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Particle size effects on the bone formation of hydroxyapatite/stem cell biocomposites

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HA has a very similar chemical composition to the inorganic part of human hard tissue, such as bone and teeth, so most of bones form a direct chemical bonding to an HA implant without forming a collagen interface layer. In this study, we tried to observe the bone formation of the biocomposite mixed with stem cells and hydroxyapatite (HA) powders with different particle sizes in rabbit long-bone defects, and investigated the size effects of hydroxyapatite particles on the formation of new bone. From the radiological photographs of the implanted biocomposites into rabbits weekly for 8 weeks, nanoscale HA biocomposites show a higher bone formation rate than that of micrometer scale HA biocomposites. Added stem cells into the biocomposite induced homogeneous bone formation and enhanced the bone formation ability. Conclusively, nanoscale HA powder with stem cells is an adequate biocomposite for new bone formation in bone defects.

Key words: Hydroxyapatite, Stem cell, Bone formation, Size effects

Introduction

The most desirable form of bone substitute is autologous bone. However, autografts are not always available and may result in morbidity at the donorsite. An allograft is preferred in some cases, but a possible immune response and disease transmission are detrimental to the recipient [1]. Bone graft substitutes have attracted much attention because of their advantages over both autografts and allografts [2].

Hydroxyapatite (HA) ceramics have been used extensively as a substitute in medical and dental applications in the form of granules, discs and coatings, because of their compositional similarities to natural human bone and teeth, and their excellent biocompatibility [3-5]. Due to the similarity of the size of nano-sized HA with the HA crystals in natural bone, many studies have been done on the synthesis of HA nanoparticles and their applications [6-10]. In the case of HA nanoparticles, their particle size, morphology, and structure have significant effects on the biological response [11].

Previous studies have demonstrated that 20-40 nm HA particles in the bone play an important role in biomineral formation, which have remarkable physical and chemical features, such as a unique mechanical strength, an insensitivity to growth/dissolution and a flexible structure [12, 13]. Therefore, nanoscale HA powder can be a better candidate in biomedical applications. But, the effects of particles size on bone formation are not understood clearly.

In this study, we tried to observe the bone formation of a biocomposite mixture of stem cells and hydroxyapatite (HA) powders with different particle sizes in rabbit long-bone defects and investigated the size effects of hydroxyapatite particles on the formation of new bone.

Experimental

Schematics of the experimental procedure from the starting powder to analysis via *in vivo* processing is shown in Fig. 1. Micrometer scale HA powder commercially obtained from Shinyo was used as starting materials in the experiment, in which the Ca/P ratio of this powder is approximately 1.67. Nanoscale HA powder with a Ca/P ratio of 1.67 was synthesized by an aqueous

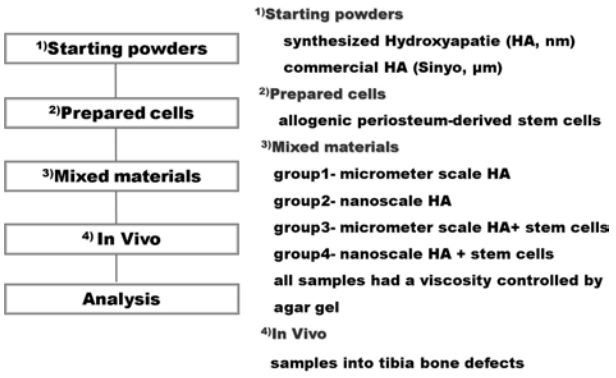

Fig. 1. Schematic of the experimental procedure from starting powders to analysis via *in vivo* processing.

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Table 1. Compositions of biocomposite specimens

Specimens	Composition
m_HAp	micrometer scale HA powder, Agar gel
n_HAp	nanoscale HA powder, Agar gel
sc/m_HAp	micrometer scale HA powder, Agar gel, stem cells
sc/n_HAp	nanoscale HA powder, Agar gel, stem cells

precipitation method from $Ca(NO_3)_2 \cdot 4H_2O$ and H_3PO_4. Stem cells were obtained from the bone marrow of a New Zealand white rabbit. Mononuclear cells containing 15 % fetal bovine serum were cultured in a 5 % CO_2 incubator at 37 ^{0}C. After 3 days, half of the culture medium was replaced and subsequently the medium was changed every other day. Confluent cells were detached with 0.25 % trypin-EDTA.

