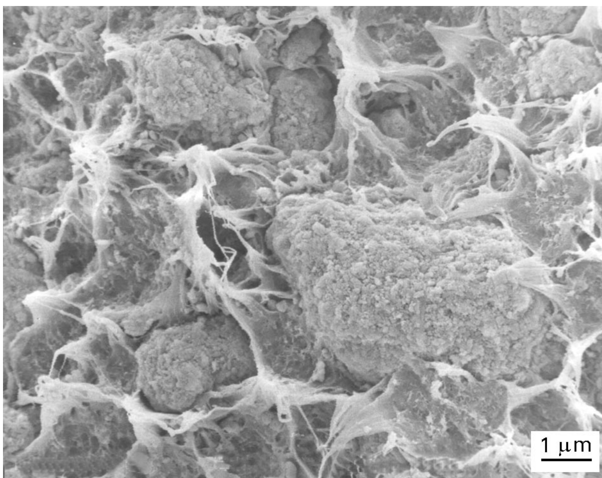


Figure 2 The accumulation of crystallites within individual HA particles from cryofracture surface, indicating the increase in surface area of HAPEX™ with the presence of HA particles.

characterized by X-ray diffraction, infrared spectroscopy and electron microscopy (Figs 1 and 2). Tensile test specimens (ISO 527) were annealed at 80 °C for 24 h before testing to eliminate the effects of thermal history. Specimens of 40 vol% HAPEX™ (HAPEX) were sterilized by gamma irradiation at a dose of 2.5 Mrad (Isotron, UK) using standard procedures for medical devices. Simulated body fluid (SBF K9) was used to immerse the specimens, as it closely resembles the inorganic ions concentrations in blood plasma [20]. The specimens were immersed at 37 and 70 °C for 1 and 3 months.

2.1. Water absorption

The water absorption of HAPEX™ in SBF was estimated by measuring weight changes. Samples were removed from the solution, excess water was wiped off with a tissue and the weight was recorded using an analytical balance. The weight change $\Delta W\%$, is given by

$$\Delta W\% = \frac{W_2 - W_1}{W_1} \times 100 \qquad (1)$$

where W_1 and W_2 are the weight of the specimen before and after incubation, respectively.

2.2. Tensile testing

Tensile testing of HAPEX™ was conducted on an Instron 6025 computer-controlled screw-driven mechanical testing machine at a crosshead speed of 0.5 mm min^{-1}. An extensometer was attached to the specimen gauge length to measure Young's modulus, and the tensile strength and strain to failure were also determined. The Young's modulus was calculated from the initial linear region of the stress–strain curve. The fracture surfaces of the composite were preserved and fixed on specimen holders by conductive carbon cement, and sputtered with gold before examination with a Jeol 6300 scanning electron microscope.

2.3. *In vitro* cell culture

Primary human osteoblast-like (HOB) cells, isolated from trabecular bone fragments of the femoral heads of patients undergoing hip surgery [19], were used. HOB cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, L-ascorbic acid (150 g ml^{-1}), 0.02 M L-glutamine, 0.01 M HEPES, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Thermanox (TMX) and unfilled PE were used as controls. HOB cells were seeded on the test materials at a density of 1×10^6 cells ml^{-1} with great care to avoid unwanted cell attachment to the surrounding surface of culture dish, and were allowed to attach for 1 h prior to flooding with 1 ml DMEM medium. The cultures were incubated at 37 °C in humidified air with 5% CO_2 for 1, 4, 7, 14 and 21 days. The culture medium was replaced carefully, at appropriate time intervals, in order to minimize disturbance of the culture conditions.

2.4. Cell growth and proliferation

The growth and proliferation of the HOB cells on the materials were determined by measuring tritiated thymidine ([^{3}H]-TdR) incorporation and total DNA content. At each time point, the cells were incubated in the presence of 1 μCi ml^{-1} of [^{3}H]-TdR (Amersham International, UK) for the final 16 h culture. Then the cells were enzymatically lysed using a 0.1% papain digest solution (Sigma, UK). Total cellular DNA was measured using the Hoechst method [21]. Hoechst 33285, a DNA specific dye, was reacted with the papain-digested cell suspension or DNA standard with concentrations of 0, 0.31, 0.62, 1.25, 2.5, 5, 10 and 20 μg ml^{-1} in saline sodium citrate buffer (pH = 7.0). The fluorescence was measured on a Fluoroskan fluorimeter at an excitation wavelength of 355 nm and an emission wavelength of 450 nm. The total DNA content for each test specimen was calculated from the standard DNA curve.

