Fabrication and evaluation of ascorbic acid phosphate-loaded spherical porous hydroxyapatite/octacalcium phosphate granules

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The spherical porous granules of octacalcium phosphate (OCP) and hydroxyapatite (HA) have the potential for applications in scaffolds for bone regeneration. The spherical porous alpha-tricalcium phosphate granules were immersed in a pH 5.0 buffer solution that contained 0.1 mM of ascorbic acid phosphate (AscP), an osteogenesis-promoting drug, at 60 °C. This resulted in the fabrication of the AscP-loaded spherical porous HA/OCP granules. The OCP and HA phases were concentrated at the surface and interior of the granules, respectively. Furthermore, the HA/OCP core/shell-like structure of the granules was observed for the first time. The loaded AscP in the HA/OCP granules was slowly released in a pH 7.4 buffer solution. The differentiation of the osteoblast-like cells was promoted by the AscP-loaded HA/OCP granules. These granules possessed the potential for applications in artificial bones to promote bone regeneration.

1. Introduction

Calcium phosphate-based ceramics are widely used as artificial bones.¹–³ Bone repairing materials with excellent bone-regeneration abilities are in high demand. Autografts are utilized in surgeries due to their higher bone-regeneration ability as compared to that of conventional calcium phosphate-based ceramics.⁴ Several strategies like the addition of osteogenesis-promoting ions in calcium phosphate-based ceramics⁵ and usage of a combination of calcium phosphate-based ceramics with cells⁶ have been adopted to increase the bone-regeneration abilities. The loading of osteogenesis-promoting drugs on porous calcium phosphate-based ceramics has been considered to be one of the most effective methods to increase the bone-regeneration ability.⁷–⁹

Octacalcium phosphate [Ca₈(HPO₄)₂(PO₄)₄·5H₂O, OCP] is a precursor of hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂, HA] in vivo.¹⁰,¹¹ OCP can be utilized to fabricate novel artificial bones that promote bone regeneration.¹²,¹³ OCP is converted to HA and supports bone regeneration in vivo.¹⁴ Miyatake et al.¹⁵ reported that a material comprising partially hydrolyzed OCP i.e., a combination of HA and OCP (HA/OCP) possesses a higher bone-regeneration ability than that of materials comprising pure HA or pure OCP. Therefore, the porous HA/OCP granules that are loaded with osteogenesis-promoting drugs exhibit high bone-regeneration abilities.

Ascorbic acid and its derivatives reportedly exhibit excellent osteogenic properties.¹⁶,¹⁷ Ito et al.¹⁸ reported the formation of an ascorbate-apatite composite layer on the surface of NaOH- and heat-treated titanium. This was achieved by the co-precipitation of ascorbic acid phosphate (AscP) and low-crystalline HA in a supersaturated calcium phosphate solution that contained AscP. The stable loading of the osteogenesis-promoting drugs on calcium phosphates can be achieved by the co-precipitation of the corresponding drugs and the calcium phosphates in a drug-laden supersaturated calcium phosphate solution.¹⁹–²¹ This method can be also be utilized for the HA/OCP granules that are prepared by dissolution–precipitation. The spherical porous HA/OCP granules can be synthesized by the dissolution–precipitation of the spherical α-tricalcium phosphate [α-TCP, Ca₃(PO₄)₂] granules in pH-controlled solutions.²²,²³ The formation of the AscP-loaded HA/OCP granules can be achieved by dissolution–precipitation in buffer solutions that contain AscP.

In this study, the AscP-loaded spherical porous HA/OCP granules were prepared. The release profile of AscP from and the biological properties of the granules were thoroughly examined.

Key-words : Octacalcium phosphate, Hydroxyapatite, Core/shell structure, Artificial bone, Ascorbic acid

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2. Experimental procedure

2.1 Preparation and characterization of the AscP-loaded HA/OCP granules

The spherical porous \( \alpha \)-TCP granules (diameter = 300–500 \( \mu \)m) were prepared according to the previously reported process.\(^{23}\) 13.5 g of the \( \alpha \)-TCP powder (Taihei Chemical Industrial Co., Ltd., Japan) was dispersed in 67.5 g of a 10 mass % gelatin solution (FUJIFILM Wako Pure Chemical Corporation, Japan). The resultant slurry was dispersed by stirring in vegetable oil that was preheated at 80°C for 10 min. The oil was then cooled with iced water to set the gelatin. The spherical granules were collected, washed in ethanol, and dried at 4°C. The dried granules were heated at 1,200°C for 30 min to produce the spherical granules of the \( \alpha \)-TCP single phase. The spherical porous \( \alpha \)-TCP granules (diameter = 300–500 \( \mu \)m) were subsequently collected with sieves.