Biocomposites of a paste type were prepared by mixing 0.7 % agar gel (300 μl) and 1cc hydroxyapatite powderwith or without 2×10^7 stem cells (300 μl) and polymerization. The compostion of the biocomposites in this study is shown in Table 1. Bone implantation for 4 biocomposite samples was performed into white rabbits from New Zealand with a weight of 3-5 kg. Thirty two white rabbits (8-10 weeks-old) were used in this study. Before the operation, four steel wires were inserted in the perpendicular direction to the long axes of tibial bone and fixed by a dynamic external fixator by a finger. And then, tibial bone 1.5 cm defects were made by fragment excision using a dental burr in each animal (Fig. 2(a)).

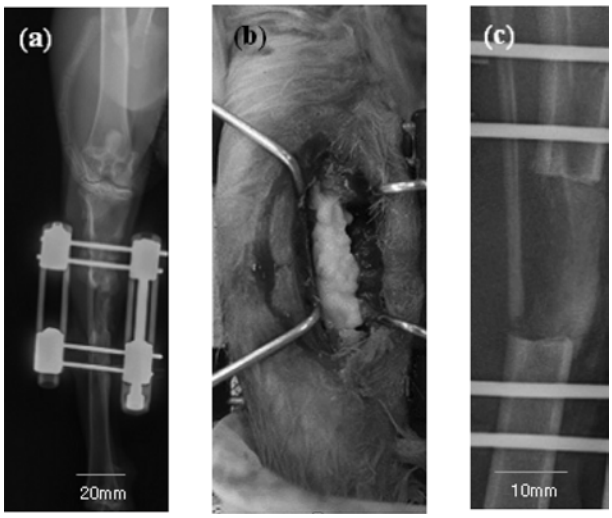

Fig. 2. (a) radiograph of excised section of a rabbit tibia with an external fixation device, (b) photograph of an inserted mixture of agar, HA powder and stem cells into a bone defect, (c) radiograph post operation.

Each paste type biocomposite was implanted into a bone defect of the eight rabbits (Fig. 2(b), (c)). After implantation, the morphology and the degree of bone formation were observed each week by radiographic and microscopic observation from post-operation to eight weeks after. Also, each microstructure of the bone tissue

