α-三リン酸カルシウム球状粒子を用いた三次元連通孔型リン酸カルシウムセメント

Kien, Pham Trung
Graduate School of Dental Science, Kyushu University

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Fully-interconnected macropore forming calcium phosphate cement using $\alpha$-TCP microspheres

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By PHAM TRUNG KIEN
Department of Biomaterials
Faculty of Dental Science
Kyushu University, Fukuoka, Japan
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CHAPTER 1
General introduction

In this thesis, fully-interconnected macropore forming calcium phosphate cement (FMF-CPC) is proposed and the behavior of FMF-CPC in simulated body fluid (SBF) at 37°C is studied. In this chapter, research background for the development of FMF-CPC is stated as a general introduction.

1.1 Role of artificial bone substitute for bone reconstruction

Bone reconstruction treatment is one of the major challenges in dental, maxillofacial surgery and orthopedic surgery. For the bone grafts used for bone reconstruction treatment, basically four graft materials are known. They are autograft, allograft, xenograft and artificial bone substitutes, which have both advantages and disadvantages as follow:

Autograft is still considered to be the "gold standard". Although they have unsurpassed biological activity even in implant site with
low osteogenic potential, harvesting of grafts require a second operative procedure associated with a specific morbidity. Also, the amount of material that can be retrieved from one individual patient is limited.

Allograft which is bone taken from somebody else’s body could be an alternative. However, allograft bone also introduces the possibilities of immune rejection and pathogen transmission from donor to host, and in some cases, infections could happen in the recipient’s body after transplantation.

Xenograft is the bone taken from another species. Although there is no limitation in the amount, the risk of immunologic reaction is high when using xenograft.

In contrast to these three kinds above, artificial bone substitute is not the bone originated materials but man-made materials. Therefore, it is free from harvesting problem and infection or immune problems. There are several artificial bone grafts. Among them, hydroxyapatite (HAp; Ca_{10}(PO_{4})_{6}(OH)_{2}) plays dominant role since its has similar chemical structure to that of bone apatite and
demonstrate excellent tissue respond and osteoconductivity (Yuasa et al. 2004, Zhou et al. 2004, Yanagida et al. 2009, Xu et al. 2009). Productions of these artificial bone grafts are clinically available as either calcium phosphate ceramic blocks or calcium phosphate granules. Despite their excellent biological behavior, calcium phosphate ceramic blocks are difficult to shape, resulting in poor contour or incomplete filling of a defect area. Calcium phosphate granules, on the other hand, still have some drawbacks. For example, they can be easily migrated or dispersed into the surrounding tissue.

1.2 Calcium phosphate cement (CPC)

In an attempt to overcome these drawbacks, calcium phosphate cement (CPC) with setting property have been invented in 1976 by Monma and Kanazawa (Monma and Kanazawa 1976). CPC was a big invention in biomaterials and has altered the surgical procedure of bone reconstruction and regeneration. Calcium phosphate cement is a mixture of one or more compound(s) of calcium phosphate powder(s) phase mixed with liquid phase, and
CPC sets at body temperature to form apatite or brushite (CaHPO$_4$·2H$_2$O) cement by interlocking of the crystals precipitating within the cement. CPC can be shaped according to the defect dimension and set \textit{in situ}. This key advantage allows CPC to fill and follow the morphology of the defect, giving stability to the defect geometry as shown in Fig.1.1.

Fig.1.1 Photographs of CPC with shaping ability.
As mentioned above, CPC is classified into 2 categories: apatite cement and brushite cement. For apatite cement, several different cement compositions have been developed. For example an equimolar mixture of tetracalcium phosphate (TTCP; Ca$_4$(PO$_4$)$_2$O) and dicalcium phosphate anhydrous (DCPA; CaHPO$_4$), $\alpha$-tricalcium phosphate ($\alpha$-TCP; $\alpha$-Ca$_3$(PO$_4$)$_2$), mixture of $\alpha$-TCP and DCPA are known as apatite cement. On the other hand, brushite cement which is invented by Mirtchi and Lemaitre (Mirtchi et al. 1989a, Mirtchi et al. 1989b), have raise remarkable interest in the last decade because it has higher solubility compared to HA cement in physiological conditions. **In vitro** and **in vivo** studies also confirmed that brushite can be degraded quickly when implanted (Frayssinet et al. 1998, Penel et al.1999, Grover et al. 2003, Theiss et al. 2005). Setting mechanism of brushite cement is also a dissolution-precipitation reaction similar to apatite cement. But the pH of the cement should be 4.2 or lower for the brushite formation. Brushite cement is sometimes called $\beta$-tricalcium phosphate ($\beta$-TCP; $\beta$-Ca$_3$PO$_4$) since its key components is $\beta$-TCP. Up to now, several formulations have
been already proposed, such as the mixture of $\beta$-TCP and monocalcium phosphate monohydrate (MCPM; $\text{Ca(H}_2\text{PO}_4\text{)}_2 \cdot \text{H}_2\text{O}$), the mixture of $\alpha$-TCP and MCPM or the mixture of $\beta$-TCP and $\text{H}_3\text{PO}_4$ solution. The setting time of brushite cement depends on the CPC formulation. For example, $\beta$-TCP + MCPM mixture has setting time around 30 to 60 sec whereas $\alpha$-TCP + MCPM mixture has setting time around a few second as shown in equations (1.1) and (1.2) (Mirtchi et al. 1989a, Mirtchi et al. 1989b). On the contrary, apatite cement set much slower than brushite cement; for example the setting time of apatite cement is longer than 30 min without using accelerator.

