Phosphoserine – a convenient compound for modification of calcium phosphate bone cement collagen composites

A. REINSTORF*, M. RUHNOW, M. GELINSKY, W. POMPE
Dresden University of Technology, Department of Materials Science, Max Bergmann Center of Biomaterials, Budapester Str. 27, 01062 Dresden, Germany
E-mail: reinst@tmfs.mpgfk.tu-dresden.de

U. HEMPEL, K.-W. WENZEL
Dresden University of Technology, Institute of Physiological Chemistry, Fiedlerstr. 42, 01307 Dresden, Germany

P. SIMON
Max Planck Institute for Chemical Physics of Solids, Nöthnitzerstr. 40, 01187 Dresden, Germany

Temporary bone replacement materials on the basis of calcium phosphates and hydroxyapatite (HAP) are used in surgery for filling bone defects. Components which are able to control the nucleation and crystal growth of HAP through their functional groups and which can additionally activate bone cells may be helpful in the development of materials with enhanced remodelling *in vivo*. In this study, the influence of *O*-phospho-L-serine (PS) on the materials properties of calcium phosphate bone cement composites was investigated. For up to an addition of 25 mg/g PS a strong increase in the stability of the cements under load was determined. The material was studied by scanning electron microscopy and transmission electron microscopy. A more dense microstructure and a plate-like morphology of the HAP-crystals were detected in the modified composites compared with the non-modified samples. By X-ray powder diffraction an inhibition of the dissolution of α-tricalcium phosphate (α-TCP) and dicalciumphosphate anhydrous (DCPA) particles was found. α-TCP and DCPA are the main constituents of the cement precursor. The results of cell culture studies using rat calvaria osteoblasts demonstrate a good viability of the cells on the PS-modified material. Furthermore, the proliferation and differentiation were found to be enhanced on the PS-modified material.

1. Introduction

Besides collagen I acting as structural framework protein in bone matrix, non-collagenous proteins play a fundamental role: these macromolecules are involved in controlling the nucleation and crystal growth as well as the cell activity associated with the biomineralisation process in bone tissue [1]. Among the most abundant non-collagenous bone matrix proteins osteopontin (OPN) is an interesting protein [2–5], as this glycosylated serine-rich phosphoprotein has been found to be an effective inhibitor of the mineralisation of bone mineral [6]. The phosphorylated residues and the carboxyl groups are involved in the binding of the protein to the hydroxyapatite (HAP) surface [7, 8] and might be jointly responsible for the typical habit of HAP crystals in bone. Furthermore, OPN is able to influence the bone cell activities [9, 10]. Biomimetic strategies observed in nature should be kept in mind when developing of temporary bone replacement materials on the basis of calcium phosphates. A modification of these materials with proteins of the organic bone matrix like OPN might be useful to generate a material with a microstructure enhancing the remodelling *in vivo*. However, an application is limited by some facts, such as the availability of a species-specific protein and the sparse information about the necessary concentration of the protein and the expected complex effects *in vivo*. Therefore, it seems to be necessary to search for model structures which are able to mimic typical functions of the native bone proteins. An interesting compound suitable as model substance is the small acidic phosphoserine molecule which has active functionalities such as phosphate and carboxyl groups. Most of the published investigations deal with adsorption of

*Author to whom all correspondence should be addressed.

phosphoserine [11–13] or related compounds [14–17] on the HPA surface.

In this study, calcium phosphate bone cement (CPBC) composites with collagen type I [18] were modified with *O*-phospho-L-serine (PS) in order to investigate its influence on the material properties and on the proliferation and differentiation of osteoblasts.