[^{3}H]-TdR incorporation was measured by trichloroacetic acid (TCA) precipitation of the cell digests. The precipitate was vacuum filtered on to a porous

membrane. Unprecipitated lysate containing the excess unbound radionucleotide was washed through the membrane with 10% TCA, the precipitate was then dissolved in 0.01 M KOH solution. The amount of radiolabel incorporated was measured using a scintillation counter.

2.5. Osteoblast phenotype expression

Alkaline phosphatase (ALP) activity of HOB cells, as an indicator of osteoblast phenotype activity, was determined biochemically. After the culture time, a freeze–thaw method was used to lyse the cells and release their content. This procedure was performed by freezing at −70 °C for 15 min and thawing at 37 °C for 20 min three times. The ALP activity was determined using a COBAS-BIO (Roche, UK) centrifugal analyser. *P*-nitro phenol phosphate in diethanolamine buffer (Merck, UK) was the substrate used. The enzyme alkaline phosphatase, cleaves the phosphate group from *p*-nitro phenol phosphate to produce *p*-nitrophenol which is yellow at alkaline pH (9.8), and is monitored at the wavelength of 405 nm.

2.6. Cell morphology

Test specimens were seeded with a density of approximately 8×10^5 HOB cells ml^{-1} and incubated at 37 °C in a humidified air with 5% CO_2. After 24 h incubation, the cultures were fixed with 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate, stained in 1% osmium tetroxide and 1% tannic acid buffer. The samples were dehydrated using a series of alcohol solutions, from 20%, 30%, 40%, 50%, 60% to 70%, then stained in 0.5% uranyl acetate and further dehydrated in 90%, 96%, 100% ethyl alcohol (containing Na_2SO_4), then dehydrated with hexamethyl-disilazane, and finally air dried overnight. The cultures were coated with a thin layer of gold before examination under a Joel scanning electron microscope at an accelerating voltage of 15 keV.

3. Results

3.1. Water absorption

No measurable dimensional change was seen for HAPEX™ after immersion in SBF. The weight of HAPEX™ increased with incubation time and temperature, but the increases were only 0.8% and 1.2% after 3 months incubation at 37 and 70 °C, respectively, indicating the low degree of water absorption by HAPEX™.

3.2. Mechanical properties

The mechanical properties of HAPEX™ following incubation at 37 and 70 °C for 1 and 3 months are shown in Fig. 3. It appeared that the tensile strength of the composite decreased with immersion time and that the decrease at 70 °C was greater than that at 37 °C, but neither decrease was statistically significant. The Young's modulus and fracture strain of HAPEX™ did not significantly alter at either temperature for 1 and 3 months. In addition, no evidence of degradation of HA particles was observed on the fracture surface.