$$\alpha\text{-Ca}_3(\text{PO}_4)_2 + \text{Ca(HP}_2\text{PO}_4\text{)}_2 \cdot \text{H}_2\text{O} + 7\text{H}_2\text{O} \rightarrow 4\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \quad (1.1)$$

$$\beta\text{-Ca}_3(\text{PO}_4)_2 + \text{Ca(HP}_2\text{PO}_4\text{)}_2 \cdot \text{H}_2\text{O} + 7\text{H}_2\text{O} \rightarrow 4\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \quad (1.2)$$

1.3 Importance of pore size and its interconnectivity in CPC

As the bone graft, CPC not only need to mimic the
composition but also morphology of cancellous bone. Cancellous bone has three dimensional interconnected porous structures as shown in Fig.1.2. The pore in the cancellous bone is connected together through the pathway between these connected pores. The pore interconnectivity and pore size of natural bone are necessary for bone tissue formation because they allow migration and proliferation of osteoblasts-like cell, as well as vascularization. In this note, it has to be emphasized that the interconnectivity of the pore size gives a strong influence on bone deposition rate when implanted. During the bone healing process, the bone tissue grows well into the interconnected pore through the connection pathway, thus increase the mechanical interlocking at the interface between implant and natural bone.

However, the size of the pores in set CPC is less than 1 μm, and thus, too small to allow fast bone ingrowth and the CPC degrades layer-by-layer. This feature is the main drawback of CPC when compared to CPC with fully-interconnected macroporous structure. Regarding to the pore size of set CPC, the pore size needs
to be larger than 100 $\mu$m for cell penetration to implanted material due to cell size, migration requirements and transport (Klawitter et al. 1971, Blitterswijk et al. 1986, Schliephake et al. 1991, Hench et al. 1998, John et al. 2007, Belyakov et al. 2008).

Fig. 1.2 Macroscopic morphology of cancellous bone with interconnected porous structure. The interconnected porous structure is necessary for vascularization and supports for cells proliferation deep into the interior of the natural bone.
1.4 Previous studies for fabrication of CPC with interconnected macroporous structure

Many researchers attempt to fabricate the CPC with fully-interconnected macroporous structure. For example, macropores can be introduced into CPC by introducing porogen into CPC. Another example is the using of water-soluble mannitol crystals (Xu et al. 2001, Xu et al. 2004a, Tajima et al. 2006, Cama et al. 2009, Shimogoryo et al. 2009), sucrose granules (Takagi et al. 2001), gelatin powder (Yin et al. 2003) or PLGA microparticles (Ruhe et al. 2005, Link et al. 2006, Habraken et al. 2006) as a porogen. After the completed hardening of the material, the porogen in CPC dissolved upon contact with a physiological solution, and created macropores. However, macropores obtained by these methods are not interconnected pores but closed pores since the porogen does not contact each other.

Xu and Quinn introduced interconnected macroporous CPC using degradable-fibers mesh with interconnected porous structure as porogen (Xu et al. 2002, Xu et al. 2004b). They found
interconnected pores after the dissolution of the degradable-fibers mesh. The drawback of using degradable-fibers mesh is the long time for its degradation. For example, the degradable-fiber mesh took 12 weeks for degrade when the CPC was immersed in saline solution at 37°C.

Karashima reported another method without using porogent for the fabrication of fully-interconnected macroporous calcium phosphate scaffold based on the setting reaction of α-tricalcium phosphate (α-TCP; Ca₃(PO₄)₂) foam granules (Karashima et al. 2009). The α-TCP foam was made and crushed to prepare α-TCP foam granules, and its setting ability was evaluated. The α-TCP foam granules set partially at 100°C and 100% relative humidity for 24h, and fully set within 24h when the temperature was raised to 200°C. Although this method can avoid using porogent for interconnected macropore forming, this method requires the high temperature (up to 200°C) and long setting time (up to 24 hours). This is thought to be caused by the slow conversion of α-TCP foam granules to HAp at physiological condition.
Fortunately, brushite cement is known to set much faster when compared to apatite cement as already stated in this chapter. Therefore, it is possible to fabricate fully-interconnected macroporous structure based on the setting reaction of cement at physiological condition.

Consequently, the objective of this study is to fabricate fully-interconnected macropore forming calcium phosphate cement based on setting reaction of brushite cement. The $\alpha$-TCP microspheres are used instead of $\alpha$-TCP powder for the fabrication of fully-interconnected pores.

In chapter 2, the fabrication of fully-interconnected macropore forming calcium phosphate cement using $\alpha$-TCP microspheres and acidic calcium phosphate solution is stated.

In chapter 3, the behavior of FMF-CPC in simulated body fluid (SBF) at 37°C is studied.
CHAPTER 2

Fabrication of fully-interconnected macropore forming calcium phosphate cement using $\alpha$-TCP microspheres and acidic calcium phosphate solution

Abstract

Fully-interconnected macropore forming calcium phosphate cement (FMF-CPC), that forms interconnected pores with diameter of 300-400 $\mu$m could be an ideal bone substitute. In this study, FMF-CPC consisting of $\alpha$-tricalcium phosphate ($\alpha$-TCP; $\alpha$-Ca$_3$(PO$_4$)$_2$) microspheres and acidic calcium phosphate solution (0.2 mol/L monocalcium phosphate monohydrate - 0.1 mol/L phosphoric acid) was proposed and its basic setting reaction was studied. When $\alpha$-TCP microspheres with 1.3 mm diameter were exposed to the acidic calcium phosphate solution, brushite (CaHPO$_4$·2H$_2$O) was formed on the surface of the $\alpha$-TCP microspheres. The formed brushite crystals interlocked with each other, resulting in a setting reaction of $\alpha$-TCP microspheres within 10
min at 37°C. As a result of this setting reaction, a fully-interconnected calcium phosphate macroporous structure was obtained. The porosity and the average pore size of the resultant FMF-CPC were 49.7 ± 2.5 % and 384 ± 250 μm, respectively.

