JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 795–801

Dynamic study of calcium phosphate formation on porous HA/TCP ceramics

Y. R. DUAN[1, 2], Z. R. ZHANG[2], C. Y. WANG[3], J. Y. CHEN[3,*], X. D. ZHANG[3]
[1]*State key Laboratory for Modification of Chemical Fibers and Polymer Material, Donghua University, Shanghai 200051, People's Republic of China*
E-mail: yrduan@dhu.edu.cn
[2]*West China School of Pharmacy, Sichuan University, Chengdu 610041, People's Republic of China*
[3]*Engineering Research Center in Biomaterials, Sichuan University, Chengdu 610064, People's Republic of China*
E-mail: jychen@scu.edu.cn

Bone-like apatite formation on porous calcium phosphate ceramics was investigated in static simulated body fluid (SBF) and dynamic SBF at different flowing rates. The results of a 14-day immersion in static SBF showed that the formation of bone-like apatite occurred both on the surface and in the pores of the samples. When SBF flowed at the physiological flow rate in muscle (2 ml/100 ml·min), bone-like apatite could be detected only in internal surface of the pores of samples. The result that bone-like apatite formation could only be found in the pores when SBF flowed at physiological flow rate was consistent with that of porous calcium phosphate ceramics implanted *in vivo*: osteoinduction was only detected inside the pores of the porous calcium phosphate ceramics. This result implicates that the bone-like apatite may play an important role in the osteoinduction of Ca-P materials. The dynamic model used in this study may be better than usually used static immersion model in imitating the physiological condition of bone-like apatite formation. Dynamic SBF method is very useful to understand bone-like apatite formation *in vivo* and the mechanism of ectopic bone formation in calcium phosphate ceramics.

1. Introduction

Approximately a decade ago, several research groups in the world reported osteoinduction of calcium phosphate ceramics. Zhang [1] and Klein [2] reported ectopic formation of bone in porous calcium phosphate ceramics implanted in muscle and subcutis of dogs. Heughebeart [3] found that bone-like material formation in porous hydroxyapatite implanted in non-bone tissue. Ripamonti [4] also observed osteoinductivity of coral derived hydroxyapatite implanted heterotopically in primates. After intensive studies of many research groups all over the world in the past decade, the osteoinductivity of some calcium phosphate ceramics has been widely accepted by biomaterial circles [5]. Owing to its excellent performance as bone substitute and its potential use as scaffold materials in bone tissue engineering [6], osteoinductive calcium phosphate ceramics has attracted much research interests. The mechanism of osteoinduction is the focus of exploration. However, it is very difficult for researchers to identify the contribution of individual factors to the osteoinductivity of materials *in vivo* due to the complexity of tissues and organs in animals and the difference between animal species. An *in vitro* study in simulated physiological environment of the body is a good choice to solve this problem. The formation of a bone-like apatite layer on biomaterials is assumed to be the precondition for their osteoinductivity to induce bone formation on the biomaterials in non-osseous site [6]. Therefore, the research of the factors effecting bone-like apatite formation is an effective approach to understand the mechanism of osteoinduction. The research method of bone-like apatite formation *in vitro* commonly is to immerse specimen in static SBF and bone-like apatite layer can be formed on all kinds of bioactive materials [7–11]. These results from the immersion experiments in static SBF could not explain why only calcium phosphate ceramics with certain microstructure and chemical composition posses osteoinductivity. The major drawback of these *in vitro* experiments is in that body fluids are always cycling in body while SBF used in *in vitro* experiments were static [11]. Therefore, an *in vitro* experiment with dynamic SBF to mimic the flowing of body fluid *in vivo* is of great significance. In this study, we investigated

*Author to whom all correspondence should be addressed.

the bone-like apatite formation on calcium phosphate ceramics in SBF flowing at a rate similar to that of body fluid of human in muscle [13]. Our objective is to evaluate the factors to effect the apatite formation and the possibility of calcium phosphates formation on surface of porous calcium phosphate ceramics.