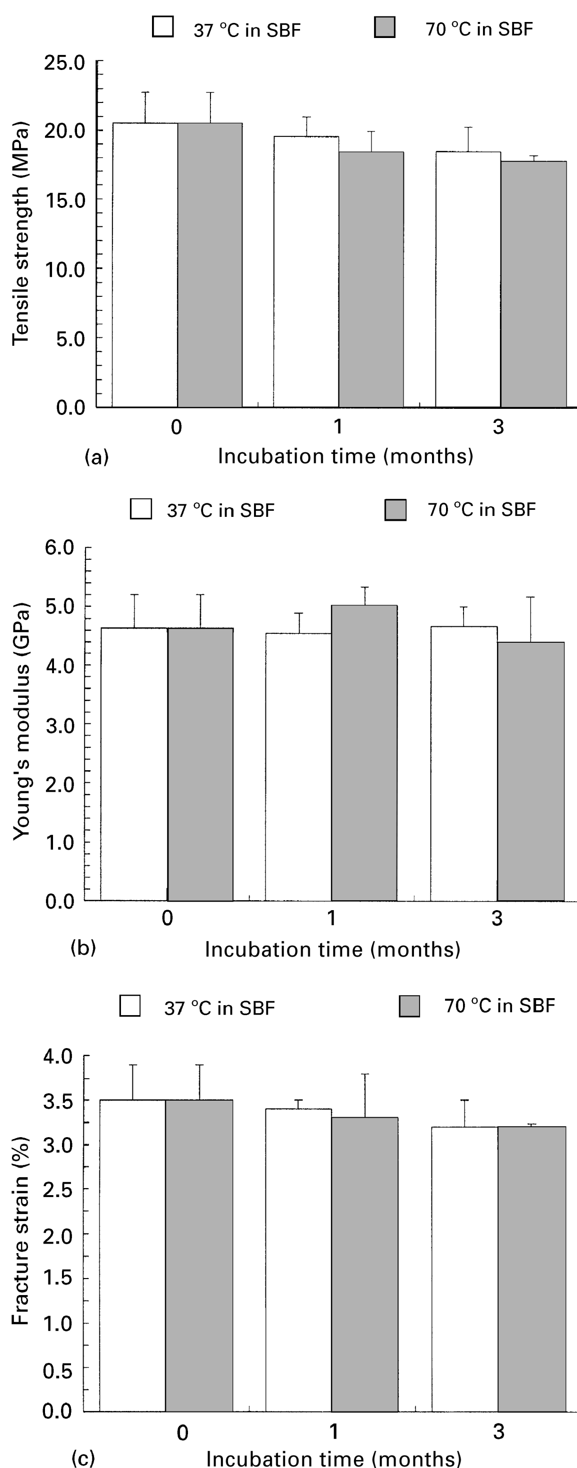


Figure 3 Comparison of the mechanical properties of HAPEX™: (a) tensile strength (b) Young's modulus and (c) strain to failure before and after immersion in SBF at 37 and 70 °C for 1 and 3 months.

3.3. Biological assessment

Continuous growth of HOB cells, expressed as the total DNA content, increased with time on HAPEX™ and PE during 21 days culture, but varying degrees of cell proliferation were observed. The highest rate of $[^3H]$-TdR incorporation was seen on day 1, when the

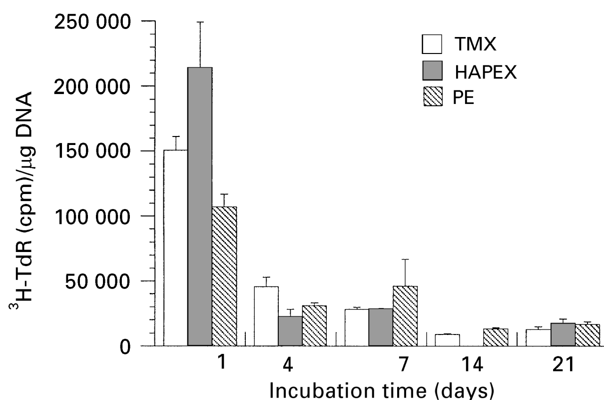


Figure 4 [^{3}H]-TdR incorporation/DNA for HOB cells on the test materials: TMX control, HAPEX™ and unfilled PE from days 1–21. The cell proliferation rate was highest on day 1, where the greatest increase was observed on HAPEX™.

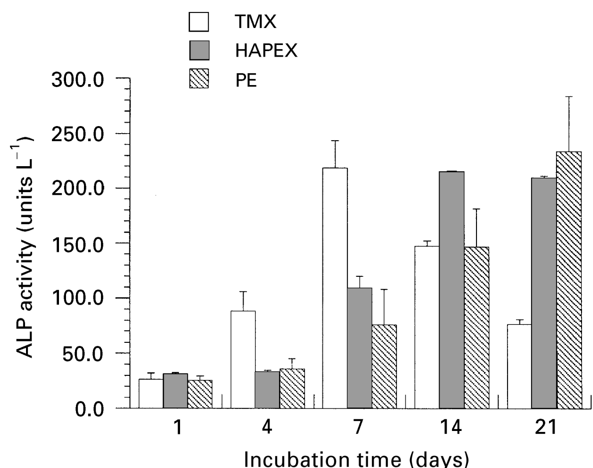
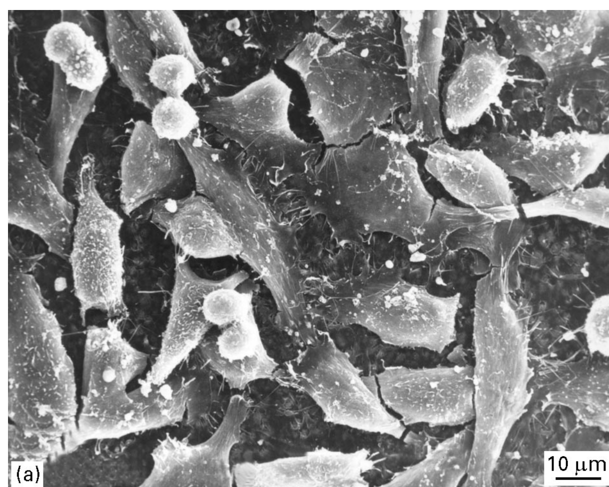


