

A study of osteocalcin production in vitro on various hydroxyapatite based biomaterials

Xu, J. L.; Khor, K. A.; Chen, William Wei Ning

2007

Xu, J., Khor, K. A., & Chen, W. N. (2007). A study of osteocalcin production in vitro on various hydroxyapatite based biomaterials. Key engineering materials, 330-332, 897-900.

<https://hdl.handle.net/10356/101849>

<https://doi.org/10.4028/www.scientific.net/KEM.330-332.897>

© 2007 Trans Tech Publications. This is the author created version of a work that has been peer reviewed and accepted for publication by Key Engineering Materials, Trans Tech Publications. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at:
[<http://dx.doi.org/10.4028/www.scientific.net/KEM.330-332.897>].

Downloaded on 13 Mar 2024 18:44:19 SGT

A Study of Osteocalcin Production *in vitro* on Various Hydroxyapatite Based Biomaterials

J.L. Xu^{1, a}, K.A. Khor^{1, b}, W.N. Chen^{2, c}

¹ School of Mechanical & Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

² School of Chemical and Biomedical Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

Emails: ^ajlxu@ntu.edu.sg, ^bmkakhor@ntu.edu.sg, ^cwnchen@ntu.edu.sg

Keywords: Osteocalcin, hydroxyapatite, *in vitro*, spark plasma sintering

Abstract.

Hydroxyapatite based biomaterials were prepared by a spark plasma sintering technology. The human limb-derived osteoblasts were cultured on the various biomaterial surfaces (HA, RF21, 1SiHA and 5SiHA) for up to two weeks to investigate the cellular behaviors. The bone gamma-carboxyglutamic protein or osteocalcin in the medium were determined at different periods of cell culture. The results indicated that a combined effect of bioceramic surface composition and surface morphology had influenced the osteoblast behaviors. The amount of osteocalcin in the medium increased in the initial periods of culture but decreased in the late periods of culture.

Introduction

Hydroxyapatite (HA) and its based biomaterials could chemically bond directly to bone when implanted, resulting in the formation of a strong bone-implant interface ^[1-2]. The *in vitro* biocompatibility of the biomaterial based on HA has been assessed by a cell line in order to test cytotoxicity of the biomaterial. It is reported ^[1] that osteoblasts appear to be very sensitive to minor variations in surface composition and topography. Once attached to the surface, osteoblasts proliferate and subsequently differentiate.

Bone osteocalcin constitutes about 15 weight % of the non-collagenous bone matrix proteins. It is the most abundant protein among the noncollagenous proteins in bone and is produced exclusively in osteoblasts and its dental counterpart. Osteocalcin is believed to be crucial in regulating osteoblast activity and binding of HA ^[3]. Because of this tissue-specific expression, the level of osteocalcin could be considered as an indicator of the overall activity of cells operating in bone formation. Thus it could be suggested that when there is increased bone formation, the serum osteocalcin concentration will also be increased. In this study, various spark plasma sintered (SPS) ceramic samples were seeded with human limb osteoblast-like cells to investigate the effect of surfaces on the production of osteocalcin *in vitro*.

Materials and Methods

An SPS system (Sumitomo Coal Mining SPS system, Dr. Sinter Modal 1050, Japan) was used to prepare consolidated compacts. The powder feedstock used for SPS were spray dried HA (ceramic: HA), RF plasma sprayed calcium phosphate prepared at power level of 21 kW (ceramic: RF21), and spray dried HA doped with 1 weight % (ceramic: 1SiHA) and 5 weight % of silica (ceramic: 5SiHA) which assembled the amounts of silicon detected in natural bone. SPS samples were prepared at the sintering temperature of 1100 °C for 3 minutes with a heating rate of 100 °C/min and a cooling rate of 100 °C/min. The surface chemistry of these prepared ceramic samples was characterized using an X-ray diffraction with Philips PW1830 (the Netherlands). The XRD scan was carried out from 20 to 50° in 0.02° steps at a step time of 1 second. The surfaces of the SPS ceramic before cell culture were characterized for roughness using a Surfpak Roughness Tester (Mititoyo, Japan).