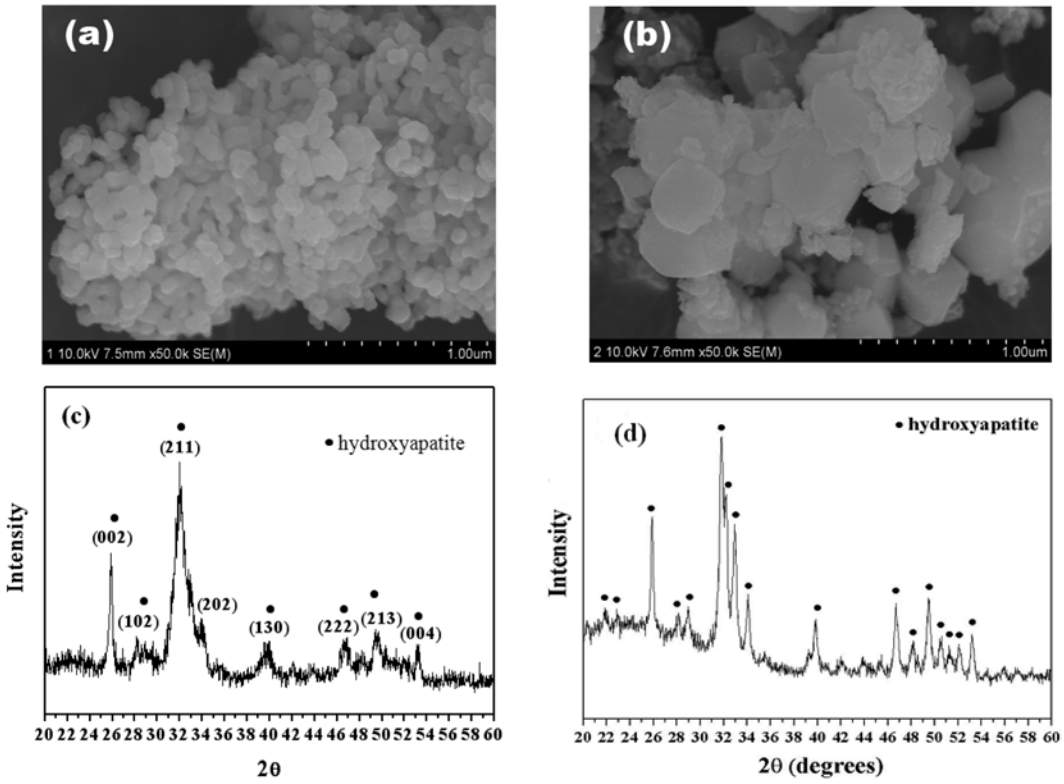

Fig. 3. Microstructure and XRD patterns of (a), (c) nanoscale and (b), (d) micrometer scale HA powders.

in the implanted area after eight weeks was observed by a photomicroscope, and the formation of new bone compared for the four biocomposite compositions.

The bone formation ability in the transplanted area within bone defects was analyzed from the photographs of the central implanted area. New bone formation are observed at five areas through the photographs magnified to 400 times using a photomicroscope and the ratio of the new bone formation area in a section using an image analyzing program of Image-proplus 5.1 was obtained.

Results and Discussion

An SEM micrograph shows that the as-dried nanoscale HA powder consists of nanoscale crystallites with a narrow size distribution (Fig. 3(a)). The powder is sphere-like with a diameter of approximately 70-100 nm. Although this is the typical morphology of particles prepared by a precipitation method [14, 15], it shows a low tendency to agglomerate. Its XRD pattern (Fig. 3(c)) has characteristic peaks consistent HA. It was noticed that the diffraction peak at $2\theta = 25.9^{o}$, corresponding to the (002) plane family, was sharper and isolated from others. This indicates crystal growth along the c-axis of the HA crystalline structure. Aging is one of the important requirements for the quality of the precipitated powder. It is considered that the long aging period, in this study, improves the quality and the characteristics of the crystals.

In the case of the commercial powder, it has a micrometer scale particle size about 0.5-2.0 μm with some agglomeration (Fig. 3(b)). Its XRD pattern (Fig. 3(d)) has characteristic peaks consistent with HA, but the HA peaks show a high intensity with a narrower width compared to the nanoscale HA powder.

Fig. 4 shows the microstructure of transplanted the n_HAp biocomposite one week after the implantation, which shows a compact microstructure. However, some new HA precipitates were observed on the surface of the biocomposite (Fig. 4(b)). This type of new HA precipitates have frequently been found in *in vitro* experiments by the mechanism of dissolution and reprecipitation [16]. Generally nanoscale HA powder has a higher solubility than that of micrometer scale HA powder, because the solubility of particle is inversely proportional to the particle radius [17].

The microstructure of sc/m_HAp biocomposites

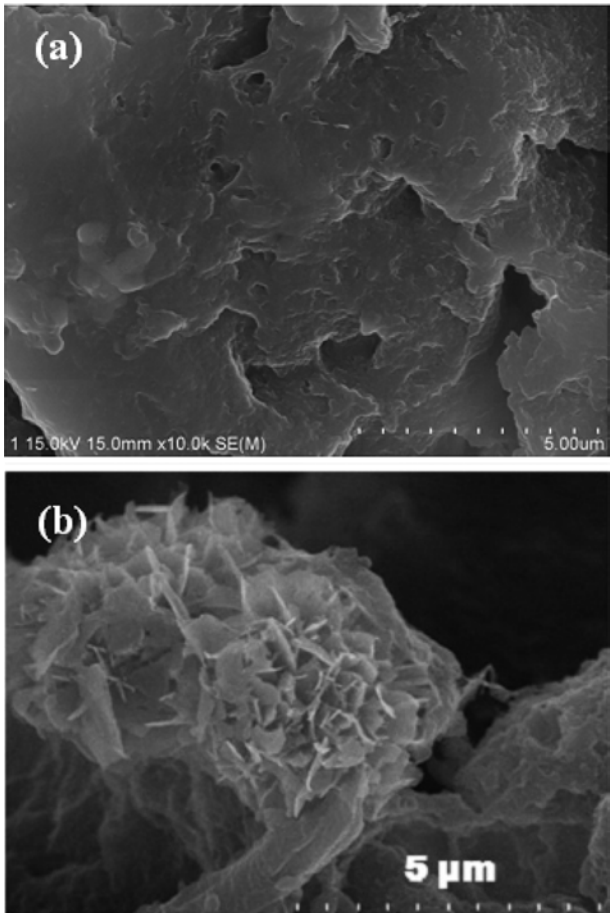

Fig. 4. Microstructure of the transplanted n_HAp biocomposite into the bone defects one week after the operation.

