Evaluation of resistance to fragmentation of injectable calcium-phosphate cement paste using X-ray microcomputed tomography

Kohei NAGATA*, Kei FUJIOKA*, Toshiisa KONISHI**, Michiyo HONDA***, Masaki NAGAYA***, Hiroshi NAGASHIMA***,**** and Mamoru AIZAWA***,†

*Department of Applied Chemistry, School of Science and Technology, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki 214–8571, Japan
**Graduate School of Natural Science and Technology, Okayama University, 3–1–1 Tsushima-naka, Kita-ku, Okayama 700–8530, Japan
***Department of Life Sciences, School of Agriculture, Meiji University, 1–1 Higashimita, Tama-ku, Kawasaki 214–8571, Japan
****Department of Bioresource Research, Meiji University International Institute for Bio-Resource Research, 1–1 Higashimita, Tama-ku, Kawasaki 214–8571, Japan
†Department of Applied Chemistry, School of Science and Technology, Meiji University, 1–1–1 Higashimita, Tama-ku, Kawasaki 214–8571, Japan

Property of resistance to fragmentation of injectable calcium-phosphate cement (CPC) pastes was evaluated. CPC pastes are widely used as bone fillers due to their biocompatibility and osteoconductivity. However, the potential for fractures due to the formation of voids and cracks in the CPC, called “fragmentation,” reduces the biomechanical strength of CPCs. To develop new CPCs that do not exhibit fragmentation, a method for assessing the presence or absence of fragmentation is required. For in vitro evaluation of the fragmentation resistance, the internal structure of cement specimens allowed to stand in pure water or blood was observed using X-ray micro-computed tomography (X-ray μ-CT) method. In the case of cement specimens derived from commercially-available β-tricalcium phosphate powder ball milled and surface modified in 3,000 ppm inositol phosphate solution, no cracks or voids in the internal structure were observed in samples allowed to set in either pure water or blood. For in vivo verification of the fragmentation resistance, the same CPC pastes were implanted into pig thigh muscle and tibiae for 4 and 24 weeks, respectively. The implanted CPC specimens formed a lump without internal voids or cracks. These data showed that the CPC pastes with the fragmentation resistance both in vitro and in vivo and are thus unlikely to generate fractures. Furthermore, the evaluation method using X-ray μ-CT could enable rapid and simple verification of the fragmentation resistance of the injectable CPC pastes. To our knowledge, this is the first report of the use of this method to evaluate resistance to fragmentation of CPC pastes. Furthermore, because of its simplicity and ease of use, the X-ray μ-CT method shows promise as a gold standard.

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Key-words : β-tricalcium phosphate, Bone graft, Calcium-phosphate cement, Fragmentation

[Received September 10, 2016; Accepted November 7, 2016]

1. Introduction

Hydroxyapatite [Ca10(PO4)6(OH)2; HAp] and β-tricalcium phosphate [β-Ca3(PO4)2; β-TCP] are used in bone grafting as bioactive and bioresorbable ceramics, respectively. Recently, injectable calcium-phosphate cement (CPC) pastes have received much attention because they enable us to realize minimally-invasive treatments for patients.1–3 However, when CPC paste comes into contact with blood or other aqueous body fluids, the set paste fracture to some pieces due to the potential occurrences of voids and cracks in it after implantation, which are called “fragmentation”.4,5 Because fragmentation can significantly lower the compressive strength of CPC, potentially necessitating a second surgery, fragmentation remains a serious problem in orthopedic medicine.6 Thus, the development of CPC with fragmentation resistance (i.e., pastes that do not undergo fragmentation) is a high priority in orthopedic research.

Despite the emphasis on development of new and improved CPC pastes, a suitable method for evaluating the fragmentation resistance of injectable CPC pastes has not been reported. Therefore, the main purpose of this study was to establish a method for evaluating fragmentation resistance of CPC specimens. X-ray micro-computed tomography (X-ray μ-CT) was used in order to assess the non-fragmentation of samples of set CPC paste. The X-ray μ-CT technique described here is an alternative to three-dimensional (3D) imaging, its primary merit being that it enables non-destructive observation of the internal structure of a sample.7 Moreover, X-ray μ-CT enables the determination of the location of voids,8 which cannot be identified using other methods. The X-ray μ-CT technique can distinguish between materials and non-materials (i.e., voids and cracks) on the basis of differences in density.9 Thus, we hypothesized that X-ray μ-CT would be an ideal tool for detecting fragmentation (voids and cracks) inside CPC.