2.1 Introduction

In this chapter, feasibility for the fabrication of fully-interconnected macropore forming calcium phosphate cement (FMF-CPC) is evaluated using α-TCP microspheres with 1.3 mm in diameter and acidic calcium phosphate solution. α-TCP microspheres are employed for the fabrication of a fully-interconnected porous structure. Diameter of α-TCP microspheres was decided as 1.3 mm since its close packing result in fully interconnected porous structure with 100 μm as the smallest diameter of inscribed circle in the center of bonded three microspheres (see Fig.2.1). As mention in chapter 1, several researches indicated that the minimum requirement for pore size is considered to be approximately 100 μm due to cell size, migration requirements and transport.
Fig. 2.1 Schematic diagram of a circle inscribed inside the bonded three microspheres. The inscribed circle has diameter $d$ and microspheres have diameter $D$. If diameter of each microsphere is 1.3 mm, the diameter of inscribed circle is 100 μm.

2.2 Materials and methods

2.2.1 Preparation of $\alpha$-TCP microspheres

For the fabrication of $\alpha$-TCP microspheres, commercially obtained $\alpha$-TCP powder (type B; Taihei, Osaka, Japan) was placed in a stainless steel mould and pressed uniaxially with an oil-pressure press machine (Riken Power, Tokyo, Japan) under 28 MPa pressure. The cylindrical compacts of 3 mm in diameter and 3 mm in height...
were calcined at 1000°C for 5 hours in an electric furnace (Superburn SC 1500D, Motoyama, Osaka, Japan) and then cooled inside the furnace. The calcined α-TCP compacts were ground between abrasive papers pasted on glass plates. Alumina balls with 2.0 mm diameter (Sogorikagaku, Kyoto, Japan) were placed at the four corners of the glass plates to regulate the final diameter of the α-TCP microspheres. The ground α-TCP microspheres were sintered again at 1400°C for 5 hours in the electric furnace and cooled down inside the furnace. The average diameter of the resultant α-TCP microspheres was 1.3 mm.

2.2.2 Preparation of liquid phase for setting reaction

Acidic calcium phosphate aqueous solution was used as a liquid phase. Commercial monocalcium phosphate monohydrate (MCPM; Ca(H₂PO₄)·H₂O) powder (Sigma-Aldrich, Saint Louis, USA) was dissolved into 0.1 mol/L H₃PO₄ solution (Wako, Osaka Japan) so that the concentration of MCPM became 0.2 mol/L. This solution was almost saturated with respect to MCPM. In the following text, this
solution is stated as MCPM-H₃PO₄ for simplicity.

2.2.3 Fabrication of fully-interconnected macroporous calcium phosphate structure and its evaluation

Fully-interconnected calcium phosphate porous structure was fabricated based on the setting reaction of α-TCP microspheres. The α-TCP microspheres were put into a split stainless steel mould (6 mm in diameter × 6 mm in height) and exposed to MCPM-H₃PO₄ solution so that the liquid to solid ratio would be 2 μL per microsphere. After being kept in an incubator (Eyela SLI-220, Tokyo, Japan) at 37°C for 10 min, the set FMF-CPC was rinsed with distilled water 3 times, followed by immersion in acetone to stop the reaction.

Interconnectivity of set FMF-CPC was evaluated using micro-computed tomography (μ-CT, Skyscan 1075 KHS, Kontich, Belgium). Based on 20 slices/mm images, a 3-dimensional structure of the set FMF-CPC was reconstructed. Also, μ-CT data was used to measure the pore size of the set FMF-CPC.

Porosity of the set FMF-CPC was also calculated by the
following equation (2.1):

\[
\text{Porosity} = 100 \times \frac{(d_1 - d_2)}{d_1}
\]  

(2.1)

where \(d_1\) is true density of set FMF-CPC, which was measured by pycnometer flask; \(d_2\) is the bulk density of the set FMF-CPC, which was calculated as the weight to volume ratio.

2.2.4 Analysis of the setting reaction of FMF-CPC

To understand the setting reaction of FMF-CPC, morphological and compositional change of the \(\alpha\)-TCP microspheres were analyzed and bonding strength was measured. Two \(\alpha\)-TCP microspheres were put side by side and exposed to MCPM-\(\text{H}_3\text{PO}_4\) solution with the liquid to solid ratio of 2 \(\mu\text{L}\) per microsphere. The microspheres set and were subsequently placed into an incubator at 37\(\degree\)C and 100\% relative humidity. After being kept in an incubator for 1, 5, 10 and 20 min, the specimens were washed with distilled water 3 times, and then immersed in acetone to stop the reaction. The characterization of the set specimens was described as follow:

\textit{Study of surface morphology:} Surface morphological change
at the interface between the $\alpha$-TCP microspheres due to exposure to MCPM-H$_3$PO$_4$ solution was observed by scanning electron microscope (SEM; JSM 5400LV, JEOL, Tokyo, Japan) at an acceleration voltage of 20 kV.

**X-Ray diffraction analysis:** Compositional change was evaluated by means of X-ray diffraction (XRD; D8 Advance, Bruker AXS, Yokohama, Japan). The set microspheres were ground to powder and then put on the sample holder for powder XRD analysis. Also, thin-film XRD analysis was performed to understand the compositional change at the surface of $\alpha$-TCP microspheres. For thin-film XRD analysis, the set microspheres were put directly on the sample holder. CuK$\alpha$ ($\lambda=1.5406$ Å) radiation generated at 40 kV and 40 mA was used for both powder and thin-film XRD analysis.