2. Materials and methods

2.1. Preparation of materials

A bone cement powder Biocement D (BioD, BIOMET Merck Biomaterials GmbH, Darmstadt, Germany) was used for the investigations. This CPBC belongs to the system tricalcium phosphate (α-TCP)/dicalcium phosphate (DCPA). The cement powder contains 58 wt % α-TCP, 24 wt % DCPA, 8.5 wt % calcium carbonate ($CaCO_3$) and 8.5 wt % precipitated HAP [19]. After mixing with aqueous solutions it is transformed to calcium-deficient carbonated HAP. For the experiments, the original cement powder was mixed with 2.5 wt % freeze-dried, mineralised collagen I [20] to prepare a collagen-reinforced cement composite, BioD/coll [18]. Crystalline PS (Fluka, Taufkirchen, Germany) was added in amounts of up to 50 mg/g BioD/coll. Finally, the powder was mixed thoroughly with the liquid accelerator solution (4% Na_2HPO_4-solution) and shaped (see below). These cement samples were allowed to set in simulated body fluid (SBF), (pH 7.4) containing 150 mM NaCl; 90 mM $NaHCO_3$; 1 mM $MgSO_4$; 1 mM NaH_2PO_4; 5 mM KCl and 1.8 mM $CaCl_2$.

2.2. Characterisation

2.2.1. Microstructure and mechanical properties

2.2.1.1. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For examination of the microstructure by SEM (DSM 982 Gemini, Zeiss, Oberkochen, Germany) the samples were coated with carbon. Samples with cells were fixed with glutaraldehyde (1%), critical point dried and gold sputtered. TEM was performed using an electron microscope Philips CM 200 FEG ST/Lorentz (FEI, Eindhoven, The Netherlands). The samples were crushed and prepared on a copper mesh.

2.2.1.2. Compressive and tensile strength. Cylindrical specimen (diameter 1 cm, length 0.8 cm) of BioD/coll without as well as with phosphoserine (BioD/coll/PS) were prepared and allowed to set at 37 °C in SBF for 100 h. The samples were loaded parallel to the cylinder axis for testing the compressive strength, and vertically for testing the tensile strength. A mechanical testing machine (Instron 5566, Instron, Darmstadt, Germany) was used. The cross head speed was 8 mm/s. The compressive strength (σ_C) was determined as the critical force (F_c) divided by the loaded area. The tensile strength (σ_T) was calculated by Equation 1 with measured F_c, diameter (D) and the length of the sample (h) [18].

$$\sigma_T = 2F_c/\pi hD \quad (1)$$

2.2.1.3. Setting times. For determining the initial (t_I) and final setting time (t_f) of the different cement composites the Gillmore needle was used [21, 22].

2.2.1.4. Specific BET-surface area. The specific surface area was determined by a multiple point BET method from a N_2-adsorption isotherm using a ASAP 2010 (Micromeritics, Norcross, USA).

2.2.2. Chemical properties

2.2.2.1. Powder X-ray diffraction (XRD). The phase composition of the material was investigated by powder X-ray diffraction (Siemens D 500). Co-K_α radiation was used. The diffractograms were recorded from $2\theta = 25°$ to 45° (step width 0.05°). The compounds were identified by comparison with Powder Diffraction File (PDF)-cards [23].

2.2.2.2. Determination of ion release. The pH-value of the solution was measured using a glass electrode (SenTix 61, wtw GmbH, Weilheim, Germany). Calcium and phosphate concentrations were determined photometrically (UV–Vis spectrophotometer Cary 50, Varian, Darmstadt, Germany) with test-kits (Calcium liquicolor and Phosphorus liquid rapid, Rolf Greiner BioChemica, Flacht, Germany). Release of phosphoserine was determined by fluorescence photometry (Fluorescence Spectrophotometer F-4500, Hitachi, Krefeld, Germany) using a method described by Roth [24].

2.2.3. Cell culture experiments

2.2.3.1. Osteoblast culture. Osteoblasts were obtained from the calvariae of newborn Wistar Kyoto rats by sequential digestion with 3.56 U collagenase P/ml and 15 U trypsin/ml in PBS and subcultured in DMEM containing 10% FCS [25]. Prior to use, the composites were rinsed twice with DMEM containing 10% FCS for 10 min each. 12 500 cells/cm^2 were placed onto the surface of the composites in 100 µl of culture medium. After 2 h, the cells were covered with 1 ml of medium. After 4 days of culture, medium was supplemented with 300 µM ascorbic acid and 10 mM β-glycerophosphate.

