Evaluation of HAp-ZrO_2 composites and monophase HAp bioceramics. *In vitro* study

A. RAPACZ-KMITA, A. ŚLÓSARCZYK, Z. PASZKIEWICZ
University of Science and Technology in Krakow, Faculty of Materials Science and Ceramics, al. Mickiewicza 30, 30-059 Krakow, Poland
E-mail: kmita@uci.agh.edu.pl
E-mail: aslosar@uci.agh.edu.pl

D. PALUCH
Medical University of Wroclaw, Department of Experimental Surgery and Research Biomaterials, Chair and Clinic Traumatology, ul. Poniatowskiego 2, 50-326 Wroclaw, Poland
E-mail: biomat@cheksp.am.wroc.pl

In recent years researchers all over the world have been interested in obtaining the evaluation and medical applications of the composites based on calcium phosphates, mainly hydroxyapatite and TCP. Hydroxyapatite (HAp)-$Ca_{10}(PO_4)_6(OH)_2$ is a material of high biocompatibility and additionally it is also one of the few bioactive materials able to create direct, and thus permanent, bonding with the bone tissue [1–3]. Full use of the unique properties of the dense hydroxyapatite ceramics is potentially possible only if their mechanical strength and fracture toughness are improved. These effects can be achieved in composite materials with HAp matrix. Among various reinforcing phases applied in the form of particles, platelets, fibers and nanoparticles, zirconia (ZrO_2) seems to be the most interesting one, due to its excellent mechanical properties [4]. Toughening mechanism utilizing the tetragonal—monoclinic transformation of ZrO_2 as well as the microcracking toughening mechanism resulting from the difference in thermal expansion coefficients of hydroxyapatite and zirconia are expected to occur in HAp-ZrO_2 composites [5]. The biological inertness and good biocompatibility of ZrO_2 sinters were confirmed by their medical applications [6].

However, in the case of composites containing substrates of excellent biocompatibility, it cannot be assumed that the final material will exhibit the same high level of biocompatibility. Numerous phenomena which occur during the materials processing may affect the phase composition, microstructure, and other properties responsible for the biological reactions. Therefore it is important to perform tests which evaluate the behavior of a composite material in conditions simulating an environment in a living organism. The objective of our study was manufacturing and comparative *in vitro* evaluation of the HAp-ZrO_2 composite and monophase HAp sinters obtained by hot pressing method.

The study was performed on dense composite sinters (HAp80 wt%-$ZrO_2$20 wt%) and pure HAp used as a reference material. Non-stabilized zirconia powder was produced by precipitation of hydrated ZrO_2 from $ZrOCl_2 \cdot 8H_2O$ aqueous solution [7]. The HAp-ZrO_2 composite powder was synthesized by precipitation of hydroxyapatite in the presence of ZrO_2 (wet process). The weighed amount of ZrO_2 powder was added to the intensively stirred aqueous suspension of $Ca(OH)_2$. Then the solution of H_3PO_4 was added slowly to the reaction medium. The precipitate produced was rinsed with distilled water, dried, calcined at 800 °C for three hours, and then ground. The composite powder obtained was an initial material for the production of dense HAp-ZrO_2 sinters. Hydroxyapatite for dense monophase HAp material was synthesized by the wet method using CaO and H_3PO_4 as reagents [8]. The precipitate was treated as described above.

The samples of monophase HAp ceramics and HAp-ZrO_2 composite were hot pressed in the temperature range of $1150 \div 1300$ °C (Thermal Technology Inc. kiln). The pressing was performed in graphite molds under argon atmosphere at a pressure of 25 MPa with a 0.5 hr soaking time, at the highest temperature.