**Evaluate the bonding strength:** Bonding strength between the set two microspheres was measured by universal testing machine (Autograph IS5000, SHIMADZU, Kyoto, Japan). Two bonded microspheres were positioned vertically so that one was on top of the other, then a metal rod was connected to the bottom of the lower
microsphere while a non-stress wire was connected to the top of the upper microsphere. The strength was measured under the tensile mode with the cross-head speed of 0.5 mm/min.

2.2.5 Statistical methods

For statistical analysis, one-way factorial ANOVA and Fisher’s LSD method as a post-hoc test, were performed using KaleidaGraph 4.0. Values are expressed as mean ± SD. A p-value of < 0.05 was considered statistically significant.
2.3 Results

Fig. 2.2 shows typical photographs (a, b) and μ-CT images (c, d) of set FMF-CPC fabricated based on the setting reaction of α-TCP microspheres and MCPM-H₃PO₄ solution at 37°C for 10 min. The μ-CT images of the cross section view confirmed that the α-TCP microspheres bonded with each other and that a fully-interconnected porous structure was obtained. Based on μ-CT data, the average pore size was calculated as 384 ± 250 μm (n=94). The porosity of the set FMF-CPC was 49.7 ± 2.5% (n=3).
Fig. 2.2 Typical photographs (a, b) and μ-CT images (c, d) of set FMF-CPC fabricated based on the setting reaction of α-TCP microspheres and MCPM-H₃PO₄ solution at 37°C for 10 min
Fig. 2.3 shows typical SEM photographs at an interface between two $\alpha$-TCP microspheres before and after being treated with MCPM-$\text{H}_3\text{PO}_4$ solution at 37°C for 1, 5, 10 and 20 min. Before treatment, the $\alpha$-TCP microspheres showed a typical smooth surface due to the sintering process. Naturally, no bonding was observed between the two microspheres (Fig. 2.3(a, b)). In contrast, plate-like precipitates were observed on the surface of the $\alpha$-TCP microspheres after being exposed to MCPM-$\text{H}_3\text{PO}_4$ solution at 37°C (Fig. 2.3(c-j)). The precipitates partially covered the surface of $\alpha$-TCP microspheres after 1 min exposure (Fig. 2.3(c, d)) and the length of plate-like precipitates was 20-30 $\mu$m. The whole surface was covered with the new precipitates and the length of the precipitates increased to 30-40 $\mu$m when treated for 5, 10 and 20 min (Fig. 2.3(e-j)). The amount of precipitates increased with reaction time. Newly formed plate-like crystals interlocked with each other, which led to the bonding of the $\alpha$-TCP microspheres.
Fig.2.3 Typical SEM photographs at the interface between two α-TCP microspheres before (a, b) and after being treated with MCPM-H$_3$PO$_4$ solution at 37°C for 1 (c, d); 5 (e, f); 10 (g, h) and 20 min (i, j).
Fig. 2.4 summarizes the powder XRD patterns of the α-TCP microspheres before and after exposure to MCPM-H$_3$PO$_4$ solution at 37°C for 1, 5, 10 and 20 min. XRD patterns of standard α-TCP (Taihei) and standard brushite (Wako) were also shown for comparison. Peaks corresponding to α-TCP were observed on all specimens. In addition, a small peak assigned to brushite was found at 2θ = 11.6° when the α-TCP microspheres were treated with MCPM-H$_3$PO$_4$ solution for 5, 10 and 20 min (Fig. 2.4(c-e)). The peak intensity of brushite was larger when the α-TCP microspheres were exposed to MCPM-H$_3$PO$_4$ solution for 10 min when compared to that for 5 min. On the other hand, the peak intensity of brushite was the same for 10 and 20 min exposures.
Fig.2.4 Powder XRD patterns of $\alpha$-TCP microspheres before (a) and after being reacted with MCPM-H$_3$PO$_4$ solution for 1 (b), 5 (c) 10 (d) and 20 min (e). Standard $\alpha$-TCP powder and brushite are also shown for comparison (●: $\alpha$-TCP crystals; ▼: brushite crystals).
Table 2.1 summarizes the amount of brushite formed when the α-TCP microspheres were exposed to MCPM-H₃PO₄ solution. Approximately 7.5 % of brushite was formed at 10 min and these brushite crystals played key role in the setting reaction of the α-TCP microspheres.

Table 2.1 Relative amount of brushite deposited on the surface of α-TCP microspheres (n=5)

<table>
<thead>
<tr>
<th>Reaction time / min</th>
<th>Amount of brushite / wt.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Cannot be detected</td>
</tr>
<tr>
<td>5</td>
<td>4.7 ± 0.5 (*)</td>
</tr>
<tr>
<td>10</td>
<td>7.5 ± 0.6 (*)</td>
</tr>
<tr>
<td>20</td>
<td>7.6 ± 0.8</td>
</tr>
</tbody>
</table>