Figure 5 The ALP activity of HOB cells cultured on the test materials: TMX control, HAPEX™ and PE from days 1–21.



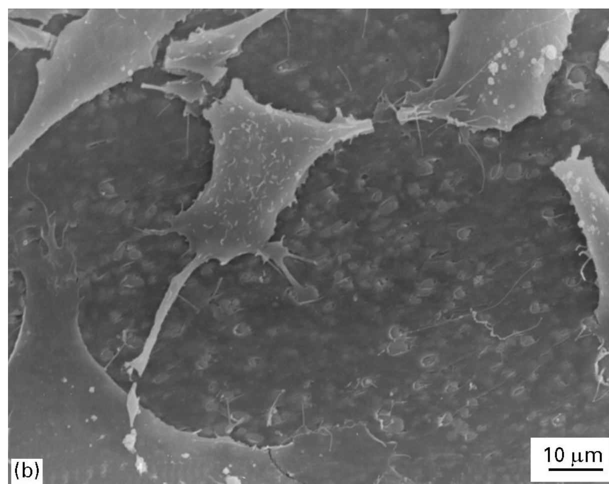


Figure 6 (a) The surface of HAPEX™ covered by a HOB cell layer after 24 h culture, and (b) the cell processes attached to HA particles.

greatest proliferation of HOB cells was found on HAPEX™ (Fig. 4). Following this initial rise in proliferation rate, a fall in cell proliferation was observed. As cell proliferation decreased, the ALP activity increased, indicating that the osteoblasts were in a state of differentiation. This process occurred more rapidly on HAPEX™ than on PE (Fig. 5). The peak ALP activity was seen on day 7 for TMX and at day 14 on HAPEX™, while a continuous increase in the ALP activity was observed on PE up to day 21.

Fewer cells adhered to the surface of PE, indicating that PE surface was not a favourable surface for HOB cell attachment and proliferation. In comparison, many cells were seen to attach on the surface of HAPEX™, a cell layer was observed covering the entire surface and cells at various stages of division were often observed (Fig. 6a). The cells appeared flattened and maintained the polygonal morphology of osteoblasts, with filapodia attached to HA particles (Fig. 6b).

4. Discussion

Bioactivity can be retained in a composite which incorporates a bioactive phase. However, the influence of the bioactive phase on the mechanical properties of the composite, particularly, those properties when the composite is in contact with body fluid, need to be considered carefully, as the long-term stability of an implant will determine its outcome, unless the implant is specifically designed to be biodegradable. The results from this study show that by careful selection of the HA filler in the composite, the tensile strength, Young's modulus and strain to fracture of HAPEX™ remained unchanged following incubation at 37 °C and accelerated ageing at 70 °C. No degradation in the properties of HAPEX™ ensures its implantation success and makes it a highly suitable implant material.

Biocompatibility of a material is considered to be its ability to perform with an appropriate host response in a specific application [22]. *In vitro* cell culture studies using the "appropriate cells", such as the HOB cells used in this study, allow the investigation of the interfacial response of the implant material in relation to its final use. The biocompatibility of HAPEX™ was demonstrated by the ability of the cells to proliferate on the material. The rapid cell attachment was followed by proliferation and differentiation of the cells. The high [^{3}H]-TdR incorporation/DNA observed on HAPEX™ on day 1 indicated an immediate stimulatory effect. When considering osteoblast expression and hence ALP activity, a greater activity was observed in those HOB cells on HAPEX™ as compared to those on unfilled PE. The increase in ALP activity

indicated that the cells had ceased to proliferate and had begun to differentiate. This process occurred earlier on HAPEX™ than on PE. Another striking feature frequently observed was that cell filapodia were embedded within HA particles on the surface of HAPEX™. It appeared that HAPEX™ provided preferential sites for cell attachment and stimulated the overall HOB cell activity. The stimulatory effect might be caused by the presence of HA particles which provide a large surface area (Fig. 2) for the absorption of proteins and growth factors, which in turn may enhance cellular activity. Therefore, HA particles in HAPEX™ may act as microanchors for developing direct bone bonding *in situ*.