2. Materials and methods

2.1. Materials and equipment

Powder of biphasic porous hydroxyapatite/tricalcium phosphate (HA/TCP = 70/30) was prepared in our lab. Previous study indicated that HA/TCP (70/30) is one of the best osteoinduction materials. Biphasic ceramics were foamed with H_2O_2 and sintered at 1200 °C. The porosity of the materials was 50–60% and the pores in the samples were interconnected. Samples were cylinders of 4 mm in diameter and 8 mm in length. The ion composition of SBF was nearly the same as that of the plasma of human [14]. The SBF of 1 liter was prepared by dissolving NaCl 7.995 g, $NaHCO_3$ 0.353 g, KCl 0.224 g; $K_2HPO_4 \cdot 3H_2O$ 0.228 g, $MgCl_2 \cdot 6H_2O$ 0.305 g, $CaCl_2$ 0.227 g, Na_2SO_4 0.0710 g into distilled water and, adjusting pH with tris-hydroxymethylaminomethane and HCl to 7.4. The cycling equipment of SBF was shown in Fig. 1. The sample chamber and storage tank of SBF solution were immersed in an incubator to keep the temperature of the solution at 36.5 ± 1 °C. The SBF solution was pumped from storage tank into the sample chamber of 100 ml and passed the sample, then returned into the tank. The peristaltic pump was used to control the rate at which SBF solution flowed through the sample chamber.

2.2. Experimental procedure

Each time 3 samples were immersed in SBF solution of the sample chamber. The volume of the sample chamber was 100 ml. The flow rate of SBF solution flowing through the sample chamber was expressed as refresh ratio of the SBF solution in the chamber by flowing. The flow rate of 2 ml/100 ml·min meant that 2 ml among 100 ml solution in sample chamber flowed out and 2 ml of solution from the store tank was pumped into the chamber at the same time. Three flow rates were chose in this study. The flow rate 2 ml/100 ml·min was chose because it is nearly the same as that of body fluid in muscle. The flow rate 0 ml/100 ml·min (static state) and 10 ml/100 ml·min (much higher than normal) were chose as contrast in two extremes of the body fluid rate. The *in vivo* experiment showed that the deposition of new bone occurred and went into active period about 14 days after implantation into bone [15]. So, experiment was conducted according to the conditions shown in Table I. The SBF was changed every other day to keep the composition of the solution constant during the immersion experiments.

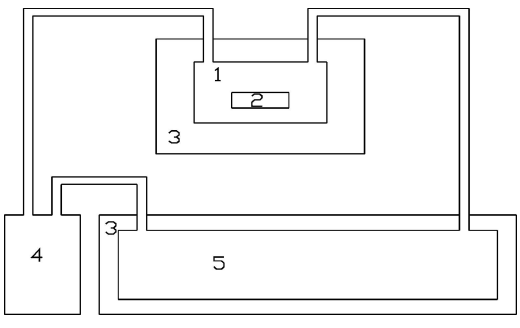

Figure 1 Schematic diagram of flow chamber system: (1) sample chamber; (2) sample; (3) water bath; (4) pump; (5) SBF storage tank.

TABLE I Experimental condition of immersion (36.5 °C)

Flow rate of SBF	Static	2 ml/100 ml·min	10 ml/100 ml·min
SBF	14 days	14 days	14 days
1.5 SBF*	7 days	7 days	7 days

*The concentration of Ca^{2+} and HPO_4^{2-} in 1.5 SBF is 150% of that in SBF.

Samples were rinsed with distilled water after immersion and dried at 50 °C. Samples without subjecting to immersion in SBF were used as control. The formation of apatite layer on the surface of samples and on the wall of pores was identified by the separate observation of the surface of samples and the section of the samples with scanning electron microscopy (SEM). The chemical composition of the sample surface was analyzed with reflectance infrared spectroscopy (RIR). The dissolution of calcium phosphate and formation of bone-like apatite on the samples may result in the changes in ion concentration of Ca, P in SBF. Detection of the changes in ion concentration of Ca, P in SBF helps us understand the mechanism of bone-like apatite formation. The changes in ion concentration of immersion solution were obtained by testing the ion concentration of 2 ml solution taking from sample chamber at time points of 4, 8, 16, 24, 48, 72, 96, 120, 144, 168 h after immersion.