* p<0.05

The formation of brushite was also confirmed by the thin-film XRD analysis as shown in Fig.2.5.
Fig. 2.5 Thin-film XRD patterns of $\alpha$-TCP microspheres before (a) and after being reacted with MCPM-H$_3$PO$_4$ solution for 1 (b), 5 (c) 10 (d) and 20 min (e). (●: $\alpha$-TCP crystals; ▼: brushite crystals).
Although brushite could not be detected by the powder XRD when \( \alpha \)-TCP microspheres were treated with MCPM-H\(_3\)PO\(_4\) solution for 1 min (Fig.2.4b), brushite formation was confirmed by thin-film XRD (Fig.2.5b). Therefore, the amount of brushite precipitated at 1 min was thought to be below the detection limit of the powder XRD method. These results together with surface morphology observation (Fig.2.3) revealed that brushite crystals were precipitated on the surface of the \( \alpha \)-TCP microspheres when exposed to MCPM-H\(_3\)PO\(_4\) solution, and the precipitated brushite crystals grew with time, resulting in the interlocking of the brushite crystals formed on the surface of the \( \alpha \)-TCP microspheres. The \( \alpha \)-TCP microspheres set and the bonding strength between two microspheres after 10 min exposure was approximately 6.3 ± 1.4 mN; no further increase was observed even when kept in an incubator for a longer time (Table 2.2).
<table>
<thead>
<tr>
<th>Reaction time / min</th>
<th>Bonding strength / mN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Cannot be detected</td>
</tr>
<tr>
<td>5</td>
<td>3.9 ± 1.2 (*)</td>
</tr>
<tr>
<td>10</td>
<td>6.3 ± 1.4 (*)</td>
</tr>
<tr>
<td>20</td>
<td>6.4 ± 1.3</td>
</tr>
</tbody>
</table>

* *p<0.05
2.4 Discussion

The results in the present study demonstrated clearly that fully-interconnected CPC can be fabricated based on the setting reaction of the $\alpha$-TCP microspheres with MCPM-H$_3$PO$_4$ solution at 37°C. The setting reaction of FMF-CPC is thought to be similar to that of the brushite cement invented by Mirtchi (Mirtchi et al. 1989a, Mirtchi et al. 1989b, Bohner et al. 2007). When the $\alpha$-TCP microspheres are exposed to MCPM-H$_3$PO$_4$ solution which is acidic solution, the $\alpha$-TCP microspheres dissolve to supply Ca$^{2+}$ and PO$_4^{3-}$ as shown in equation (2.2) (Bohner et al. 2001, Chai et al. 2006, Han et al. 2009). The dissolution of $\alpha$-TCP and resulting supply of Ca$^{2+}$ and PO$_4^{3-}$ lead to the MCPM-H$_3$PO$_4$ solution becoming supersaturated with respect to brushite and thus Ca$^{2+}$ and PO$_4^{3-}$ are precipitated as brushite on the surface of $\alpha$-TCP microspheres (equation (2.3)). Precipitation of brushite from the liquid results in the decrease in super saturation and thus, the $\alpha$-TCP microspheres further dissolves to supply Ca$^{2+}$ and PO$_4^{3-}$ to the liquid phase.

$$\text{Ca}_3(\text{PO}_4)_2 \rightarrow 3\text{Ca}^{2+} + 2\text{PO}_4^{3-}$$  \hspace{1cm} (2.2)

$$\text{Ca}^{2+} + \text{H}^+ + \text{PO}_4^{3-} + 2\text{H}_2\text{O} \rightarrow \text{CaHPo}_4\cdot 2\text{H}_2\text{O}$$  \hspace{1cm} (2.3)
Based on this dissolution-precipitation reaction (equations (2.2), (2.3)), the brushite crystals on the surface of the $\alpha$-TCP microspheres grew with time and interlock with each other to set and form a fully-interconnected porous structure.

In the case of brushite cement, it is reported that $\beta$-TCP and MCPM dissolved to supply $\text{Ca}^{2+}$ and $\text{PO}_4^{3-}$ and precipitated as brushite since brushite is the most stable phase thermodynamically at acidic condition (Lundager et al. 1984, Ferreira et al. 2003, Arifuzzaman et al. 2004, Lee et al. 2010, Nancollas et al. 2010).

The key difference between FMF-CPC and brushite cement is the use of $\alpha$-TCP microspheres instead of $\beta$-TCP powder. Microspheres are employed for the fabrication of a fully-interconnected porous structure; however, the surface area of $\alpha$-TCP microsphere with 1.3 mm diameter is extremely small when compared to $\beta$-TCP powder. The small surface area results in a small amount of the dissolution of $\alpha$-TCP to supply $\text{Ca}^{2+}$ and $\text{PO}_4^{3-}$. As a result, FMF-CPC takes 10 min to set even though brushite cement set in 30 sec when free from retarder.

The pore size of the set FMF-CPC, $384 \pm 250 \mu\text{m}$, seems to be ideal for cell penetration and revascularization. It need to be
emphasized that the interconnected porous structure advances bone tissue ingrowth and nutrition of bone cells for the optimal bone growth (Werner et al. 2002, Rose et al. 2004, Itoh et al. 2006, Gan et al. 2008, Yoshikawa et al. 2009). In addition, the pores size of 200-500 μm is recommended for new bone formation (Holmes et al. 1988, Kuhne et al. 1994, Tsuruga et al. 1997, Karageorgiou et al. 2005). Although the pore size obtained in the present study seems to be ideal for bone tissue penetration, it should be emphasized that the pore size can be regulated easily by changing the diameter of the α-TCP microspheres. In addition, the pore size and porosity of the set FMF-CPC is much larger than the theoretical value of a closed-packing structure (with the pore size of 100 μm and porosity of 26%). The reason is thought to be caused by the loose-packing of the α-TCP microspheres, thus increase the pore size and porosity of set FMF-CPC.