The samples were sterilized via autoclaving before cell seeding on the surfaces. Human limb-derived osteoblast cells were cultured on the samples in the Dulbecco's modified Eagle's

medium/F12 supplemented with 10 volume % of fetal bone bovine serum and 5 volume % of antibiotics for 2 weeks with an initial cell density of $2 \times 10^4/\text{cm}^2$ and incubated at 37°C in a 5% CO_2 atmosphere. After fixation, the cell morphology after attachment on the surfaces was observed under a scanning electron microscope (SEM, JEOL JSM-5600 LV, Japan). The amount of osteocalcin in the conditioned medium was determined by a Gla-OC ELSIA Kit (USA) which utilized a novel set of monoclonal antibodies highly reactive to the osteocalcin. The osteocalcin production was measured for absorbance at 490 nm wavelength on a micro-plate reader machine (Benchmark Plus, Bio-Rad Laboratories Inc.). Every plate was read for 5 times.

Results and discussion

The surface phase compositions of the various ceramic samples were shown in Fig. 1. As observed, HA was the dominated phase in all the samples. β -TCP was also observed in the samples RF21, 1SiHA and 5SiHA. The presence of β -TCP in the sample RF 21 has been discussed in reference [4]. The decomposition of HA into TCP in the presence of silica occurred, as shown in Fig. 1. Referring to the relative peak intensities compared with the peak intensities of HA, there were higher amount of β -TCP in the RF21 samples.

As the topographical features of these samples influenced the cell attachment and proliferation, the roughness were characterized and tabulated in Table 1. It could be found that the sample RF21 gave the highest roughness value of 104.8 nm (arithmetic average roughness, Ra).

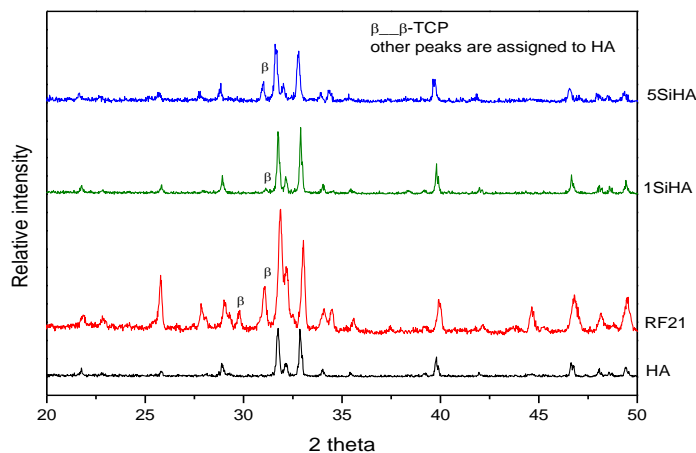


Fig. 1 XRD patterns of various hydroxyapatite based ceramic samples.

Table 1 Surface roughness of various SPS ceramic samples.

Ceramic	Surface roughness (Ra; nm)
HA	27.4 ± 2
RF21	104.8 ± 20
1SiHA	74.3 ± 26
5SiHA	40.8 ± 8

The typical osteoblast morphologies under SEM were exhibited in Fig. 2 after fixation. It was found that the cells had attached on the sample surface after 2 days of cell culture. No apparent differences in the cell morphology on any of the ceramic surfaces were found. The majority of the cells had a flattened appearance with a predominantly polygonal morphology, which indicated a high affinity to the substrate surface. A rough texture was observed due to the presence of numerous blebs on the surface of the cells.