3. Results

Table II shows the examination results of SEM, which clearly indicates that the apatite formation relies on the flow rate and concentrations of solution. The morphology of the samples obtained with SEM was shown in Figs. 2 and 3. There was no change could be observed on the sample surface after 14 days immersion in SBF flowing at normal physiological rate of tissue fluid while flake-like crystal could be found on the internal surface of pores (Fig. 2(d)). On the contrary,

TABLE II Morphology of the porous ceramics after immersion in SBF and 1.5 SBF

Flow rate of SBF	Static	2 ml/100 ml·min	10 ml/100 ml·min
SBF (14ds)			
Surface	Needle-like (Fig. 2(b))	No changes	No changes
Section	Litter potion (Fig. 2(c))	Flake -like (Fig. 2(d))	No changes
1.5 SBF (7ds)			
Surface	Flake-like (Fig. 3(a))	Flake-like (Fig. 3(c))	No changes
Section	Flake-like (Fig. 3(b))	Flake-like (Fig. 3(d))	Flake-like (Fig. 3(e))

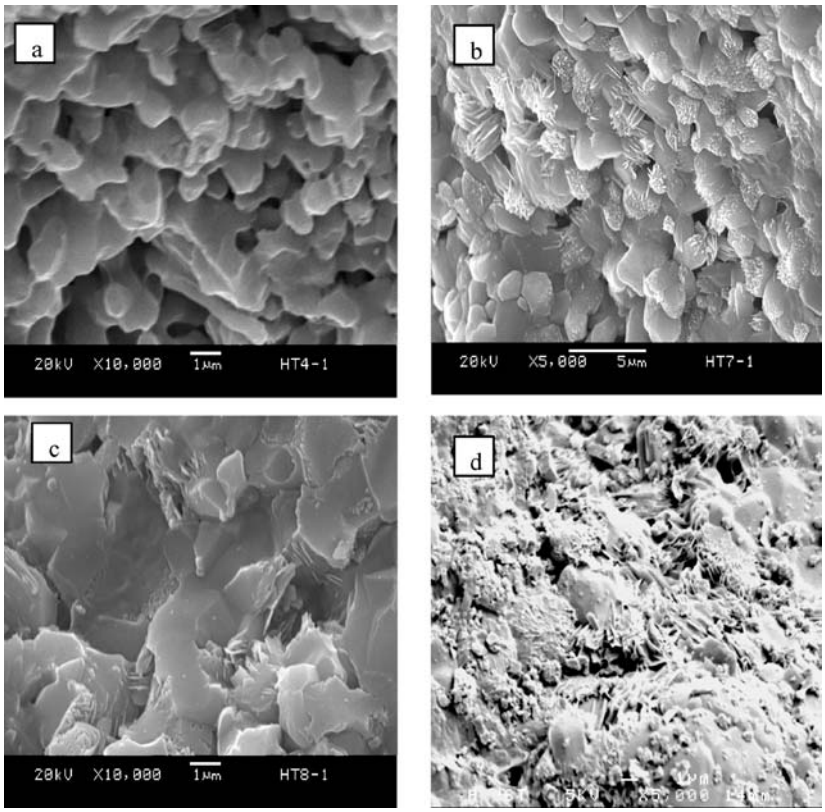

Figure 2 SEM micrograph of calcium phosphate ceramics before and after immersion for 14 days in SBF: (a) the outer surface of HA/TCP before immersion; (b) apatite formed on the surface in static SBF; (c) apatite formed in the internal surface of the pores in static SBF; (d) apatite formed in the internal surface of pores in SBF flow at rate 2 ml/100 ml·min.

crystal deposition appears on the surface of the samples in static SBF (Fig. 2(b)); formed little crystal can be also observed in pores (Fig. 2(c)). No obvious changes could be found both on the surface and in the pores of the samples when SBF flowed at the rate of 10 ml/100 ml·min.

Fig. 3 shows the apatite formed on calcium phosphate ceramics immersed for 7 days in 1.5 SBF. When samples were immersed in 1.5 SBF, flake-shaped crystal could be found both on pore wall and outer surface of materials immersed in static solution and in solution flowing at a physiological rate of tissue fluid. When 1.5 SBF flows at a rate (10 ml/100 ml·min) higher than physiological rate, evenly distributed flake-shaped crystal could also be found in pores (Fig. 3(e)), but no changes could be found on the outer surface of the materials.