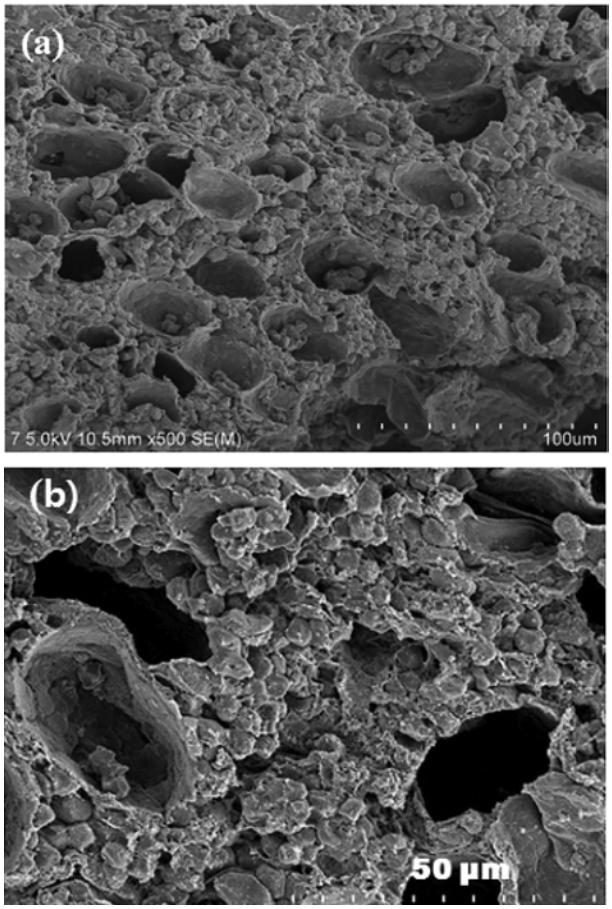

Fig. 5. Microstructure of the transplanted sc/m_HAp biocomposite into bone defects one week after the operation.

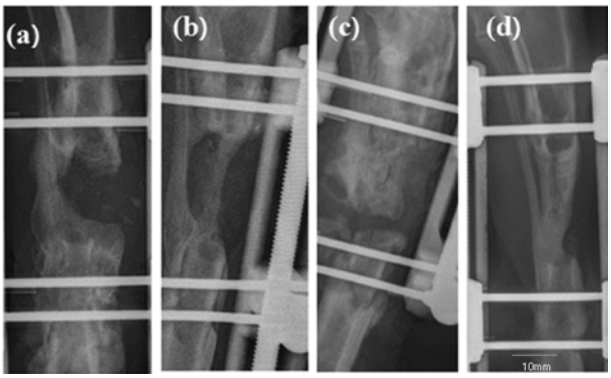

Fig. 6. Radiographs for the bone formation of (a) m_HAp biocomposite, (b) n_HAp biocomposite, (c) sc/m_HAp biocomposite and (d) sc/n_HAp biocomposite by implantation into bone defects eight weeks after the operation.

implanted in bone defects is shown in Fig. 5 after the operation one week. Micrometer scale HA particles were homogeneously dispersed with the stem cells, however, a trace of dissolution in the HA particles had not appeared at that time. Bonding between the HA particles is very weak compared to n-HAp biocomposite.

Fig. 6 shows the radiographs for bone formation of the biocomposites in bone defects. In the case of m_HAp biocomposite, diffuse bone formation was seen at the edge of the old bone, but the consolidation just in the lateral position of the defect appeared at eight weeks. The degree of bone formation and consolidation of n_HAp biocomposite is higher than that of m_HAp biocomposite as shown in Fig. 6(b), which indicates that nanoscale HA powder have an advantage compared to the micrometer scale HA powder.