2.5 Conclusion

In summary, FMF-CPC consisting of α-TCP microspheres and MCPM-H₃PO₄ solution is fabricated in the present study. When α-TCP microspheres were exposed to MCPM-H₃PO₄ solution, fine
brushite crystals were formed on the surface of $\alpha$-TCP microspheres and the precipitated brushite crystals interlocked with each other to set.
CHAPTER 3

Behavior of fully-interconnected macropore forming calcium phosphate cement in simulated body fluid at 37°C

Abstract

In this chapter, behavior of set fully interconnected macropore forming calcium phosphate cement (FMF-CPC) with brushite layer in simulated body fluid (SBF) solution at 37°C was studied. The result clearly showed that brushite layer could be transformed to apatite layer after immersion into SBF solution for 3 days by dissolution-precipitation reaction. The apatite layer formed was calcium deficient hydroxyapatite (CDHA) with the Ca/P ratio of 1.5. Since apatite layer can be bond directly with the host bone, the set FMF-CPC is expected to be used for the application in bone reconstruction and bone regeneration.

3.1 Introduction

As stated in chapter 2, we have reported that FMF-CPC can be fabricating by setting reaction of α-TCP microspheres with a
mixture of monocalcium phosphate monohydrate solution (MCPM; Ca(H₂PO₄)₂·H₂O) and H₃PO₄ solution. After setting for 10 min, FMF-CPC is composed of brushite layer at the surface whereas the inside is still α-TCP core. In case FMF-CPC is implanted, at first the brushite layer exposes to the bone defect and has chemical response. In this note, it is well known that brushite is the metastable phase under physiological conditions (Vereecke et al. 1990, Rousseau et al. 2003, Grover et al. 2003, Grover et al. 2006, Pina et al. 2010), and it shows quick replacement to bone in vivo study (Apelt et al. 2004, Kuemmerle et al. 2005, Wen et al. 2009, Cheol et al. 2010). After resorption, brushite has the ability to be replaced by new bone. In this note, it should be noted that the behavior of FMF-CPC when implanted is an important factor for the bonding ability of implanted materials with host tissue. However, it is difficult to examine the behavior of FMF-CPC when expose to the living plasma of the animal because in vivo study is quite expensive and require a lot of strictly procedures. Simulation the behavior of FMF-CPC in the condition that mimics the living plasma is quite useful in examining
the behavior of FMF-CPC when implanted.

Kokubo et al. introduced the use of SBF solution that mimic the condition of human blood plasma as described in Table 3.1 (Kokubo et al. 2006, Bohner et al. 2009). Thus, it is quite useful to study the behavior of implanted materials in SBF solution as a preliminarily to animal experiments. For this reason, the aim of this research is to study the behavior of FMF-CPC with brushite layer in SBF solution at 37°C. To easily understand the behavior of FMF-CPC when immersed into SBF solution, the set two-microsphere specimens were employed as the simple model of FMF-CPC.
Table 3.1 Concentration (mM) and pH of SBF solution

<table>
<thead>
<tr>
<th>Ion</th>
<th>SBF (mMol concentration)</th>
<th>Human plasma (mMol concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>142.0</td>
<td>142</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
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<td>Ca²⁺</td>
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<td>2.5</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>147.8</td>
<td>103.0</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>HPO₄²⁻</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>pH at 37°C</td>
<td>7.40</td>
<td>7.40</td>
</tr>
</tbody>
</table>
3.2 Materials and methods

3.2.1 Fabrication of set specimens

As we described in chapter 2, set specimens were prepared based on setting reaction of $\alpha$-TCP microspheres and MCPM-H$_3$PO$_4$ solution. The setting reaction for set specimens is designed 10 min since it showed no difference in setting behavior between 10 min and 20 min.

3.2.2 Immersion set specimens in SBF solution

SBF solution with ion concentrations nearly equal to those of human blood plasma was prepared as described previously. The pH value of SBF solution was adjusted to pH 7.4 by adding HCl. Subsequently, the set specimens were immersed into 20 mL SBF solution at 37°C for 1, 3 and 7 days. The SBF solution to microsphere ratio would be 2 mL/microspheres. After immersion, the specimens were rinsed with distilled water 3 times, followed by immersion in acetone to stop the reaction.
3.2.3 Characterization of the set specimens before and after immersion into SBF solution

*Study of surface morphology:* Surface morphology change and apatite formation ability of set specimens before and after immersion into SBF solution was observed by scanning electron microscope (SEM; JSM 5400 LV, JEOL, Tokyo, Japan) at an acceleration voltage of 20 kV after gold coating.

*Powder X-ray diffraction analysis:* Compositional change of set specimens before and after immersion into SBF solution was evaluated by means of X-ray diffraction (XRD; D8 Advance, Bruker ASX, Yokohama, Japan). The set specimens were ground to powder, then put on the sample holder for powder XRD analysis. CuK$_\alpha$, ($\lambda=1.5406$ Å) radiation generated at 40 kV and 40 mA was used for powder XRD analysis. Data set was collected from $2\theta = 5-40^\circ$ with a step size of 0.02$^\circ$.

*Ca/P ratio difference:* The set specimens before and after immersion into SBF solution were fixed into resin, followed by cutting at the centre of microsphere. Energy dispersive X-ray spectroscopy
(EDS) (Genesis XM4, EDAX, New Jersey, USA) was used to identify Ca/P ratio at cross section surface of the set specimens.

**FT-IR analysis:** The surface of set specimens before and after immersion into SBF solution was scratched to get the fine powder, followed by mixing with KBr at the ratio 1:200. Infrared spectra were measure at a resolution of 2 cm\(^{-1}\) using a Fourier transform infrared (FT-IR) spectrometer (PerkinElmer-spectrum 2000, Massachusetts, USA).

**Measurement of Ca and P ion concentration in SBF solution:** Ca and P concentration of SBF solution before and after immersed was measure using inductively coupled plasma (ICP) equipment (PerkinElmer-Optima 7300DV, Massachusetts, USA). The concentration of Ca and P give information about dissolution-precipitation reaction during immersion process in SBF medium.