The composition of sample surface was analysis by a reflection infrared (R-IR). Fig. 4 shows the results of R-IR. Before immersion, a peak appears in 870 cm^{-1}, which is specific for HPO_4^{2-} (Fig. 4(a)). After immersion, two peaks, which represent CO_3^{2-}, appear at 1320–1530 cm^{-1}(Fig. 4(b) and (c)), and while the peak of 870 cm^{-1} specific for HPO_4^{2-}also appears (Fig. 4). The C─O absorption bands due to CO_3^{2-} group were presented in the porous HA/TCP samples. This result indicated the CO_3^{2-} ions in the simulated body fluid substituted a part of HPO_4^{2-}. The calcium phosphate formed on the HA/TCP ceramics surface is apatite. This result is similar to bone apatite. So the apatite was called bone-like apatite. Similar results were reported by other researcher [7–10].

As shown in Fig. 5, the profiles of Ca concentration change of SBF solution during immersion both in dynamic and static condition were somewhat similar each other. The concentration of Ca ions in SBF increases with the immersion time in the first several hours, then the increment rate is getting slow in the following 30 h. After 2 days immersing, the concentration of Ca ions decreases with time. The difference between static and dynamic SBF is that the concentration of Ca ions in static condition increases faster in first several hours and decreases slower after two-day immersion than that in dynamic condition. The variation of P ion concentration with immersion time is different from that of Ca ion concentration in first two days. After the fast increment in first several hours, the Ca ion concentration is no much change in the following 40 h, while the concentration of P ion concentration appears a continued increment.

Fig. 3(f) shows the examination results of SEM, which clearly indicates that flake-like crystal could be found on the wall of pores of HA/TCP porous ceramic implanted in muscle of dogs for 15 days. Crystal deposition could be also observed on the surface of the concave regions of the samples when samples were implantation in dogs' body.

4. Discussion

4.1. Mechanism of bone-like apatite formation

The process of bone-like apatite formation on the surface of calcium phosphate ceramics actually is a process