2.2.3.2. Determination of proliferation of osteoblasts. At indicated time points, cell cultures were supplemented with 1.2 mM MTT followed by incubation at 37 °C for 4 h. After removing the medium, formazane dye converted from MTT by mitochondrial dehydrogenases was solubilised with 36 mM HCl in isopropanol containing 17.3 mM SDS and determined photometrically at 570 nm.

2.2.3.3. Determination of collagen synthesis. Collagen synthesis was determined according to Scutt *et al.* [26] with some modifications. Cells were incubated in DMEM with 10% FCS, 2.5 µCi [3,4-^{3}H]proline/ml, 300 µM ascorbate and 200 µM β-aminopropionitril for 24 h. The extracellular collagen was extracted by the addition of 10 M acetic acid to give a final concentration of 0.5 M, followed by incubation at 4 °C for 24 h. After that, 10 µg

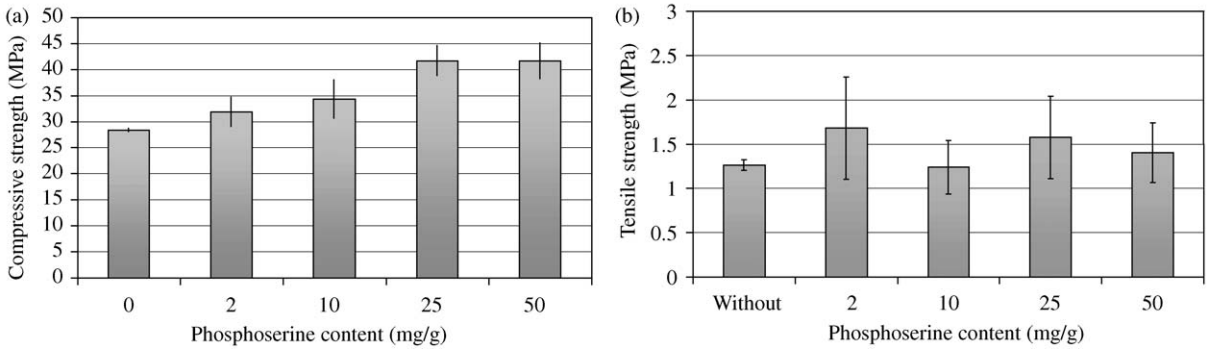

Figure 1 Influence of the amount of added phosphoserine on the stability under loading: (a) compressive strength and (b) tensile strength of the composites (after setting for 100 h at 37 °C in SBF).

of rat-tail collagen per ml in 0.5 M acetic acid were added as carrier, and collagen was precipitated by the addition of 4.3 M NaCl in 0.5 M acetic acid to give a final concentration of 0.85 M NaCl. The samples were thoroughly shaken and incubated at 4 °C for 1 h. After centrifugation at 20 800 × g for 30 min, the pellet was washed with 0.85 M NaCl and dissolved in 0.5 M acetic acid. Aliquots were used for scintillation counting. Cell number was assessed by methylene blue staining according to Currie [27].

2.2.3.4. Determination of alkaline phosphatase activity. For determination of the catalytic activity of alkaline phosphatase, osteoblasts were washed with PBS and lysed in 1.5 M Tris-HCl, pH 10 containing 1 mM $ZnCl_2$, 1 mM $MgCl_2$ and 1% Triton X-100 at 4 °C for 10 min. The lysates were clarified by centrifugation at 20 800 × g at 4 °C for 30 min and incubated with 3.7 mM *p*-nitrophenylphosphate in 0.1 M diethanolamine, pH 9.8 containing 0.1% Triton X-100 at 37 °C for 30 min. Released *p*-nitrophenolate was determined photometrically at 405 nm. Protein concentration was determined using the BioRad assay with bovine serum albumin as a standard.