**Measurement of pH changes in SBF:** The change in the pH of SBF solution was measured at pre-determined time intervals using a pH meter (TPX-90, Tokyo, Japan).
Evaluate the bonding strength: Bonding strength of the set specimens before and after immersion into SBF solution was measured by universal testing machine (Autograph IS5000, SHIMADZU, Kyoto, Japan). Two bonded microspheres were positioned vertically so that one was on top of the other, then a metal rod was connected to the bottom of the lower microsphere while a non-stress wire was connected to the top of the upper microsphere. The strength was measured under the tensile mode with the cross-head speed of 0.5 mm/min.

3.2.4 Statistical methods

For statistical analysis, one-way factorial ANOVA and Fisher’s LSD method as a post-hoc test, were performed using KaleidaGraph 4.0. Values are expressed as mean ± SD. A p-value of < 0.05 was considered statistically significant.

3.3 Results

Fig.3.1 shows typical SEM photograph of FMF-CPC with brushite layer at the interface of two microspheres before (a, b) and
after immersion into SBF solution at 37°C for 1 (c, d), 3 (e, f); and 7 days (g, h). The connection between microspheres can be maintained. Before immersion, brushite crystals on the surface of FMF-CPC have plate-like shape (Fig.3.1(a, b). Naturally, these plate-like crystals have smooth morphology and these crystals interlock with each other, which make to the bonding of the α-TCP microspheres. The morphology of brushite crystals on the surface of set specimens is maintained the plate-like shape after immersion into SBF solution up to 1 day (Fig. 3.1 (c, d). However, the morphology of plate-like shape changes to spherical-like shape after immersion into SBF solution for 3 and 7 days (Fig.3.1(e-h)). The precipitated spherical-like particles are fully found on the surface of set specimens when the specimens are immersed into SBF solution for 3 and 7 day.
Fig.3.1 Typical SEM photographs of set specimens at the interface between two microspheres before (a, b) and after immersion into SBF solution at 37°C for 1 (c, d); 3 (e, f); and 7 days (g, h).
Fig. 3.2 summarizes the powder XRD patterns of FMF-CPC before (a) and after immersion into SBF solution at 37°C for 1 (b); 3 (c); and 7 days (d). Before immersion into SBF solution, the XRD pattern of set specimens shows the peak corresponding to \( \alpha \)-TCP \((2\theta=30.7^\circ)\) and brushite \((2\theta=11.6^\circ)\) (Fig 3.2 (a)). The peak of brushite appears after 1 day immersion into SBF (Fig. 3.2 (b)) and disappears after 3 and 7 days immersion (Fig 3.2 (c, d)). On the other hand, the broaden diffraction peaks are detected around 26° and 32° in 2\( \theta \) which corresponding to apatite peak when the specimens are immersed into SBF solution for 3 and 7 days. This simply indicates that brushite layer is completely transformed to apatite layer after 3 days immersion.
Fig. 3.2 Powder XRD patterns of set specimens before (a) and after immersion into SBF solution at 37°C for 1 (b); 3 (c); and 7 days (d) 

(●: α-TCP crystals; ▼: brushite crystals; ◆: apatite crystals).
Fig. 3.3 shows typical EDS line analysis of set specimens at cross-section surface before (a) and after immersion into SBF solution at 37°C for 1 (c); 3 (e); and 7 days (g). Based on EDS line analysis, the Ca/P ratio at cross-section surface of set specimens before (b) and after immersion into SBF at 37°C for 1 (d); 3 (f); and 7 days (h) were established. Ca/P ratio of specimen before and after immersion into SBF solution for 1 day is quite similar (Fig. 3.3(b, d)), with Ca/P ratio of 1.0 and 1.5 for the brushite layer at the outside and α-TCP layer at the inside, respectively. However, the Ca/P ratio at the outside increases from 1.0 to approximately 1.5 after immersion specimen in SBF solution for 3 and 7 days (Fig. 3.3(f, h)). On the other hand, the new layer formed after 3 and 7 days immersion in to SBF solution can easily identify by white arrow (Fig. 3.3(e, g)). This new layer suggests that the new apatite-layer formed only on the surface of α-TCP microspheres.
Fig. 3. EDS line analysis to establish Ca/P ratio at cross section surface of the specimen before (a, b) and after immersion into SBF solution at 37°C for 1 (c, d); 3 (e, f); and 7 days (g, h). White arrow indicates new apatite layer formed on the surface of α-TCP microsphere.
Fig. 3.4 shows FT-IR spectra of the precipitate removed from the surface of set specimens before (a) and after immersion into SBF solution at 37°C for 1 (b); 3 (c); and 7 days (d). The spectra of α-TCP, brushite and HAp standard are also listed for comparison. Before immersion into SBF solution, the surface of set specimens is brushite (Fig. 3.4(a)), characterized by HPO$_4^{2-}$ peak at 955 cm$^{-1}$, 1030 cm$^{-1}$ and 1120 cm$^{-1}$. After immersion into SBF solution for 3 and 7 days, HPO$_4^{2-}$ peak is disappeared. In addition, peaks assigned to PO$_4^{3-}$ are detected at 570 and 1030 cm$^{-1}$ and the features of these spectra are similar to that of HAp standard (Fig. 3.4(c, d)). This result indicates that the brushite on the surface of set specimens is transformed to apatite upon immersion into SBF solution for 3 days.
Fig. 3.4 FT-IR spectra of the precipitate removed from the surface of FMF-CPC with brushite layer before (a) and after immersion into SBF solution at 37°C for 1 (b); 3 (c); and 7 days (d) (♦: PO$_4^{3-}$ peak; ●: HPO$_4^{2-}$ peak)
Fig. 3.5 shows change in Ca or P concentration (mmol/L) as a function of immersion time in SBF solution at 37°C. At first, both Ca and P ion increase initially, then gradually decrease until the leveling off at approximately after 3 days immersion.