We previously developed a novel CPC using inositol phosphate [C6H6(OPO3H2)6; IP6] as a chelating agent.10–12 This newly-developed cement can be fabricated by mixing β-TCP powders that were simultaneously ball-milled and surface-modified with IP6 and sodium phosphate solution on the basis of the chelate-setting mechanism of IP6. IP6 is present in wheat, rice, corn, and soybean and strongly chelates calcium ions.13 Recently, we also fabricated chelate-setting cements exhibiting anti-washout property.14 Here, the washout rate of the CPCs was defined based on the Japanese Industrial Standard (JIS) T 0330-4.
However, non-fragmentation of CPCs has not been defined in the JIS and the other global standards. In the present study, fragmentation was defined as follows: (i) the CPC does not become a lump after setting, and (ii) cracks and voids are generated in the CPC during setting. However, until now, no method was available to evaluate fragmentation resistance (i.e., non-fragmentation property) of CPC specimens, as mentioned above.

In the present study, we describe for evaluating the fragmentation resistance of injectable CPC paste by the X-ray μ-CT technique. To evaluate the fragmentation resistance of cement specimens in vitro and in vivo, the internal structure of CPC specimens allowed to set in pure water or blood for up to 24 h was observed by X-ray μ-CT, together with the same CPC pastes implanted in the thigh muscles and tibiae of pigs for 4 and 24 weeks, respectively.

2. Materials and methods

2.1 Preparation and characterization of starting CPC powders

Starting CPC powders were prepared according to our previous report.14) In brief, commercially-available β-TCP (β-TCP-100, Taihei Chemical Industrial Co. Ltd., Japan) powder (10 g) was placed into a 3,000 ppm 40-cm3 IP6 (50% phytic acid, Wako Pure Chemical Industries Co., Japan) solution or pure water in a zirconia pot. The mixture was simultaneously ground and surface-modified using a planetary ball mill (Pulverisette 6, Fritsch Japan Co. Ltd., Japan) under the following conditions: (i) ZrO2 pot with a volume of 250 cm3, (ii) 180 g of ZrO2 beads with a diameter of 2 mm, and (iii) a rotation rate of 300 rpm for 3 h. The slurry was then centrifuged at 37°C. After setting for 24 h, the mixture was washed out using a syringe into pure water (30 cm3, 37°C). After 24 h of immersion, the washout ratio of the sample was evaluated. According to reference,15) we considered that the cement paste had the anti-washout property if it did not visibly disintegrate. The anti-washout rate was determined according to the following formula:

\[
\text{Washout rate} \% = \left( \frac{\text{Washout sample}(g)}{\text{Washout sample}(g) + \text{Antiwashout sample}(g)} \right) \times 100.
\]

The resulting cement paste was packed in a cylindrical Teflon® mold (6 mm in diameter, 12 mm in height) and placed in 30 cm3 of sheep aseptic conservation blood (Kojin Bio Co. Ltd., Japan) with a 5 kN load cell at a cross-head speed of 0.5 mm·min⁻¹. After compressive strength testing, the XRD patterns of the CPC specimens were measured, and the HAp content was calculated as follows:

\[
\text{HAp content} \left( \% \right) = \left( \frac{I_{\text{HAp}(211)}}{I_{\beta\text{-TCP}(220)} + I_{\text{HAp}(211)}} \right) \times 100, \tag{3}
\]

where, \( I_{\text{HAp}(211)} \) and \( I_{\beta\text{-TCP}(220)} \) represent the XRD intensity of the peaks for HAp \( (20 = 31.7°) \) and β-TCP \( (20 = 31.03°) \), respectively.

2.2 Preparation of cement pastes and characterization of their material properties

To prepare cement pastes, the IP6/β-TCP(0)-3h and IP6/β-TCP(3000)-3h powders were mixed with 2.5 mass% Na2HPO4 solution at a powder/liquid (P/L) ratio of 1/1.0, 1/1.1, or 1/1.2 g cm⁻³.