The cytotoxicity analyses performed in this study constitute one of the stages of biological analyzes used for evaluation of broadly understood material biocompatibility. Although *in vitro* systems may not reflect normal *in vivo* conditions accurately, a cell-culture system is useful to observe phenomena at the cellular level. Such systems have been used to evaluate biocompatibility of implant materials and to study the interactions occurring at the cell-material interface [9]. The whole range of biocompatibility analyzes depends on the duration and kind of contact between an implant material and an organism, and is determined according to the PN-EN ISO 10993-1:1997 standard. The cytotoxicity analyzes are fast, standardised tests which can determine if a given material designed for medical applications contains significant amounts of biologically harmful substances. High sensitivity of these tests result from the isolation of cell cultures and thus lack of defence mechanisms, which accompany the cells within the body.

The cytotoxicity tests were performed on the samples according to the standard PN-EN ISO 10993-5 "Biological evaluation of medical products. Cytotoxicity investigations: *in vitro* methods". A method of direct contact with a layer of mouse fibroblasts 3T3/Balb evenly

0022–2461

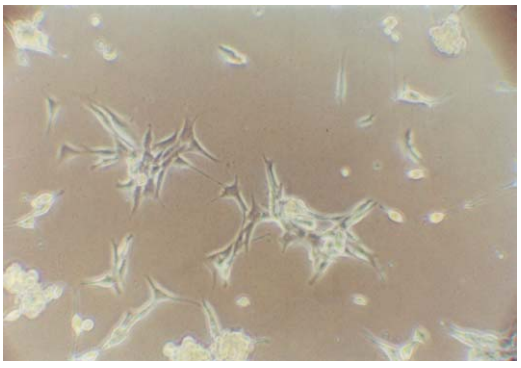

Figure 1 A control culture of mouse fibroblasts after 48 hrs.

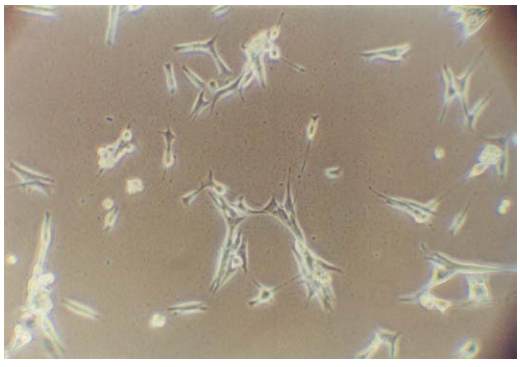

Figure 3 A culture of mouse fibroblasts after 48 hrs contact with the HAp 80 wt%-ZrO_2 20 wt% sinter surface.

spread and growing in a monolayer, was used. The cell cultures were incubated at 37 °C and then observed in an inverted contrast-phase microscope. Cytotoxic effect was evaluated after 24, 48, and 72 hrs of cell culture growth. The cells were cultivated in direct contact with the monophase HAp ceramics as well as the HAp-ZrO_2 composite (HAp 80 wt%-ZrO_2 20 wt%). Additionally, six control cultures with no presence of studied materials were grown. The mouse fibroblast cultures were grown on Petri dishes of 2.5×10^5 cells each.

The behavior of cells in contact with the surface of the HAp-ZrO_2 composite, as well as monophase HAp ceramics after 24, 48, and 72 hrs was similar. Cells adhered to the substrates and had regular morphological characteristics in each sample studied. Neither agglutination, vacuolisation, detachment from the medium, nor cell membrane lyses, were found. After 24 hrs the percentage of dead cells was identical for all cultures. The greatest cell growth was found in control and reference cultures. After 48 hrs the amount of dead cells in control, HAp and HAp-ZrO_2 cultures, was equal to 4%. In the photographs of cell cultures (Figs 1–3) regular fibroblast patterns are visible, which were characteristic for all tests conducted on sinters of materials studied. After 72 hrs the amount of dead cells in HAp and HAp-ZrO_2, as well as in the control cultures was equal to 5%. During the study, after 72 hrs the greatest growth of fibroblasts were found in cultures with monophase HAp ceramics. The growth was only slightly lower in the other cultures. In all the cultures examined and at all times of examination, the toxicity grade was zero according to standard evaluation (Table I).