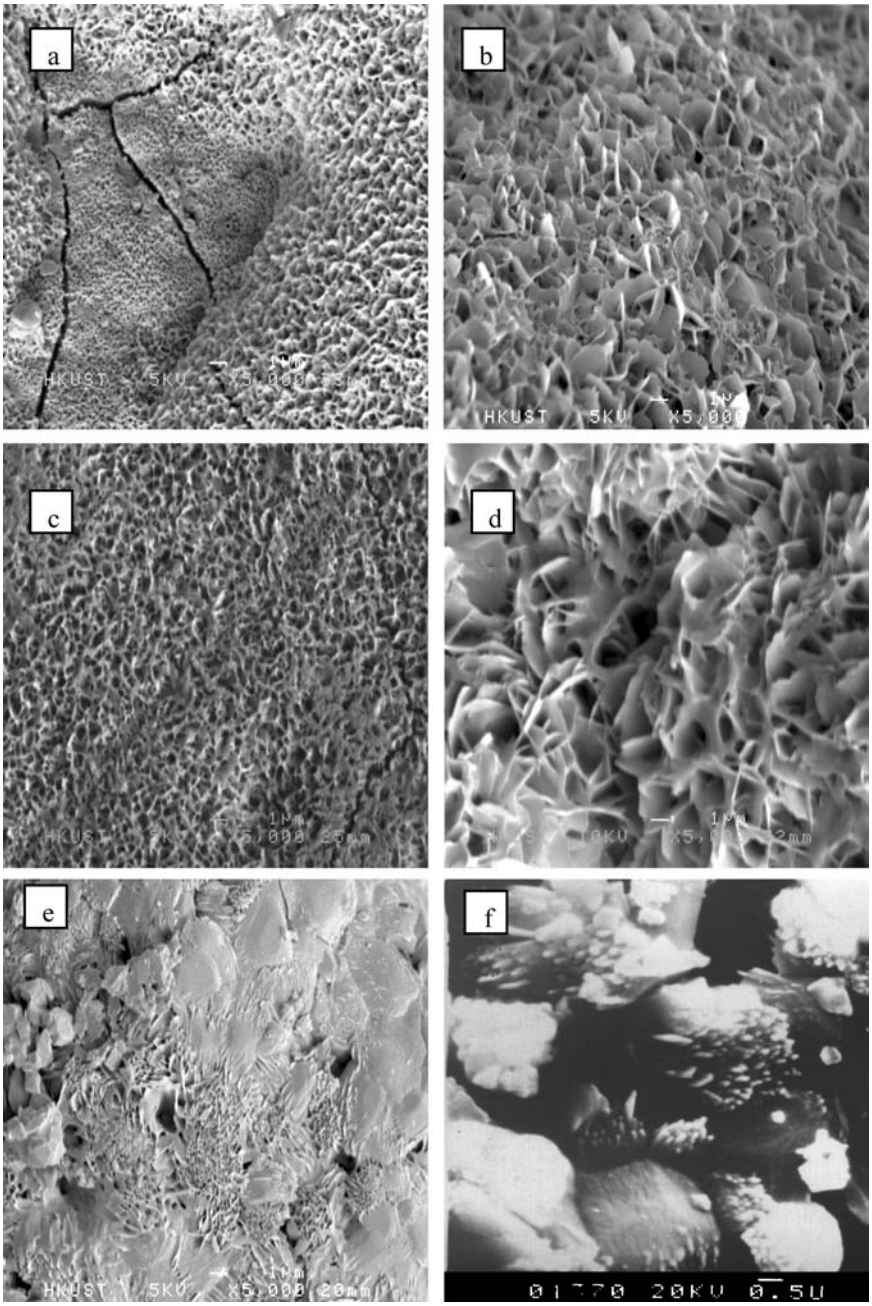

Figure 3 SEM micrograph of apatite formed in HA/TCP ceramics immersing in 1.5 SBF for 7 days: (a) on the surface in static; (b) in pores in static; (c) on the surface at flow rate 2 ml/100 ml·min; (d) in the pores at flow rate 2 ml/min; (e) in the pores at flow rate 10 ml/min. (f) Flake-like Crystal formed on the wall of pores of HA/TCP porous ceramic implanted in muscle of dogs for 15 days.

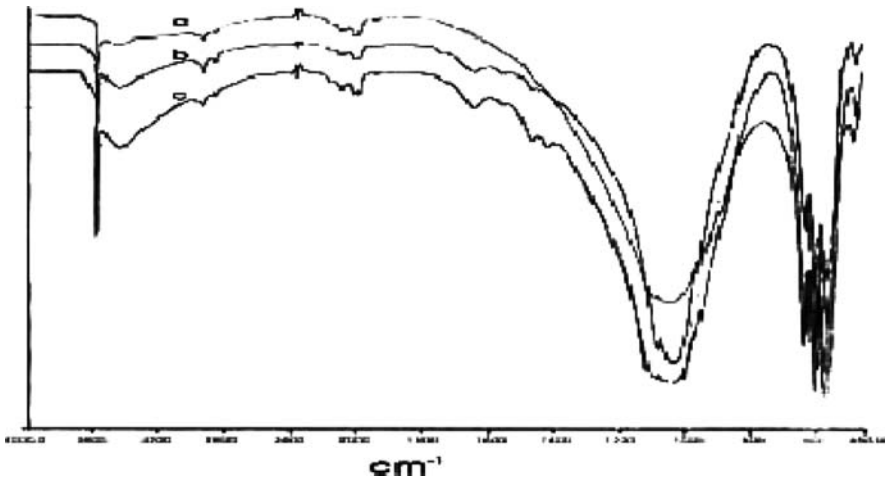

Figure 4 RIR spectra for the HA/TCP before and after immersion in 1.5 SBF: (a) before immersion; (b) after immersion for 7 days in 1.5 SBF; (c) after immersion for 7 days in 1.5 SBF flows at rate 2 ml/100 ml·min.

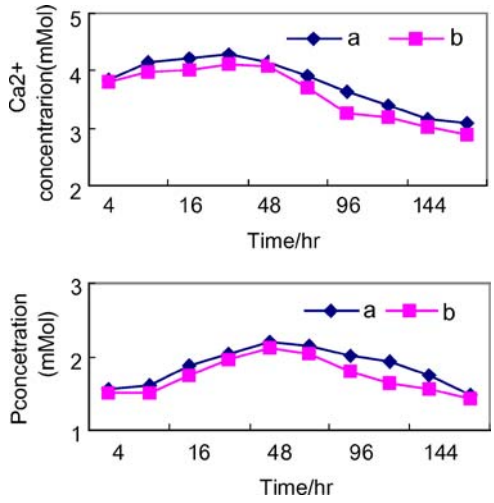

Figure 5 Changing profile of Ca, P in the immersion solution: (a) in static condition; (b) in dynamic condition (2 ml/100 ml·min).