Fig. 3.5 Change in Ca or P concentration (mmol/L) as a function of immersion time SBF solution at 37°C.
Fig. 3.6 shows the pH change of SBF solution before and after immersion set specimens into SBF solution. The pH of SBF solution is gradually decreased when set specimens was immersed into SBF solution. This is thought due to the release of $H^+$ from brushite to SBF solution according to the equation (3.1) and (3.2):

$$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \rightarrow \text{Ca}^{2+} + \text{HPO}_4^{2-} + 2\text{H}_2\text{O}$$  \hspace{1cm} (3.1)

$$\text{HPO}_4^{2-} \rightarrow \text{H}^+ + \text{PO}_4^{3-}$$  \hspace{1cm} (3.2)

![Graph showing pH change over time](image)

Fig. 3.6 The pH change of SBF solution in the presence of set specimens at 37°C.
Fig.3.7 shows the bonding strength between calcium phosphate microspheres as a function of immersion time in SBF solution at 37°C. The bonding strength values exhibited comparable increases from day 1 to day 3, then show no significantly difference from day 3 and day 7.

Fig.3.7 Bonding strength between calcium phosphate microspheres as a function of immersion time in SBF solution at 37°C.
3.4 Discussion

The obtained results in the present study demonstrated clearly that the brushite layer of FMF-CPC can be converted to apatite layer upon immersion into SBF solution for 3 days. The connection between each microsphere can be maintained due to the interlocking of apatite crystal on the surface of \( \alpha \)-TCP microspheres (Fig.3.1). The morphology of interlocked crystals is still maintained plate-like shape at low magnification; however we can observe the spherical-like shape on the interlocked crystals at high magnification. The apatite layer formed in SBF is stable and it enhances fast bone-bonding at the interface. Bone tissue may accept it more easily without a foreign body response because of its similarity to bone apatite. It is reported that a bone-like apatite layer plays an important role in establishing the bone–bonding interface between biomaterials and living tissue. Thus, the set FMF-CPC with apatite layer is expected to become the ideal material for bone tissue regeneration.

XRD patterns reveal that the conversion of brushite layer to apatite layer happens only on the surface of \( \alpha \)-TCP microspheres (Fig.3.2). Since the Ca/P ratio of apatite formed on the surface of \( \alpha \)-TCP microspheres after 3 and 7 days is 1.5, this result clearly
suggest that apatite formed is calcium deficient hydroxyapatite (CDHA). Since apatite layer can bond directly with the host bone, therefore FMF-CPC is expected to be used for the application in bone reconstruction and bone regeneration.

The increase of Ca and P ion in SBF solution upon immersing set FMF-CPC in SBF solution for 1 day indicates that the immersed set FMF-CPC initially undergo partial dissolution. Brushite layer on the surface of α-TCP microspheres is most probably the major source for the Ca and P increase, since brushite is metastable phase in SBF solution. At first, brushite layer dissolve to releases Ca and P into SBF solution, then the subsequent fall of Ca and P concentration in SBF solution suggests that the initially dissolution process was quickly followed by the precipitation of new material on the surface of FMF-CPC.

An important factor in the utilization of calcium phosphate cement as an implant is mechanical strength. As shown in Fig.3.7, the bonding strength between microspheres increases as a function of immersion time in SBF solution. This is thought due to the conversion of brushite to apatite at the surface of microspheres. One explanation is that the conversion from α-TCP to brushite
happens faster than the conversion from brushite to apatite. Thus, the interlocking of brushite crystals is less contact than that of apatite crystals and thus increases the bonding strength between $\alpha$-TCP microspheres. These results suggest that the establishment of apatite layer enhance the bonding strength of $\alpha$-TCP microspheres by mechanically strengthening the interface between them.

### 3.5 Conclusion

In summary, we have studied the behavior of set FMF-CPC in SBF solution at 37°C. The result indicated that brushite surface layer can be transformed to apatite layer after 3 days immersion into SBF solution by dissolution-precipitation reaction. The apatite layer formed at the surface of $\alpha$-TCP microspheres was CDHA with the Ca/P ratio of 1.5. Since apatite layer can bond directly with the host bone, therefore the set FMF-CPC is expected to be used for the application in bone reconstruction and bone regeneration.
CHAPTER 4

General summary

The present research is concerned with method on fabrication of fully interconnected macropore forming calcium phosphate cement (FMF-CPC) based on the setting reaction of $\alpha$-TCP microspheres. As mentioned previously in chapter 2, $\alpha$-TCP microspheres were exposed to MCPM-$\mathrm{H}_3\mathrm{PO}_4$ solution, fine brushite crystals were formed on the surface of $\alpha$-TCP microspheres and the precipitated brushite crystals interlocked with each other to set. In chapter 3, the behavior of FMF-CPC when expose to simulated body fluid (SBF) solution that mimic the condition of human blood plasma is studied. The result indicated that brushite surface layer can converted to apatite layer after 3 days immersion into SBF solution by dissolution-precipitation reaction. The apatite layer formed at the surface of $\alpha$-TCP microspheres is calcium deficient hydroxyapatite (CDHA) with the Ca/P ratio of 1.5. Since apatite layer can bond directly with the host bone, FMF-CPC is expected to be used for the application in bone reconstruction and bone regeneration.
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