The study of cytotoxicity of dense HAp-ZrO_2 composite and monophase HAp sinters conducted *in vitro* shows very high biocompatibility of materials produced. All samples were considered nontoxic (toxicity grade = zero) with the level of dead cells comparable to the control. Also the numbers of living cells in the samples examined increased regularly, and were comparable to the controls. In the case of HAp culture the increase was even higher than that in the controls, which confirms the known reports on excellent biocompatibility of this material [10].

The present data reveals no signs of cytotoxicity in the case of the all investigated materials. As expected, the monophase HAp ceramics was the most biocompatible. After careful analysis of the results of cell response it can be concluded that no negative influence of ZrO_2, as a phase reinforcing the HAp matrix, was observed. The X-ray diffraction analysis in the range of 2θ from 25 to 37 degree was used to determine the qualitative phase composition of the investigated powders and materials, (XRD7 Rich. Seifert & Co. diffractometer).

Lack of hydroxyapatite decomposition products, in the range of sintering temperature of 1150–1300 °C, indicates that the studied HAp-ZrO_2 composite shows high phase and thus biological stability. Only the process of phase transformation of monoclinic ZrO_2 into the tetragonal form occurring above 1200 °C can be observed. It manifests itself in an X-ray diffraction pattern by a loss of reflections originating from monoclinic ZrO_2 form at $2\Theta = 28^\circ$ and 31°, and the appearance of peaks at angles $2\Theta = 30^\circ$ and 34° corresponding to the tetragonal form of ZrO_2 (Figs 4–5). The process

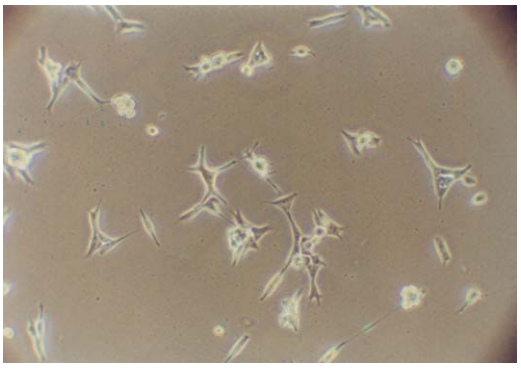

Figure 2 A culture of mouse fibroblasts after 48 hrs contact with the HAp sinter surface.

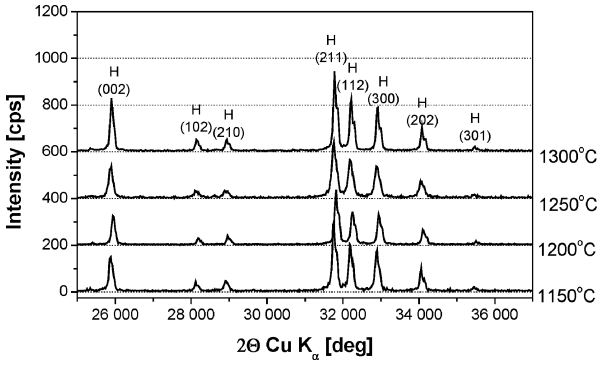

Figure 4 X-ray diffraction patterns of HAp sinters for various sintering temperatures (H—hydroxyapatite).

TABLE I Evaluation of cytotoxic activity of HAp and HAp 80 wt%-ZrO_2 20 wt% samples on mouse fibroblasts culture

	24 h			48 h			72 h			
Material	Morphological changes	Number of living cells	Percentage of dead cells	Morphological changes	Number of living cells	Percentage of dead cells	Morphological changes	Number of living cells	Percentage of dead cells	Evaluation Grade of toxicity
HAp	none	3.42×10^5	2	none	4.75×10^5	4	none	6.20×10^5	5	0
HAp-ZrO_2	none	3.10×10^5	2	none	4.37×10^5	4	none	5.40×10^5	5	0
Control culture	none	3.50×10^5	2	none	4.70×10^5	2	none	5.70×10^5	5	0