3. Results and discussion

The modification of the biocement collagen composites with PS leads to a rise of compressive strength (Fig. 1(a)). An increase of about 30% was found for an addition of 25 mg and 50 mg PS per g cement collagen composite. The tensile strength was influenced only weakly by PS. However, a slight increase might be assumed for an addition up to 25 mg. For the modification with 50 mg/g a decrease of tensile strength was found (Fig. 1(b)). An increased scattering of the strength has been found to correlate with rising PS concentration. This could be caused by a larger variation of the critical flaw size. Such flaws can be formed by inclusions, e.g. non-transformed α-tricalcium phosphate (α-TCP) particles. Small amounts of added PS can already result in such particles as can be seen in the X-ray diffraction pattern (Fig. 2). This is due to the inhibition of the dissolution of the precursor by PS. Additionally, by means of SEM a different microstructure of the modified material compared with the pure cement collagen composite was observed (Fig. 3). PS modification leads

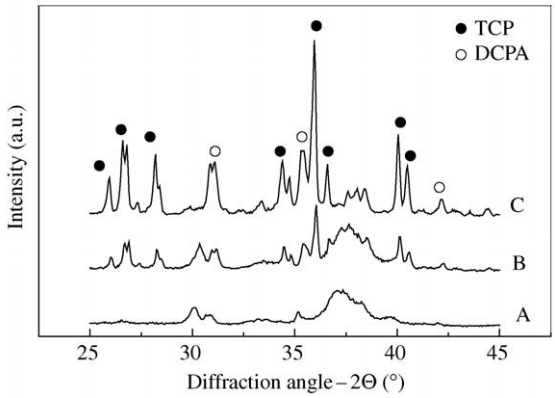

Figure 2 X-ray powder diffraction pattern of cement collagen composites after storage for 4 days in SBF at 37 °C (a = without PS, b = with 2 mg/g PS, c = 50 mg/g PS). Additional to the HAP pattern (not marked), the patterns of α-TCP and DCPA can be found in the samples with PS.

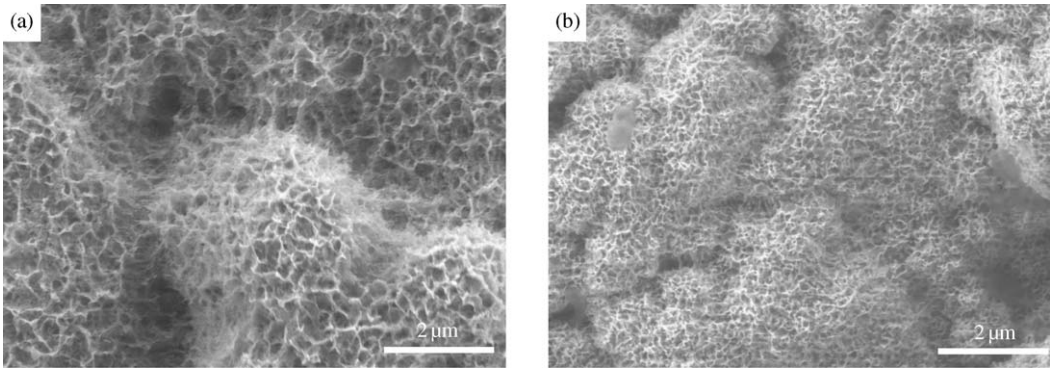

Figure 3 SEM image of the microstructure of the cement collagen composite (a) without and (b) with 25 mg/g *O*-phospho-L-serine; 10 000 × .