The consistency of the prepared cement pastes was evaluated by the following method. Glass plates (200 g) were placed on the cement paste (0.5 cm³), and the spread area was measured after 3 min using image analysis software (Win ROOF®; Mitani Co. Ltd., Japan) according to the following formula:

\[
\text{Consistency} = \frac{\text{Original area (pixel)}}{\text{Spread area (pixel)}} \times 100.
\]

To evaluate the anti-washout property of the cement pastes, 5 min after mixing, 0.5 cm³ of cement paste was pushed out via a syringe into pure water (30 cm³, 37°C). After 24 h of immersion, the washout ratio of the sample was evaluated. According to reference,15) we considered that the cement paste had the anti-washout property if it did not visibly disintegrate. The anti-washout rate was determined according to the following formula:

\[
\text{Washout rate} \% = \left( \frac{\text{Washout sample}(g)}{\text{Washout sample}(g) + \text{Antiwashout sample}(g)} \right) \times 100.
\]

The cement paste was packed in a cylindrical Teflon® mold (6 mm in diameter, 12 mm in height) and placed in 30 cm³ of sheep aseptic conservation blood (Kojin Bio Co. Ltd., Japan) with a 5 kN load cell at a cross-head speed of 0.5 mm·min⁻¹. After compressive strength testing, the XRD patterns of the CPC specimens were measured, and the HAp content was calculated as follows:

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2.3 In vitro evaluation of the non-fragmentation property of cement specimens

In order to evaluate the fragmentation resistance of the injectable cement pastes in vitro, the prepared powders were mixed with 2.5 mass% Na2HPO4 solution at a P/L ratio of 1/1.1 and 1/1.2 [g·cm⁻³]. The resulting cement pastes were injected into the above-mentioned Teflon® mold via a syringe through an 18G needle and immersed in 30 cm³ of pure water or blood at 37°C. After immersion for 24 h, the internal structure of the cement specimens was observed using X-ray μ-CT (InspeXio SMX-90CT, Shimadzu Co., Japan) at an X-ray voltage of 90 kV and 110 μA with an isotropic voxel size of 0.08 μm. As a control, the cement pastes were also observed immediately after immersion. VGstudio MAX2.1 software (Volume Graphics GmbH, Japan) was used for data analysis.

Generally, the resolution of X-ray μ-CT will be at 3–5 times of voxel size; thus, the resolution in the present work may be in the range of 0.24 to 0.4 μm. Therefore, we cannot detect the voids or cracks with the sizes of 0.4 μm or less in the present experimental conditions. However, as this X-ray μ-CT technique can be...
detected the fragmented pieces of set CPC specimens, the present test method will be effective for the reconfirmation of the presence or non-presence of fragmentation. In addition, although we can see many dots with dark gray color in the X-ray μ-CT images, these will be air and water in the pores. Thus, we have estimated that these dots are not voids and cracks due to the fragmentation in this work.

2.4 In vivo evaluation of the fragmentation property of cement specimens
In vivo experiments were performed to verify the fragments generated by long-term implantation in living tissue. The animal experimental protocol was approved by guidelines of the Animal Care and Use Committee at Meiji University. Two female pigs (about 110 kg) were used as experimental animals. IP6 (an original commercially-available β-TCP cement) pastes were then injected into the cavities. After implantation into tibiae, the right side of the tibiae were exposed and the samples with surrounding tissue were excised. X-ray images of the samples were captured to evaluate fragmentation in a similar manner to 2.3 sections.

3. Results and discussion
3.1 Characterization of starting powders
The XRD patterns of the prepared powders shown in Fig. 1 indicate that the IP6/β-TCP(0)-3h and IP6/β-TCP(3000)-3h powders were typical single phase β-TCP. However, the crystallinity of the prepared β-TCP powders was lower than that of the original β-TCP-100. It is known that ball-milling decreases the crystallinity of β-TCP powders. This phenomenon suggests that mechanochemical energy converts β-TCP from a crystalline to an amorphous phase.