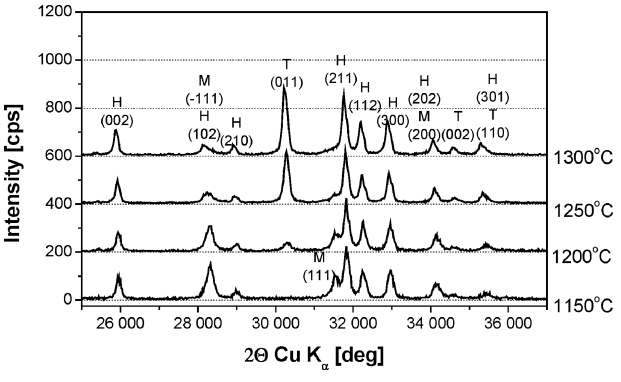

Figure 5 X-ray diffraction patterns of HAp–ZrO_2 composites for various sintering temperatures (H—hydroxyapatite, T—tetragonal ZrO_2, M—monoclinic ZrO_2).

described undoubtedly results from the formation of solid CaO-ZrO_2 solution. Probably slightly decaying hydroxyapatite is a source of calcium here [11]. Results similar to ours were reported by Heimann and Vu [12]. However, in contrast to the results obtained by these researchers, in our study no presence of $CaZrO_3$, as an additional phase which may affect the material biocompatibility, was found. This is probably due to the hot pressing conditions used in the production of our samples. Neither the solid CaO-ZrO_2 solution formed, nor an increase in the content of tetragonal ZrO_2 phase, caused any changes in cell response for the HAp-ZrO_2 composites in comparison with the monophase HAp ceramics. This suggests that hydroxyapatite, being an excellent biomaterial of bioactive properties, forms with zirconia a two-phase material which shows a similar, and a very high biocompatibility.

Acknowledgments

The present work was supported by grant no. 7T08D03019 from the State Committee for Scientific Research of Poland. The authors thank Professor K. Haberko and Doctor W. Pyda for preparing the ZrO_2 powder.

References

1. L. L. HENCH, *J. Amer. Ceram. Soc.* **74** (1991) 1487.
2. Z. LEGEROS, J. P. LEGEROS, G. DACULSI and R. KIJKOWSKA, "Encyclopedic Handbook of Biomaterials and Bioengineering" (1995) Vol. 2, p. 1429.
3. P. DUCHEYNE and Q. QIU, *Biomaterials* **20** (1999) 2287.
4. J. LI, L. HERMANSSON and R. SÖREMARK, *J. Mater. Sci. Mat. Med.* **4** (1993) 50.
5. M. TAKAGI, M. MOCHIDA, N. UCHIDA, K. SAITO and K. UEMATSU, *ibid.* **3** (1992) 199.
6. C. PICONI and G. MACCAURO, *Biomaterials* **20** (1999) 1.
7. M. M. BUĆKO and K. HABERKO, *J. Amer. Cer. Soc.* **78** (1995) 3397.
8. E. ANDRONESCU, E. STEFAN, E. DINU and C. GHITULICĂ, "*Key Engineering Materials*" (Trans Tech Publications, Switzerland, 2002) p. 1595.
9. M. F. BASLÉ, D. CHAPPARD, F. GRIZON, R. FILMON, J. DELECRIN, G. DACULSI and A. REBEL, *Calcif. Tissue Int.* **53** (1993) 348.
10. S. M. SCHNURER, U. GOPP, K. D. KUHN and S. J. BREUSCH, *Bone Substitutes: Orthopade* **32**(1) (2003) 2.
11. W. PYDA, A. ŚLÓSARCZYK, Z. PASZKIEWICZ, A. RAPACZ-KMITA, M. HABERKO and A. PYDA, *Composites* **1** (2001) 2.
12. R. B. HEIMANN and T. A. VU, *J. Mater. Sci. Lett.* **16** (1997) 437.

Received 26 January
and accepted 14 April 2004