The recruitment of osteoblastic cells played a crucial role in osteogenesis (bone growth) [5] since the bone formation was mainly dependent on the number of osteoblastic cells rather than the osteoblast activity. The increase in the surface roughness led to increased surface area. An increase in surface area correlated to an increase in osteoblast adhesion and proliferation. The rougher surface of RF21 helped adsorb more proteins compared with the other samples due to the rougher surface (Data not shown). Previous result has been reported by Loty et al. [1] showing better cell attachment on rougher surfaces compared with the smoother ones. Webster [5] et al. discussed that the dimensions of nanometer or fine surface features gave rise to larger amounts of interparticulate

voids (with fairly homogeneous distribution). Deligianni et al. [6] also found that the surface roughness affected cellular response, enhancing cell adhesion and proliferation. In addition, Matsuura et al [7] suggested that osteoblasts might exhibit different affinities for various surfaces, and these different surfaces might adsorb similar and unique extracellular matrix proteins found in the serum.

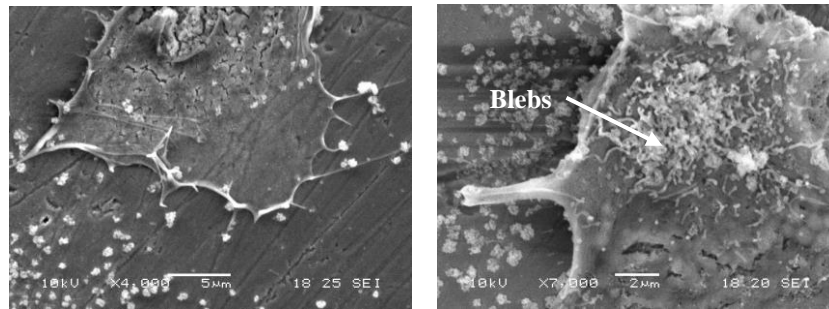


Fig. 2 SEM images of human osteoblast-like cells on the SPS ceramic 5SiHA.

The results of osteocalcin production (Fig.3) showed that the amount of osteocalcin in the conditioned medium was time and surface composition dependent though it was comparable among the various samples. The highest concentration of osteocalcin was detected in conditioned medium after culturing for 4 days except the samples doped with 5 weight % of silica whose maximum value of 15.5 ng/ml was obtained in the supernate after culturing for 2 days. Moreover, the osteocalcin were slightly higher on the ceramic 5SiHA and 1SiHA when compared with that of ceramic HA and ceramic RF21. This finding suggested that osteoblasts differentiation on all these HA based biomaterials, which was consistent with the ability of HA to support osteoblast attachment and promote bone formation within implants. It was also confirmed that osteoblast differentiation was enhanced when compared with the control group whose cells were directly cultured on the Petri dish surfaces.

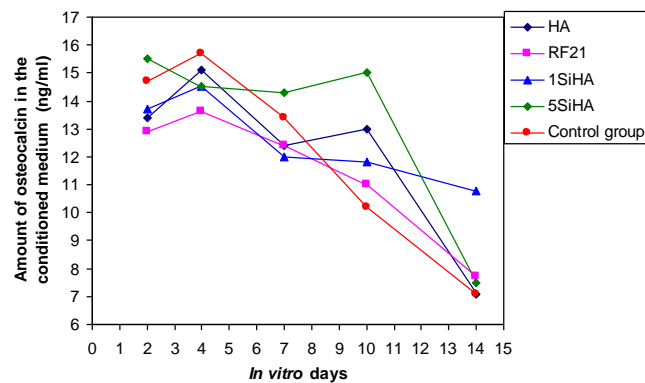


Fig. 3 Effect of various hydroxyapatite based ceramic samples on osteocalcin production by osteoblast at different culture periods.

The instability of β -tricalcium phosphate (β -TCP) and even minor tetracalcium phosphate (TTCP), calcium oxide (CaO) and amorphous calcium phosphate phase (ACP) in the RF21 ceramic samples was thought to be a factor that accelerated osteoconductivity, and improved the chemical affinity and connectivity with the bone tissue *in vivo* [4]. The comparative dissolution behavior of HA, β -TCP, TTCP, ACP and CaO in increasing order as shown below:



In the present study, the dissolution of Ca ions from the ceramic surfaces resulted in an interfacial supersaturated condition with the already present Ca ions in the medium. The super-saturation of Ca ions consequently stimulated the proliferation of osteoblast compared with the sample HA. While, no obvious precipitation of carbonated apatite was observed on the sample surface after the cell

culture. This may be ascribed to the more acidic microenvironment produced by osteoblasts, in which the amorphous phase dissolved at an accelerated rate ^[8]. However, we must consider that excessively high solubility and reactivity of bioceramic surfaces may result in damage to adherent cells, which may correspondingly stimulate inflammatory responses in surrounding tissues. The high solubility of the TTCP and TCP was found to be the dominant factor. They would decrease the viability of the cells by causing their rupture during initial anchoring phase. The failure of the initial attachment between the surrounding tissues and the implant materials might cause acute inflammation, and thus delay wound repair.