From a previous study, the bioactivity of nanoscale HA particles is higher than that of micrometer scale HA particles because nanoparticles may promote the adhesion, proliferation and synthesis of alkaline phosphatase of osteoblasts and lead to a more rapid repair of a hard tissue injury [18-20]. Recent research suggests that better osteoconductivity would be achieved if synthetic materials could resemble bone minerals in composition, size, and morphology [21]. Therefore, nanoscale biomaterials may also have other special properties due to the small particle size and enormous specific surface area. For example, nano-sized ceramic materials have shown a significant increase in protein adsorption and osteoblast adhesion [22, 23].

Better new bone formation and consolidation was found in the HAp specimens mixed with stem cells, as shown in Fig. 6(c) and (d). HAp biocomposites with added stem cells have a higher bone formation ability than those of specimens without stem cells, and then most of the bone defects were filled with new bone in these specimens eight weeks after the operation. In particular, a complete bone consolidation is shown in the case of sc/n_HAp biocomposite. Many previous reports have shown excellent bone formation by stem cells on scaffolds with coculture [24-27]. Also, it is well known that the bone marrow contains stem cells called mesenchymal stem cells and stromal stem cells,

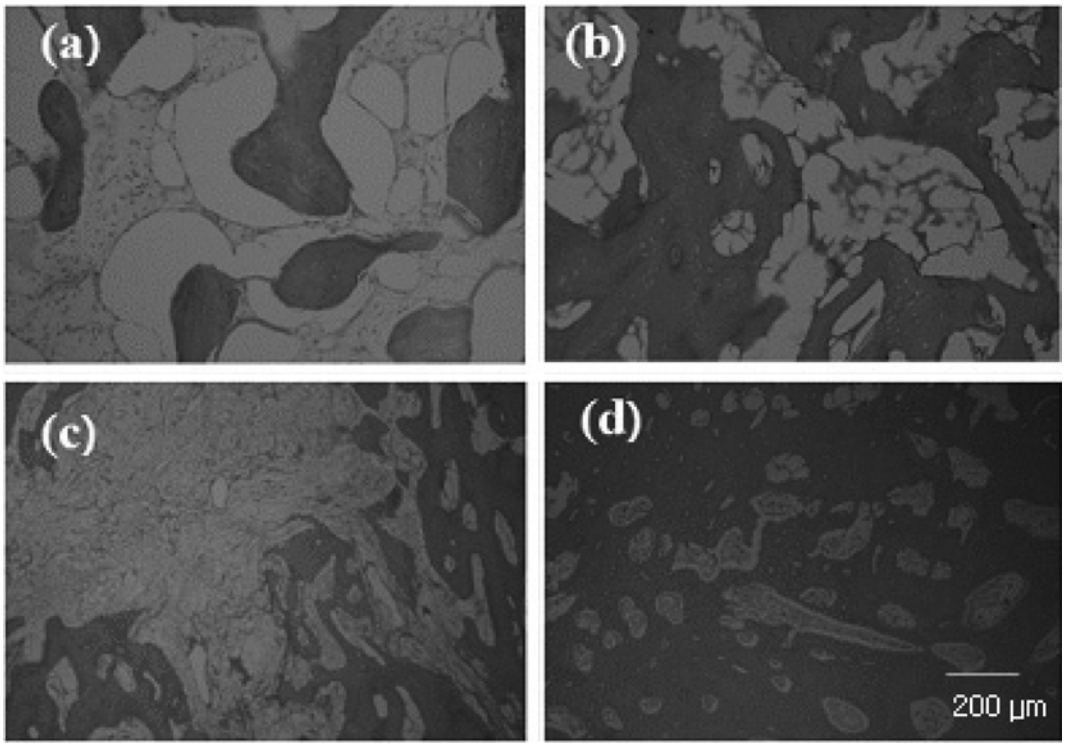

Fig. 7. Micrographs showing the new bone formation in the transplanted areas within bone defects compacted with (a) m_HAp biocomposite, (b) n_HAp biocomposite, (c) sc/m_HAp biocomposite and (d) sc/n_HAp biocomposite eight weeks after the operation.

and mesenchymal stem cells can differentiate into osteoblasts, chondrocytes, adipocytes, and muscle cells, and can also differentiate into osteoblastic cells after culturing in an osteoinductive medium [28]. Based on these results, it is suggested that the implanted stem cells with hydroxyapatite powders could survive, and differentiate into osteoblasts, or release various kinds of cytokines for bone formation in the presence of an immunosuppressive agent [29].