The acetic acid-sodium acetate buffer solution (pH 5.0) was prepared by mixing the solutions of 0.1 M acetic acid (FUJIFILM Wako Pure Chemical Industries, Japan) and 0.1 M sodium acetate (FUJIFILM Wako Pure Chemical Industries, Japan) at 60°C. Furthermore, the L-ascorbic acid 2-phosphate trisodium salt (FUJIFILM Wako Pure Chemical Industries, Japan) was dissolved in the solution to prepare the acetic acid-sodium acetate buffer solutions that contained 0–1 mM (0, 0.01, 0.1, 0.5, and 1 mM) of AscP. The obtained \( \alpha \)-TCP spherical granules were placed in a polypropylene tube with the AscP-containing buffer solutions (pH 5.0, 60°C). The tube was shaken in an incubator at 60°C for 3 h. The granules were immersed in the buffer solutions that contained different concentrations of AscP; furthermore, the concentration of the granules in the solutions was 0.1 g/10 mL. The samples were named G-0 mM, G-0.01 mM, G-0.1 mM, G-0.5 mM, and G-1 mM according to the respective concentrations of AscP in the buffer solutions. The granules were washed with distilled water and ethanol, and dried at room temperature.

The structures of the granules were observed by scanning electron microscopy (SEM; SU8000, Hitachi, Japan). The crystalline phases were examined by powder X-ray diffraction (XRD; RINT-2200VL: Rigaku, Japan) using Cu K\(_\alpha\) radiation. The ratio of the HA and OCP phases in the HA/OCP granules was estimated by the calibration curve that was plotted by measuring the respective concentrations in a mixture of the HA powder (Taihei Chemical Industrial, Japan) and OCP powder prepared by the method reported previously.\(^{24}\) The slice around the center of the granules was examined by Fourier transform infrared (FT-IR) spectroscopy (IRT-7000; JASCO Corporation, Japan). The slice was held on KBr plates to facilitate the measurement by FT-IR spectroscopy. The peak areas of the absorption band at 917 cm\(^{-1}\), which corresponded to HPO\(_4^{2-}\) of OCP, and 3560 cm\(^{-1}\), which corresponded to OH\(^-\) of HA,\(^{25}\) were used for the FT-IR spectral mapping. The amount of AscP loaded on the granules was examined by dissolving 10 mg of the granules in 5 mL of 0.1 M HCl solution and measuring the concentration of AscP. The absorbance of the solutions at 233 nm was measured to determine the concentrations of AscP.

2.2 Evaluation of the AscP-loaded HA/OCP granules

The release profile of AscP from the G-0.1 mM granules in a 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer solution (pH 7.4) at 37°C was examined. Forty milligram of the granules was placed in a cell culture insert that comprised a filter (pore size = 1 \( \mu \)m) and loaded into a 24-well cell culture plate that contained 800 \( \mu \)L of the HEPES buffer solution. The plate was kept at 37°C for 3 and 7 d. The amount of the released AscP was determined by measuring the absorbance of the solution at 260 nm.