5. Conclusions

The mechanical properties of HAPEX™ were not altered by contact with physiological solution. Furthermore, HAPEX™ provided a favourable environment for HOB cell attachment, proliferation and differentiation. A combination of these properties will lead to HAPEX™ having the long lifetime required for permanent implants, which make it a highly suitable second generation implant material.

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References

1. L. L. HENCH, *J. Amer. Ceram. Soc.* **74** (1991) 1487.
2. W. BONFIELD, M. D. GRYNPAS, A. E. TULLY, J. BOWMANN and J. ABRAM, *Biomaterials* **2** (1981) 185.
3. W. BONFIELD, in "Annals of New York Academy of Sciences", Vol. 523, edited by P. Ducheyne and J. E. Lemons (New York Academy of Science, New York, 1988) p. 173.
4. R. N. DOWNES, S. VARDY, K. E. TANNER and W. BONFIELD, *Bioceramics* **4** (1991) 239.
5. K. E. TANNER, R. N. DOWNES and W. BONFIELD, *Br. Ceram. Trans.* **93** (1994) 104.
6. J. ABRAM, J. BOWMAN, J. C. BEHIRI and W. BONFIELD, *Plast. Rubb. Process. Applic.* **4** (1984) 261.
7. W. BONFIELD, J. C. BEHIRI, C. DOYLE, J. BOWMAN and J. ABRAM, in "Biomaterials and Biomechanics", edited by P. Ducheyne, G. Van der Perre and A. E. Aubert (Elsevier, Amsterdam, 1983) p. 421.
8. G. P. EVANS, J. C. BEHIRI, J. D. CURREY, W. BONFIELD, *J. Mater. Sci. Mater. Med.* **1** (1990) 38.
9. K. HEMACHANDRA, PhD thesis, University of London (1992).
10. M. WANG, D. PORTER and W. BONFIELD, *Br. Ceram. Trans.* **93** (1994) 91.
11. P. S. THEOCARIS, G. C. PAPANOCOLAOU and E. A. KONTOU, *J. Polym. Sci.* **28** (1983) 3145.
12. G. C. PAPANOCOLAOU and R. MERCOGLIANO, *Plast. Rubb. Process. Applic.* **6** (1986) 229.
13. J. SUWANPRATEEB, PhD thesis, University of London (1996).
14. W. BONFIELD, C. DOYLE and K. E. TANNER, in "Biological and Biomechanical Performance of Biomaterials", edited by P. Cristel, A. Meunier and A. J. C. Lee (Elsevier, Amsterdam, 1986) p. 153.
15. Y. ITAKURA, A. KOSUGI, H. SUDO, S. YAMAMOTO and M. KUMEGAWA, *J. Biomed. Mater. Res.* **22** (1988) 613.
16. F. B. BAGAMBISA and U. JOOS, *Biomaterials* **11** (1990) 50.
17. W. C. A. VROUWENVELDER, C. G. GROOT and K. DE GROOT, *J. Biomed. Mater. Res.* **27** (1993) 465.
18. C. FAUCHEUX, R. BAREILLE, F. ROUAIS, J. AMEDEE, A. LIEBENDORFER and M. DARD, *J. Mater. Sci. Mater. Med.* **5** (1994) 635.
19. L. DI SILVIO, PhD thesis, University of London (1995).
20. T. KOKUBO, H. KUSHITANI, S. SAKKA, T. KITSUGI and T. YAMAMURO, *J. Biomed. Mater. Res.* **24** (1990) 721.
21. R. RAGO, J. MITCHEN and G. WILDING, *Anal. Biochem.* **191** (1990) 31.
22. D. F. WILLIAMS, *J. Biomed. Engng* **11** (1989) 185.

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