Fig. 7 shows the histological sections taken 8 weeks after implantation of specimens showing the new bone formation in the transplanted areas within bone defects. In the case of the m_HAp biocomposite (Fig. 7(a)), cartilaginous components were predominant in the defect, and well-formed lamellar bone was found in the n_HAp biocomposite (Fig. 7(b)). But, in the case of the sc/m_HAp biocomposite, most of the defects were filled with loose connective tissue with a small amount of woven and lamellar bones (Fig. 7(c)). On the other hand, the major part of the tibial defects were filled with new bone and inflammatory cells in the case of the sc/n_HAp biocomposite (Fig. 7(d)).

The bone formation ability in the transimplanted area within bone defects is shown in Fig. 8 by analyzing the photographs of the implanted central areas. The ratio of the new bone formation area for a section was calculated using the image analyzing program of Image-proplus 5.1. As shown the results of radiographs for bone formation shown in Fig. 6, the bone formation ability of nanoscale HA biocomposites is higher than that of micrometer scale HA biocomposites. Also, biocomposites with stem cells show a two times higher bone formation ability than those of biocomposites without stem cells, as shown in Fig. 8.

The significance of cells for bone formation in hydroxyapatite implants has been demonstrated previously [24-29]. Bone forming capacities of ectopically implanted hydroxyapatite scaffolds show a significant difference according to the addition of stem cells, which indicates the crucial role of the cells in bone formation. In this study, the sc/n_HAp biocomposite has better bone repair properties than that of the others. So, it is important to add the stem cells and use the nanoscale HA particles in an hydroxyapatite-based scafford to enhance the bone formation and repair ability of the biocomposite.

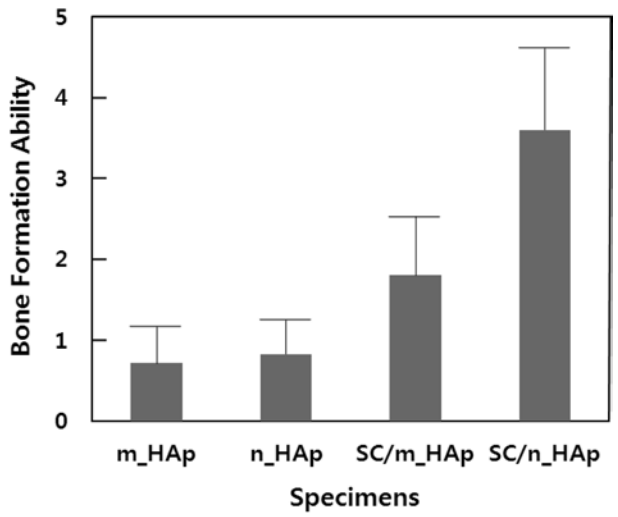

Fig. 8. Bone formation ability in the transimplanted areas within bone defects compacted with (a) m_HAp biocomposite, (b) n_HAp biocomposite, (c) sc/m_HAp biocomposite and (d) sc/n_HAp biocomposite eight weeks after the operation.

Summary and Conclusions

Bone formation and repair characteristics were studied in biocomposites composed of hydroxyapatite (HA) powders and stem cells in rabbit long-bone defects and also the size effects of hydroxyapatite particles on the formation of new bone was investigated. Nanoscale hydroxyapatite added biocomposites show a higher bone formation ability than that of micrometer scale HA biocomposites. Stem cells added into the biocomposite induced homogeneous bone formation and enhanced the bone formation ability. Conclusively nanoscale HA powder with stem cells is an adequate biocomposite for new bone formation in the bone defects and it could provide a powerful scaffold for bone tissue engineering.