The effect of the granules on the proliferation and differentiation of the osteoblast-like MC3T3 cells (Riken BioResource Research Center, Japan) was determined. Initially, the MC3T3 cells were cultured with the G-0 mM and G-0.1 mM granules. The cell culture media used in the experiment were Dulbecco’s Modified Eagle’s Medium (FUJIFILM Wako Pure Chemical Corporation, Japan) with 20 and 10 vol% fetal bovine serum (Thermo Fisher Scientific, US) to evaluate the proliferation and differentiation, respectively, of the MC3T3 cells. The cells that were cultured in the media without granules and with 0.25 mM of AscP served as the reference. The cells were seeded in a 24-well cell culture plate at a density of 10,000 cells per well and exposed to an atmosphere of 5% \( \text{CO}_2\) at 37°C for 1 d to allow the adhesion of the cells. Subsequently, the medium was removed and the cell culture insert that contained 40 mg of the granules was loaded into the 24-well cell culture plate that contained 800 \( \mu \)L of the medium. The cells were cultured for 3 and 7 d; subsequently, the DNA of the cells was collected using a QIAshredder Spin Column and AllPrep DNA Spin Column (QIAGEN, Germany) to analyze the cell proliferation. The absorbance of the solutions (50 \( \mu \)L) was measured to determine the concentrations of the collected DNA. The cells were cultured for 7 d to determine the alkaline phosphatase (ALP) activity; thus, the cell differentiation was evaluated. A buffer solution containing an ALP substrate (MilliporeSigma, US) and Triton X was added to the cells and the ALP activity was determined after an incubation period of 15 min. Subsequently, a 2 M NaOH solution was added to the solution to measure the absorbance at 405 nm. The test number was 5 for each condition (\( n = 5 \)). The obtained data were statistically evaluated by the analysis of variance (ANOVA).

3. Results

3.1 Characterization of the AscP-loaded HA/OCP granules

The analysis of the XRD patterns (Fig. 1) of the granules before and after the treatment in the buffer solutions with different concentrations of AscP revealed that the granules were composed of \( \alpha \)-TCP crystal phase before
immersion. Both the HA and OCP phases were detected in the G-0 mM, G-0.01 mM, and G-0.1 mM granules. The α-TCP crystal phase was retained in the G-0.5 mM and G-1 mM granules. Therefore, the HA/OCP granules were obtained when the concentration of AscP was less than or equal to 0.1 mM. The G-0 mM granules comprised approximately 50 mass % of HA and 50 mass % of OCP. Figure 2 shows the SEM images of the granule surfaces before and after the treatment in the buffer solutions with different concentrations of AscP. When the concentration of AscP was less than or equal to 0.1 mM, the formation of plate-shaped particles was observed in the images. When the granules were treated with the AscP-containing buffer solutions, the decrease in the AscP concentration was observed. In the case of G-0.1 mM granules, the AscP concentration was changed from 0.1 to 0.058 mM. The decrease in the AscP concentration provides the indication of the AscP loaded on the granules. The amount of the loaded AscP in the G-0.1 mM granules was determined to be 3.1 μmol/g after washing and drying. Hereafter, the G-0.1 mM granules were deemed to be the primary subject of the study because of the high amount of the loaded AscP in these HA/OCP granules. Figure 3 shows the low-magnification SEM image of the G-0.1 mM granule. The spherical shape of the granules was maintained even after the treatment in the buffer solution. The G-0.1 mM granules had diameter about 300-500 μm. The size of the granules did not change much by the transformation from α-TCP to OCP/HA.

Figure 4 shows the FT-IR mapping of the cross-sections of the G-0 mM and G-0.1 mM granules. When the sliced G-0.1 mM granules were held on KBr discs, cracks were observed. The peak area of the absorption band at 917 cm⁻¹ that corresponded to HPO₄²⁻ of OCP was high in the outer shell of the granules. Conversely, the peak area of the absorption band at 3560 cm⁻¹ that...
corresponding to OH\(^-\) of HA was high in the interior of the granules. Despite the vague boundary, the HA/OCP core/shell-like structure of the granules was detected.

### 3.2 Evaluation of the AscP-loaded HA/OCP granules

The AscP concentration change of the pH 7.4 buffer solution due to immersion of the G-0.1 mM granules is shown in **Fig. 5**. The AscP concentration increased with the release period, and the concentration reached 0.019 mM after 7 days. As the amount of AscP loaded on the G-0.1 mM granules was 3.1 µmol/g, the concentration would have been 0.16 mM if all the amount of AscP loaded on the granules had been released from the granules. This indicates that AscP was released slowly in the buffer solution.

**Fig. 5.** AscP concentration change of the pH 7.4 buffer solution due to immersion of the G-0.1 mM granules.

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**Figure 6** shows the DNA content of the osteoblast-like cells that were cultured with the granules for 3 and 7 d (n = 5).

**Figure 7** shows the ALP activity of the osteoblast-like cells that were cultured with the granules. The ALP activity of the cells that were cultured with the G-0.1 mM granules was lower than that of the cells that were cultured in the AscP-added medium with 0.25 mM AscP. However, the ALP activity of the cells that were cultured with the G-0.1 mM granules was significantly higher than that of the
cells that were cultured with the G-0 mM granules and without any granules. Therefore, the release of AscP from the G-0.1 mM granules increased the ALP activity of the cells.