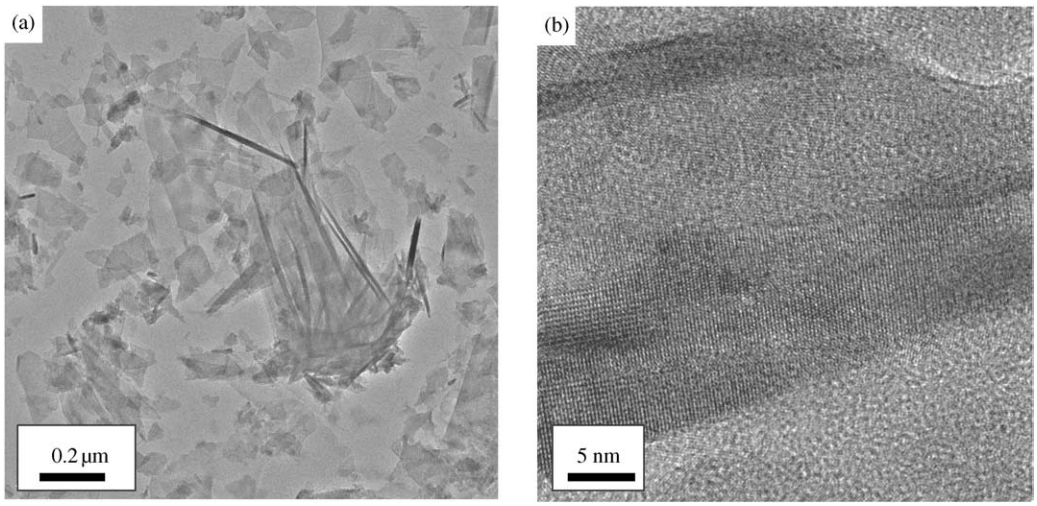

Figure 4 TEM images of a sample with 10 mg/g *O*-phospho-L-serine added; (a) overview image of particles and (b) high resolution micrograph of a single convoluted sheet (display detail of 4(a)).

to more closely packed HAP crystals. This phenomenon could be responsible for the enhancement of mechanical properties and the higher specific surface area of PS-modified samples. For the specific surface area (after setting 100 h at 37 °C in SBF) of BioD/coll/25PS a value of $77.96 \pm 0.3\,m^2/g$ was determined. That is about 1.5 times greater than measured for unmodified composites (BioD/coll $= 51.79 \pm 0.2\,m^2/g$). The lower surface area of the composite with 50 mg/g PS (BioD/coll/50PS $= 37.39 \pm 0.2\,m^2/g$) is due to the high amount of big particles of α-TCP and DCPA. However, after longer setting (for 30 days) the specific surface area for BioD/coll/50PS increased and reached $71.07 \pm 0.2\,m^2/g$. TEM investigations were performed to specify the HAP-crystal morphology of the PS-modified material. Compared to non-modified species, significant more large platelets, sometimes showing parallel microcracks, were found. Additionally, some needles have been observed, which seem to be convoluted platelets (Fig. 4). These findings could be explained by the interaction of PS with calcium phosphate surfaces via the functional groups (preferable phosphate groups) [28, 29]. Presently, the detailed mechanism of both adsorption and inhibition is being investigated.

For medical application a good workability within a limited time range ($8\text{ min} < t_f < 15\text{ min}$) is necessary [30]. Though the addition of PS shortens the setting times slightly, the workability can be optimised by changing the liquid to powder ratio for the preparation of the cement pastes from 0.32 to 0.42 ml/g.

Investigations of the chemical stability were performed by testing the ion concentration in the surrounding fluid (SBF). Nearly 10% of the added phosphoserine was found to be released within 30 days, with the highest quantity of PS being released within the first 3 days. That indicates a strong binding capacity of the cement composite for PS. The addition of phosphoserine has no adverse influence on the pH value, the calcium and phosphate ion concentration of the surrounding fluids. The solution has been found to be stable In the physiological range, which is the precondition for a good tissue compatibility [31, 32].

For assessment of the *in vitro* behaviour of the composites, osteoblasts were cultured on these materials, and their adhesion, proliferation and differentiation were investigated. As observed by SEM, osteoblasts cultured on phosphoserine-modified composites spread very well. The proliferation rate of osteoblasts cultured within 25 days on the different composites was determined. Osteoblasts cultured on PS-modified composites proliferate comparable to cells on polystyrene, whereas proliferation rate of osteoblasts cultured on BioD/coll