It has been reported that nucleation of bioactive apatite around silicate-substituted HA was enhanced by increased dissolution of calcium and phosphate ions from the implant ^[9]. It was also important to emphasize the effect of silica on cell behaviors on these SPS bioceramic samples. The presence of silica played an integral role during the bone mineralization processes. It was a fundamental constituent of collagen and as being an essential element in the formation in the formation of collagen. The effects of orthosilicic acid on collagen type I synthesis have demonstrated the enhanced differentiation of osteoblastic cell lines exposed to orthosilicic acid. Other study has elaborated the role of silicon or silica in osteoblast function ^[10]. It has been reported that increased Si content would cause HA based ceramic to be more soluble, releasing more Ca^{2+} , PO_4^{3-} and SiO_4^{4-} ions into the culture medium ^[10]. The released Si could have bound with oxygen, forming a silicate network structure on the surface, which might be capable of holding elements of the proteins together in an organized fashion, thus contributing to the architecture of connective tissue ^[10]. It was possible that these proteins were adsorbed onto the bound silicate network, thus promoting better cell differentiation via the interaction with the integrins on the osteoblast cells. Present results indicated that the presence of soluble silicate in ceramic 5SiHA might cause the rapid release of silicate and Ca^{2+} ions leading to rapid bone cell differentiation after 2 days of culture.

Conclusions

The cellular activity of osteoblasts was dependent on materials surface. It was obvious that from the data, the difference on the ceramic surface chemistry affected the cell differentiation as the SPS samples consisted of various amounts of calcium phosphates which included hydroxyapatite and beta tricalcium phosphate. In addition, the presence of silicon had enhanced the osteoblast maturation. This might be attributed to that the incorporation of silicate ions into HA led to an increased rate of dissolution of SiHA following by an increase in bone apposition on the surface of the ceramic. Moreover, the released Si promoted better cell differentiation process than the samples without Si via the interaction between the proteins and osteoblasts.

References

- [1]. C. Loty, J.M. Sautier, H. Boulekbache, T. Kokubo, et al. (2000) *J Biomed Mater Res* 49: 423-434.
- [2]. D.D. Deligianni, N.D. Katsala, P.G. Koutsoukos, Y.F. Missirlis. (2001) *Biomater*, 22: 87-96.
- [3]. S. Rammelt, M. Neumann, U. Hanisch, A. Reinstorf, et al. (2005) *J Biomed Mater Res*, 73A: 284-294.
- [4]. J.L. Xu, K.A. Khor, Y.W. Gu, R. Kumar, et al. (2005) *Biomater* 26:2197-2207.
- [5]. T.J. Webster, J.U. Ejiofor. *Biomater* (2004) 25: 4731-4739.
- [6]. D.D. Deligianni, N.D. Katsala, P.G. Koutsoukos, Y.F. Missirlis. (2001) *Biomater* 22: 87-96.
- [7]. T. Matsuura, R. Hosokawa, K. Okamoto, T. Kimoto, et al. (2000) *Biomater* (2000) 21:1121-1127.
- [8]. J. Weng, Q. Liu, J.G.C. Wolke, X. Zhang, et al. (1997) *Biomater* 18: 1027-1035.
- [9]. A.E. Porter, N. Patel, J.N. Skepper, S.M. Best, et al. (2004) *Biomater* 25: 3303-3314.
- [10]. E.S. Thian, J. Huang, S.M. Best, et al. (2006) *Biomater* 27: 2692-2698.