4. Discussion

The spherical porous granules with HA/OCP core/shell-like structures were obtained when the α-TCP granules were treated with the pH 5.0 buffer solution. This is the first report of the formation of HA/OCP core/shell-like structures, although we had prepared granules comprising the HA and OCP phases by a similar method in previous studies. Monna reported the formation of a monophasic OCP powder by the treatment of the α-TCP powder under the identical experimental conditions; however, the HA/OCP core/shell-like structure was obtained in this study. The diffusion of ions was prevented by the porous structure of the granules; therefore, HA was formed inside the granules by the following reactions.

\[
3Ca_3(PO_4)_2 + 7H_2O \rightarrow Ca_8(HPO_4)_2(PO_4)_3 \cdot 5H_2O + Ca(OH)_2
\]

Ca(OH)_2 was formed by the hydrolysis of α-TCP and existed in the form of Ca^{2+} and OH^{-}. The low diffusion rate of Ca^{2+} and OH^{-} inside the porous granules increased the local concentration of these ions that resulted in the supersaturation of HA. It was speculated that HA was formed by the following reactions in the interior of the porous granules:

\[
Ca_8(HPO_4)_2(PO_4)_3 \cdot 5H_2O + 2Ca(OH)_2 \rightarrow Ca_{10}(PO_4)_{6}(OH)_2 + 7H_2O
\]

\[
3Ca_3(PO_4)_2 + Ca(OH)_2 \rightarrow Ca_{10}(PO_4)_{6}(OH)_2
\]

It was reasonably estimated that the formed HA was non-stoichiometric and Ca-deficient. Conversely, Tripathi et al. reported the synthesis of pure OCP spheres. The difference in the crystal phase was attributed to the differences in the microstructure of the porous α-TCP granules that were used as the starting materials.

The HA/OCP granules were obtained only when the concentration of AscP was less than or equal to 0.1 mM. When the concentration of AscP in the buffer solution was high, the HA/OCP granules were not formed. Several organic substances inhibit the formation of the HA crystals. It was speculated that AscP had a similar inhibitory effect on the formation of the HA and OCP crystals. Ito et al. also mentioned the inhibitory effect of AscP on the formation of the HA crystals from the supersaturated solution. AscP was added during the formation of the HA/OCP granules. This ensured the relatively stable loading of AscP on the HA/OCP granules.

The ALP activity of the cells that were cultured with the AscP-loaded HA/OCP granules was lower than that of the cells that were cultured in the AscP-added medium with 0.25 mM AscP. The concentration of AscP can reach only 0.16 mM even if all the amount of AscP loaded on the AscP-loaded HA/OCP granules is released from the granules. Moreover, it was estimated that the amount of AscP that was released into the AscP-supplemented medium from the AscP-loaded HA/OCP granules was also low although the precise comparison of the amounts of the released AscP was difficult due to the difference in the nature of the media. Even this situation, the ALP activity was significantly enhanced for G-0.1 mM granules. AscP is hydrolyzed to ascorbic acid and the resultant ascorbic acid enhances the differentiation of the osteoblast-like cells, but the stability of ascorbic acid is low. It is speculated that all the AscP is hydrolyzed to ascorbic acid and the ascorbic acid degraded in the short period when AscP is directly added to the medium once. On the other hand, the HA/OCP granules loaded with AscP can release AscP slowly, the released AscP is hydrolyzed to ascorbic acid in the occasion and the continuous supply of active ascorbic acid is achieved. The AscP loaded on the surface of the particles consisting the granules are speculated to be released by the desorption of AscP and the dissolution of the granules.

The HA/OCP granules are expected to be scaffolds for bone regeneration and the loaded AscP enhanced the differentiation of the osteoblast-like cells. Therefore, the AscP-loaded HA/OCP granules possessed the potential for applications in artificial bones to promote bone regeneration.

5. Conclusions

The spherical porous AscP-loaded HA/OCP granules were prepared and their properties were examined. The following conclusions were derived:

1) The AscP-loaded spherical porous HA/OCP granules were obtained by immersing the spherical porous α-TCP granules in a buffer solution containing 0.1 mM of AscP.
2) A HA/OCP core/shell-like structure was obtained.
3) The AscP loaded on the granules was slowly released into the pH 7.4 buffer solution.
4) The AscP-loaded HA/OCP granules promoted the differentiation of the osteoblast-like cells.