of phase transformation in which a new solid phase grows up from liquid phase [16]. This process can be divided into two stages: nucleation and crystal growth. The apatite formation from SBF solution usually is considered as a process of heterogeneous nucleation on calcium phosphate ceramics following the spontaneous growth of crystals. The enough supersaturability of Ca and P ion concentration in the immersion solution and suitable nucleation sites on the specimen is the prerequisite for heterogeneous nucleation. So the first stage of the phase transformation, the nucleation, is critical to apatite formation. The induction period for the apatite nucleation depends largely on the parameters of SBFs and conditions of experiments. Normal SBF does not provide the sufficient supersaturation of Ca and P ions required for spontaneous nucleation, but it is sufficient for the growth of apatite crystal. Thus, higher super-saturation of Ca and P in the SBF near the solid surface is required for nucleation than for crystal grow. The nucleation can only happened when the local Ca ion concentration in SBF near the surface of samples reaches to a certain level, which is higher than that of the standard SBF. This phenomenon was observed by examining concentration changes in SBF. Either dissolution of HA/TCP ceramics or adding Ca and P ions to the SBF can provide the super-saturation for nucleation. Only when Ca^{2+} and HPO_4^{2-} concentration on the surface of calcium phosphate ceramics reaches the threshold for nucleation, could crystal nucleation occur on the surface of material. The induction period became shorter and thus apatite formation is accelerated when Ca^{2+} and/or HPO_4^{2-} concentrations rise in SBF. The local concentration of Ca^{2+} and HPO_4^{2-} on the surface of materials is higher, the faster the speed of nucleus formation is. In closed static solution, nucleation formation is faster than in dynamic solution. When the crystal nucleus grows to a certain size, it becomes stable and will grow naturally if sufficient ions needed are available. Once the apatite nuclei were formed they grew [17], taking a spherulitic form by consuming the calcium and phosphate ions from the surrounding fluid. Each spherulite consisted of a lot of flake that clustered into a petal-like morphology. The flake was carbonate-containing hydroxyapatite of small-crystallites and/or defective structure. The Ca/P ration of the apatite was estimated as 1.5–1.67. Thus, the apatite formed was able to induce secondary nucleation of the apatite.

4.2. The precipitation sequence of Ca^{2+} and HPO_4^{2-} in the process of nucleation

The adhesion of ions to solid surface is related to the pH of medium. When pH of the medium is higher than the isoelectric point of the surface, solid surface would be negative electrically, which showed strong adsorption to positive ions. The isoelectric point of calcium phosphate ceramics is lower than that of SBF and therefore, the surface of calcium phosphate ceramics is electrically negative in SBF [18, 19]. So, calcium phosphate ceramics shows strong adsorption to positive ions in SBF solution at the first stage of bone-like apatite formation. Then, opposite ions, i.e. negative ions are attracted to the solid-liquid interface. We deduce that Ca^{2+} is appeared on the surface of calcium phosphate ceramics first and subsequently HPO_4^{2-} is attracted.

4.3. The changing for Ca, P concentration in immersion solution

The release of Ca^{2+}, HPO_4^{2-} and PO_4^{3-} form the surface of calcium phosphate ceramics would result in the increase of concentration of Ca and P in the solution as shown in Fig. 5. These ions, together with ions in SBF, react directly with the solid surface of the material through static electricity and finally result in adhesion of ions in solution to the solid surface. When the concentration of related ions reaches a certain value in SBF, nucleation would occur and subsequently the second stage of bone-like apatite formation began. Once crystal nucleus forms, crystal grows rapidly, which result in decrease of concentration of Ca and P in the solution as observed in Fig. 5.

4.4. Bone-like apatite formation in static SBF

When SBF is static, Ca^{2+}, HPO_4^{2-} released from the material surface can not easily disperse and the resultant relatively high concentration of Ca^{2+} and HPO_4^{2-} near the surface of the samples may reach the threshold of nucleation. After nucleation, growth of crystal consumes great amount of ions from the SBF solution, which results in the decrease of ion concentration near the sample surface to a level lower than standard SBF. Because SBF is static, ions could only be driven to move by ion concentration gradient. In a certain time period, the ions needed for crystal growth are more than that dissolved from the surface and thus, once nucleation occurs on some spot of the surface, new nucleation at the near spot of established crystal nucleus will be suppressed, newly released ions from the samples surface, together with those in SBF, are used for the growth of crystal. This results in the vertical outgrowth of the crystal from the surface as shown in Fig. 2.