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References

1. S. Best, B. Sim, M. Kayser, and S. Downes, J. Mater. Sci. Mater. Med. 8 (1997) 97-103.
2. X. Wang, Y. Li, J. Wei, and K de Groot, Biomaterials 23 (2002) 4787-4791.
3. J. Gallo, I. Landor, I. Cechova, and D. Jahoda, Acta Chir. Orthop. Traumatol. Cech. 75 (2008) 339-346.
4. A.A. Shetty, R. Slack, A. Tindall, K.D. James, and C. Rand, J. Bone Joint Surg. Br. 87 (2005) 1050-1054.
5. M. Wei, A.J. Ruys, M.V. Swain, B.K. Milthorpe, and S.C. Sorrell, J. Mater. Sci. 16 (2005) 101-106.
6. X. Guo, J.E. Gough, P. Xiao, J. Liu, and Z. Shen, J Biomed Mater Res A. 82 (2007) 1022-1032.
7. W.M. Laschke, K. Witt, T. Pohlemann, and M.D. Menger, J. Biomed. Mater. Res. Part B: Appl. Biomater. 82B (2007) 494-505.
8. Q. Fu, M.N. Rahaman, N. Zhou, W. Huang, D. Wang, L. Zhang, and H. Li, J. Biomater. Appl. 23 (2008) 37-50.
9. Y. Cai, Y. Liu, W. Yan, Q. Hu, J. Tao, M. Zhang, Z. Shic, and R. Tang, J. Mater. Chem. 17 (2007) 3780-3787.
10. M. Thorwarth, S. Schultze-Mosgau, P. Kessler, J. Wiltfang, and K.A. Schlegel, J. Oral Maxillofac. Surg. 63 (2005) 1626-1633.
11. T.J. Webster, C. Ergun, R.H. Doremus, R.W. Siegel, and R. Bizios, Biomaterials 21 (2008) 1803-1810.

12. R.K. Tang, L.J. Wang, C.A. Orme, T. Bonstein, P.J. Bush, and G.H. Nancollas, Angew. Chem. Inter. 43 (2004) 2697-2701.
13. Z. Shi, X. Huang, Y. Cai, R. Tang, and D. Yang, Acta Biomater. 5 (2009) 338-345.
14. Y.X. Pang and X. Bao, J. Eur. Ceram. Soc. 23 (2003) 1697-1704.
15. L.Y. Cao, C.B. Zhang, and J.F. Huang, Ceram. Int. 31 (2005) 1041-1044.
16. H. Wang, J.K. Lee, A.M. Moursi, and J. Lannutti, J. Biomed. Mater. Res. 67A (2003) 599-608.
17. Y. Cai, Y. Liu, W. Yan, Q. Hu, J. Tao, M. Zhang, Z. Shic, and R. Tang, J. Mater. Chem. 17 (2007) 3780-3787.
18. N. Fukui, T. Satoa, Y. Kuboki, and H. Aoki, Bio-Med. Mater. Eng. 18 (2008) 25-33.
19. J.S. Sun, F.H. Lin, T.Y. Hung, Y.H. Tsuang, W.H.S. Chang, and H.C. Liu, J. Biomed. Mater. Res. 45 (1999) 311-321.
20. X.H. Xie, X.W. Yu, S.X. Zeng, R.L. Du, Y.H. Hu, Z. Yuan, E.Y. Lu, K.R. Dai, and T.T. Tang, J. Mater. Sci.: Mater. Med. 21 (2010) 2165-2173.
21. H. Shigematsu, M. Akahane, Y. Dohi, A. Nakamura, H. Ohgushi, T. Imamura, and Y. Tanaka, Open Tissue Eng. Regener. Med. J. 2 (2009) 63-70.
22. T.J Webster and J.U. Ejiofor, Biomaterial 25 (2004) 4731-4739.
23. L.B. Jeng, H.Y. Chung, T.M. Lin, J.P. Chen, Y.L. Chen, Y.J. Wang, S.C.N. Chang, and Y.L. Lu, J. Biomed. Mater. Res. 91A (2009) 886-893.
24. E.M. Fischer, P. Layrolle, C.A. Van Blitterswijk, and J.D. De Brujin, Tissue Eng. 9 (2003) 1179-1188.
25. H.H.K. Xu, L. Zhao, and M.D. Weir, J. Dent. Res. 89 (2010) 1482-1488.
26. Y. Ito, N. Tanaka, Y. Fujimoto, Y. Yasunaga, O. Ishida, M. Agung, and M. Ochi, J. Biomed. Mater. Res 69A (2004) 454-461.
27. C. Du, F.Z. Cui, X.D. Zhu, and K. de Groot, J. Biomed. Mater. Res. 44 (1999) 407-415.
28. D.S. Zhou, K.B. Zhao, Y. Li, F.Z. Cui, and I.S. Lee, J. Bioactive Compatible Polymer. 21 (2006) 373-384.
29. W. Zhu, X. Zhang, D. Wang, W. Lu, Y. Ou, Y. Han, K. Zhou, H. Liu, W. Fen, L. Peng, C. He, and Y. Zeng, Micro & Nano Lett. 5 (2010) 19-27.