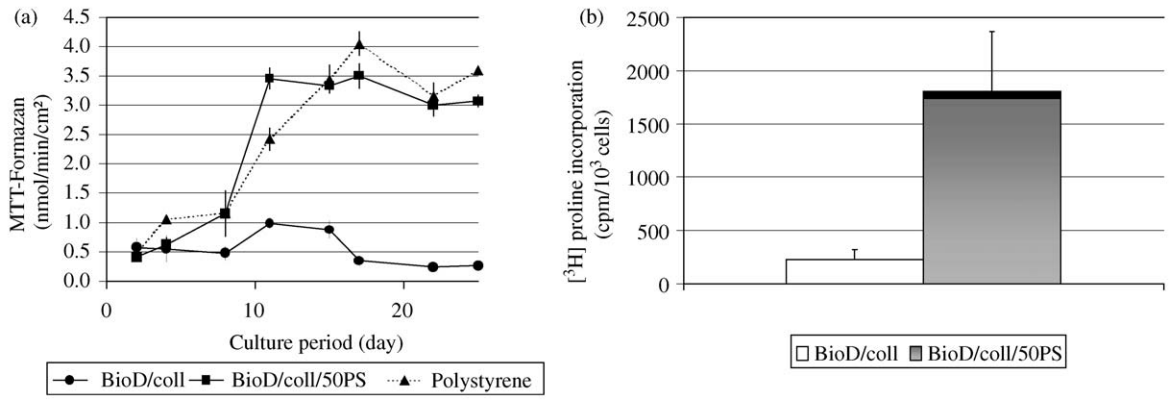

Figure 5 Osteoblasts cultured on cement composite modified with 50 mg/g PS: (a) Proliferation rate was assessed with the MTT-test and (b) collagen synthesis rate was determined after incorporation of [^{3}H] proline into collagen for 24 h. Data represent mean ± SD, $n = 9$.

was inhibited (Fig. 5(a)). The rate of collagen synthesis by osteoblasts cultured on the different composites was determined at day 4 of culture. Osteoblasts cultured on PS-modified composites (BioD/coll/50PS) showed about sevenfold increase of collagen synthesis rate compared to osteoblasts on BioD/coll (Fig. 5(b)). Differentiation of osteoblasts is characterised by the continuous increase in enzymic activity of alkaline phosphatase from day 8 to day 18 as a prerequisite for mineralisation [33]. Cells cultured on PS-modified composites (BioD/coll/50PS) revealed alkaline phosphatase activity at day 11 of culture (0.48 ± 0.07 mU/mg protein), whereas nearly no alkaline phosphatase activity was found in cells on BioD/coll (0.012 ± 0.001 mU/mg protein). The results of the cell culture experiments revealed that modification of BioD/coll with PS improve *in vitro* differentiation of osteoblasts cultured on these composites. It can be assumed that PS, beside the HAP crystal growth, also influences the osteoblasts activity.

4. Conclusion

O-phospho-L-serine was found to be a convenient compound for the modification of the calcium phosphate bone cement collagen composites. By an addition of phosphoserine in the range of 25–50 mg/g an enhancement in the materials properties, in particular in the strength, was observed. The results obtained by the cell culture studies indicate an improvement for proliferation and differentiation of osteoblasts on these phosphoserine-modified composites. Further cell culture studies with osteoclasts are being undertaken to investigate the potential of the material to be resorbed.

Furthermore, a significant influence of phosphoserine on the α-TCP dissolution and the morphology of the newly formed HAP was observed. Phosphoserine leads to an inhibition of α-TCP dissolution. The HAP crystal morphology changes in the presence of phosphoserine, culminating in a plate-like habit. These phenomena should be investigated further on, in respect to clarify the role of phosphoserine.