4.5. Bone-like apatite formation in dynamic SBF

In dynamic solution, ions are driven to move by two different mechanisms transmission. The first is the concentration-gradient-driven ion dispersion. The second is the stress-gradient-driven ion transportation. The resultant ion movement in solution is called convective dispersion [11]. The flowing rate caused by convection is in direct proportion to the flowing rate and ion concentration of the solution [14]. In cycling SBF, ions dissolved from sample surface can easily leave the sample surface to enter the SBF solution under the function of concentration-gradient-driven dispersion and stress-gradient-driven transportation. Ca and P concentration near the sample surface can only be slightly higher than that in solution and thus, ions can not easily concentrate on the surface. The threshold for nucleation is not easily reachable. The SBF flowing may take away the excessive Ca and P ions from the surface of samples and destroy supersaturating of Ca and P caused by the dissolution of HA/TCP ceramics, thus the nucleation on the surface will be prevented. In our experimental condition that SBF flowed at physiological rate, there was no bone-like apatite formation on the outer surface of the samples. On the contrary, in the internal surface of the pore of the sample, relatively high concentration of Ca and P could be reached due to the dissolution of Ca and P from the large internal surface and slower rate caused by sinus internal structure and so, the Ca and P concentration in internal surface of the pore of the sample is higher than that on the outer surface and nucleation and subsequent crystal growth could eventually occur there.

The bone-like apatite was formed on the bioactive ceramics before bone tissues were induced on or near the ceramics. The apatite plays a key role in bone bonding of bioactive materials [23]. If the bone-like apatite also plays an important role in osteoinduction of biphasic calcium phosphate ceramics? The fact that both the apatite formation in dynamic SBF and the osteoinduction *in vivo* in biphasic porous calcium phosphate ceramics happened only in the pores of the ceramics implicated that the bone-like apatite may play an important role in the osteoinduction of Ca-P materials. This is our research project under way.

The result that the apatite formed on the surface (Fig. 2(b)) when the flow rate was 0 ml/min was in agreement with other results obtained from immersion studies in static SBF [7–10], static SBF was a special case of dynamic SBF with flow rate 0ml/min. The result also demonstrated that local ion concentration in solution near the nucleation site on the samples played a key role in nucleation.

4.6. Bone-like apatite formation in 1.5 SBF

Increase Ca^{2+} and HPO_4^{2-} concentration in SBF facilitates the maintenance of high concentration near the surface so that the threshold of ion concentration for nucleation can be easily reachable. Nucleation and crystal growth can easily occur. Evenly distributed bone-like apatite was observed on both outer surface and in internal surface of in the internal surface of the pore of the sample in most circumstances. Even when the flowing rate is faster than the normal physiological rate of tissue fluid, bone-like apatite could form inside the sample. Immersion experiment in 1.5 SBF confirmed that sufficient ion concentration near the material surface to reach the threshold of nucleation is the key to bone-like apatite formation.

Obviously, the SBF flow effect is much less effective in the internal surface of the pore of the sample, and the formation of apatite on the internal surface of the pore wall is easy to be understood. This implies that *in vivo* bone-like apatite formation is more difficult on the HA/TCP implant surfaces contacting body fluid flow directly. This inference has been confirmed by our observation of apatite formation in porous CaP implanted in dogs: bone-like apatite formed only on the pore's wall inside (Fig. 3(f)). On the other hand, artificially increasing the Ca and P concentrations of SBF can compensate the flow effects as indicated in Table II. The 1.5SBF provides super-saturation concentration for nucleation when SBF flows at the physiological rate (2 ml/100 ml·min). When SBF flowed at 10 ml/100 ml·min, the apatite could not form at the surface. The possible reason is that the fast flowing of solution destroyed the stability of the nucleation sites and prevented ions in solution from being precipitated.

Experiments of *in vitro* immersion in dynamic SBF can better simulate conditions of bone-like apatite formation in human body than that in static SBF, which was used in most *in vitro* research. Thus, such experiments are useful for the research of the osteoinduction of biomaterials and the formation mechanism of bone-like apatite, and may enable us more exactly to foresee osteoinductivity of bioceramics.