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References

1) L. L. Hench, J. Am. Ceram. Soc., 74, 1487–1510 (1991).
2) R. Z. LeGeros, Clin. Orthop. Relat. R., 395, 81–98 (2002).
3) S. V. Dorozhkin, J. Mater. Sci., 42, 1061–1095 (2007).
4) W. Wang and K. W. K. Yeung, Bioact. Mater., 2, 224–247 (2017).
5) S. Bose, G. Fielding, S. Tarafder and A. Bandyopadhyay, Trends Biotechnol., 31, 594–605 (2013).
6) M. Kamitakahara, E. Tatsukawa, Y. Shibata, S. Umemoto, T. Yokoi, K. Ioku and T. Ikeda, J. Mater. Sci. Mater. Med., 27, 97 (2016).
7) A. Ho-Shui-Ling, J. Bolander, L. E. Rustom, A. W. Johnson, F. P. Luyten and C. Picart, Biomaterials, 180, 143–162 (2018).
8) S. Bose and S. Tarafder, Acta Biomater., 8, 1401–1421 (2012).
9) A. Saito, Y. Suzuki, M. Kitamura, S. Ogata, Y. Yoshihara, S. Masuda, C. Ohtsuki and M. Tanihara, J. Biomed. Mater. Res. A, 77A, 700–706 (2006).
10) W. E. Brown, J. P. Smith, J. R. Lehr and A. W. Frazier, Nature, 196, 1050–1055 (1962).
11) H. Tohda, M. Yamada, Y. Yamaguchi and T. Yanagisawa, J. Electron Microsc., 46, 97–101 (1997).
12) O. Suzuki, M. Nakamura, Y. Miyasaka, M. Kagayama and M. Sakurai, Tohoku J. Exp. Med., 164, 37–50 (1991).
13) O. Suzuki, Acta Biomater., 6, 3379–3387 (2010).
14) O. Suzuki, S. Kamakura, T. Katagiri, M. Nakamura, B. Zhao, Y. Honda and R. Kamiyo, Biomaterials, 27, 2671–2681 (2006).
15) N. Miyake, K. N. Kishimoto, T. Anada, H. Imaizumi, E. Ito and O. Suzuki, Biomaterials, 30, 1005–1014 (2009).
16) J. Zhang, B. A. Doll, E. J. Beckman and J. O. Hollinger, J. Biomed. Mater. Res. A, 67A, 389–400 (2003).
17) S. Takamizawa, Y. Maehata, K. Imai, H. Senoo, S. Sato and R. Hata, Cell Biol. Int., 28, 255–265 (2004).
18) A. Ito, Y. Sogo, Y. Ebihara, M. Onoguchi, A. Oyane and N. Ichinose, Biomed. Mater., 2, S181–S185 (2007).
19) X. Wang, A. Ito, X. Li, Y. Sogo and A. Oyane, Biofabrication, 3, 022001 (2011).
20) X. Wang, A. Ito, Y. Sogo, X. Li, H. Tsurushima and A. Oyane, Acta Biomater., 5, 2647–2656 (2009).
21) K. Sasaki, A. Oyane, K. Hyodo, A. Ito, Y. Sogo, M. Kamitakahara and K. Ioku, Biomed. Mater., 5, 065008 (2010).
22) N. Ito, M. Kamitakahara and K. Ioku, Funct. Mater. Lett., 5, 126009 (2012).
23) N. Ito, M. Kamitakahara and K. Ioku, Mater. Lett., 120, 94–96 (2014).
24) M. Kamitakahara, H. Okano, M. Tanihara and C. Ohtsuki, J. Ceram. Soc. Jpn., 116, 481–485 (2008).
25) B. O. Fowler, M. Markovic and W. E. Brown, Chem. Mater., 5, 1417–1423 (1993).
26) H. Monma, J. Mater. Sci., 15, 2428–2434 (1980).
27) G. Tripathi and T. Miyazaki, Int. J. Appl. Ceram. Tec., 17, 372–379 (2020).
28) G. Williams and J. D. Sallis, Calcified Tissue Int., 34, 169–177 (1982).