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References

1. S. MANN, in ''Biomineralisation – Principles and Concepts in Bioinorganic Materials Chemistry'' (Oxford University Press, 2001).
2. W. T. BUTLER, A. L. RIDALL and M. D. MCKEE, in ''Principles of Bone Biology'' (Bilezikian, Academic Press, New York, 1996) 167.
3. B. GANSS, R. H. KIM and J. SODEK, *Crit. Rev. Oral Biol. Med.* **10** (1999) 79.
4. J. SODEK, B. GANSS and M. D. MCKEE, *ibid.* **11** (2000) 279.
5. E. S. SORENSEN, P. HORUP and T. E. PETERSEN, *Protein Sci.* **4** (1996) 2040.
6. G. K. HUNTER, P. V. HAUSCHKA, A. R. POOLE, L. C. ROSENBERG and H. A. GOLDBERG, *Biochem. J.* **317** (1996) 59.
7. G. K. HUNTER and H. A. GOLDBERG, *ibid.* **302** (1994) 175.
8. R. A. SAAVEDRA, *BioEssays* **16** (1994) 913.
9. T. UEMURA, A. NEMOTO, Y. LIU, H. KOJIMA and J. DONG, *Mater. Sci. Eng. C* **17** (2001) 33.
10. A. L. BOSKEY, L. SPEVIAK, E. PASCHALIS, S. B. DOTY and M. D. MCKEE, *Calcif. Tissue Int.* **71** (2002) 145.
11. T. AOBA and E. C. MORENO, *J. Coll. Interface Sci.* **106** (1985) 110.
12. D. N. MISRA, *ibid.* **194** (1997) 249.
13. L. BENAZIZ, A. BARROUG, A. LEGROURI, C. REY and A. LEBUGLE, *ibid.* **238** (2001) 48.
14. A. L. BOSKEY and B. L. DICK, *Calcif. Tissue Int.* **49** (1991) 193.
15. N. SPANOS, P. G. KLEPITSIANIS and P. G. KOUTSOUKOSON, *J. Coll. Interface Sci.* **236** (2001) 260.
16. S. DAHLIN, J. ANGSTRÖM and A. LINDE, *Eur. J. Oral. Sci.* **106** (1998) 239.
17. N. L. HUQ, K. J. CROSS and E. C. REYNOLDS, *J. Mol. Model* **6** (2000) 35.
18. B. KNEPPER-NICOLAI, A. REINSTORF, I. HOFINGER, K. FLADE, R. WENZ and W. POMPE, *Biomol. Eng.* **19** (2002) 227.
19. F. C. M. DRIESSENS, I. KHAIROUN, M. G. BOLTONG and J. A. PLANELL, in Proccedings of the 10th Internatinal Symposium on Ceramics in Medicine (Seydel & Rey, Paris) 1997, p. 279.
20. J. H. BRADT, M. MERTIG, A. TERESIAK and W. POMPE, *Chem. Mater.* **11** (1999) 2694.
21. F. C. M. DRIESSENS, M. G. BOLTONG, O. BERMUDEZ and J. A. PLANELL, *J. Mater. Sci. Mater. Med.* **4** (1993) 503.
22. Standard Test Method for Time of Setting of Hydraulic-Cement Paste by Gillmore Needles, Document Number: ASTM C266–99, ASTM International, 01-Nov-1999.
23. Powder Diffraction File – cards of the International Centre for Diffraction Data (ICDD).
24. M. ROTH, *Anal. Chem.* **43** (1971) 880.
25. U. GEIßLER, U. HEMPEL, C. WOLF, D. SCHARNWEBER, H. WORCH and K.-W. WENZEL, *J. Biomed. Mater. Res.* **5** (2000) 752.
26. A. SCUTT, A. BERG and H. MAYER, *Anal. Biochem.* **203** (1992) 290.
27. G. A. CURRIE, *Br. J. Cancer* **43** (1981) 335.
28. M. TANAHASHI and T. MATSUDA, *J. Biomed. Mater. Res.* **34** (1997) 305.
29. M. S. MOHAN and E. H. ABBOTT, *Inorg. Chem.* **17** (1978) 2203.
30. F. C. M. DRIESSENS, J. A. PLANELL, M. G. BOLTONG, I. KHAIROUN and M. P. GINEBRA, *Proc. Inst. Mech. Eng. Part: H J. Eng. Med.* **207** (1993) 87.
31. D. H. KOHN, M. SARMADI, J. I. HELMAN and P. H. KREBSBACH, *J. Biomed. Mater. Res.* **60** (2002) 292.
32. C. KNABE, F. C. M. DRIESSENS, J. A. PLANELL, R. GILDENHAAR, G. BERGER, D. REIF and R. FITZNER, *ibid.* **52** (2000) 498.
33. D. BECKER, U. GEIßLER, U. HEMPEL, S. BIERBAUM, D. SCHARNWEBER, H. WORCH and K.-W. WENZEL, *ibid.* **59** (2002) 516.

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