5. Conclusions

This study has analyzed some key factors affecting bone-like apatite formation. It is helpful to the understanding of the mechanism of bone-like apatite formation, the control of its growth and furthermore, the understanding of the osteoinductivity of calcium phosphate ceramics. Bone-like apatite can only formed in the internal surface of the pores when SBF flows at physiological rate of tissue fluid. Bone-like apatite formation in dynamic SBF is a better model than that in static SBF for it is more similar to the real condition of the body.

Acknowledgments

This work was financially supported by the key basic research project of China, contract No.G1999064760.

References

1. X. ZHANG, W. CHEN, Y. LI, C. KLEIN and K. DE GROOT, in "Trans of 19th Annual Meeting of the SOCIETY FOR BIOMATERIALS" (Birmingham, AL, USA. 1993) 299.
2. C. KLEIN, K. DE GROOT, J. ZHOU, Y. LI and X. ZHANG, in "Trans of Fourth World Biomaterials Congress" (Berlin, Germany, 1992) 261.

3. M. HEUGHEBEART, R. Z. LEGEROS, M. GINEST and G. BONEL, *J. Biomed. Mater. Res.* (*Appl. Biomater.*) **22** (1988) 257.
4. U. RIPAMONTI, in "CRC Handbook Ceramics" (Boca Raton, FL, CRC Press, 1990) 245.
5. X. ZHANG, H. YUAN and K. DE GROOT, in "Notebook: Workshop1#, Biomaterials With Intrinsic Osteoinductivity. The 6th World Biomaterials Congress" (Hawaii, USA, 2000).
6. J. D. DE BRUIJN, H. YUAN, R. DEKKER, P. LAYROLLE, K. DE GROOT and VAN C. A. BLITTERSWIJK, in "Bone Engineering" (Toronto, Canada, em Squared Incorporated 2000) 421.
7. T. KOKUBO, *Mater. Sci*. Foru. **293** (1999) 65.
8. H. M. KIM, H. TAKADAMA, F. MIYAJI, T. KOKUBO, S. NISHIGUCHI and T. NAKAMURA, *J. Mater. Sc.: Mater Med*. **11** (2000) 555.
9. T. KASUGA, Y. HOSOI, M. NOGAMI and M. NIINOMI, *J. Amer. Ceram. Soc.* **84** (2001) 450.
10. J. M. BOULER and G. DACULSI, *Key Engng. Mater.* **192–195** (2001) 119.
11. Z. L. TAO, in "Biohydrodynamics" (Beijing, Science Publisher, 1984) (in Chinese).
12. Y. LENG, J. Y. CHEN and S. X. QU, *Biomaterials* **24** (2003) 2125.
13. X. H. WU, in "Physiological Data of Human Body" (Beijing, Science Popularization Publisher, 1987) 68 (in Chinese).
14. T. KOKUBO, S. ITO and T. YAMAMURO, *J. Biomed. Mater. Res*. **24** (1990) 331.
15. W. Q. CHEN, S. X. QU, Z. J.YANG, X. D.ZHANG and M. Q.YUAN, *Key Eng. Mater.* **115** (1996) 233.
16. I. V. MARKOV, in "Crystal Growth for Beginners: Fundamentals of Nucleation, Crystal Growth and Epitaxy" (Singapore, World Scientific Publishing Co. Pte. Ltd, 1995) 62.
17. P. LI, C. OHTSUKI, T. KOKUBO, K. NAKANISHI and N. SOGA, *J. Am. Ceram. Soc.* **75** (1992) 2094.
18. R. M. KOWALCHUK, S. R. POLLACK, P. DUCHEYNE and L. A. KING, *J. Biomed. Mater. Res.* **27** (1993) 783.
19. S. A. MEZZASALMA, *J. Colloid Inter. Sci.* **190** (1997) 302.
20. U. RIPAMONTI, in "Bone Engineering" (Tornto, Canada,em sqared incorporated, 2000) 215.
21. U. RIPAMONTI, *Matrix* **13** (1993) 491.
22. C. KLEIN, K. DE GROOT, W. Q. CHEN, Y. B. LI and X. D. ZHANG, *Biomaterials* **35** (1994) 31.
23. T. NAKAMURA, M. NEO and T. KOKUBO, in "Mineralization in Natural and Synthetic Biomaterials" (Materials Research Society, Warrendale, Pennsylvania, 2000) 15.

Received 4 February
and accepted 20 May 2004