Table 1 lists various properties of the prepared powders, such as median particle size, amount of IP6 adsorbed and surface zeta potential. The median particle size of the prepared powders was smaller than that of β-TCP-100. Takahashi et al. reported that ball-milling decreases the median particle size of β-TCP powder. Compared to IP6/β-TCP(0)-3h and IP6/β-TCP(3000)-3h powder, the median particle size was almost the same. This result suggests that surface modification by IP6 does not affect particle size. IP6 adsorption onto β-TCP powder results in surface modification, and as a result, the surface charge of the modified powder decreases. The results of the present study agree with those of a previous study. Surface charge of the starting β-TCP-100 powder was decreased after ball-milling in the IP6 solution. This result may be due to the presence of low crystalline HAp particles which cannot be detected by XRD, together with surface modification of IP6 on the starting CPC powder.

3.2 Material properties of the CPC pastes
The consistency of the CPC pastes increased with decreasing P/L ratio (Fig. 2). The consistency of the IP6/β-TCP(3000)-3h paste was higher than that of the P6/β-TCP(0)-3h paste at the same P/L ratio. This result will be caused by difference in surface charge of prepared powders. When the surface of starting CPC powder is shifted to negative charge, dispersion of the CPC paste

![Fig. 1. XRD patterns of the starting powders, together with those of the original commercially-available β-TCP-100. (A) β-TCP(3000)-3h, (B) β-TCP(0)-3h, and (C) β-TCP-100. Black circles indicate typical β-TCP peaks.](Image)

![Fig. 2. Consistency of the resulting β-TCP cement pastes (n = 4). Error bars represent the standard deviation.](Image)
not only by hydration but also chelate-setting of the PO$_4^{3-}$ hand, hardening of the IP$_6$ phosphate calcium-phosphate phase to the HAp phase. On the other hand, hardened following hydration-induced transition from the amorphous calcium-phosphate phase to the HAp phase. Both CPC pastes exhibited anti-washout property resistance in pure water. The IP$_6$ and Ca$_2^+$/PO$_4^{3-}$ pastes was almost the same as that of the IP$_6$ pastes. The washout rate of the IP$_6$ specimens were of the cement pastes. The compressive strength was comparable to that of human cancellous bone.20) The setting time of CPC paste and the compressive strength of the cement specimens. The compressive strength was 14.8 MPa for the IP$_6$/β-TCP(3000)-3h cement specimens, respectively, after compressive strength testing. These results revealed that hydrolysis of β-TCP powder during the setting reaction is inhibited in the presence of IP6 on the surface of the β-TCP particles.14) The entry of water or blood into the IP$_6$/β-TCP(3000)-3h powder was higher than that of IP$_6$/β-TCP(0)-3h powder. At a consistency of 2.0 or higher, the CPC paste was injectable by syringe through an 18G needle. Thus, the optimum P/L ratio for the IP$_6$/β-TCP(0)-3h and IP$_6$/β-TCP(3000)-3h pastes was determined to be 1/1.2 and 1/1.1 [g cm$^{-3}$], respectively.

Overviews of the cement pastes after anti-washout testing are shown in Fig. 3, together with the corresponding washout rates of the cement pastes. The washout rate of the IP$_6$/β-TCP(3000) pastes was almost the same as that of the IP$_6$/β-TCP(0)-3h paste. Both CPC pastes exhibited anti-washout property resistance in pure water. The IP$_6$/β-TCP(0)-3h paste could have tightened and hardened following hydration-induced transition from the amorphous calcium-phosphate phase to the HAp phase. On the other hand, hardening of the IP$_6$/β-TCP(3000)-3h paste was induced not only by hydration but also chelate-setting of the PO$_4^{3-}$ ions in IP$_6$ and Ca$_2^+$ ions in the powder.

The setting time of CPC paste and the compressive strength of fabricated CPC specimens after immersion for 24 h in blood are shown in Table 2. Generally, the setting time is long P/L ratio is low.19) This experimental result was in agreement with those of the previous report. The compressive strength of the IP$_6$/β-TCP(3000)-3h cement specimens was higher than that of the IP$_6$/β-TCP(0)-3h cement, suggesting that the ability of IP6 to form chelate bonding enhances the compressive strength of the cement specimens. The compressive strength was comparable to that of human cancellous bone.20) The proportion of CPC specimens set in pure water that exhibited fragmentation was almost the same as that of specimens set in blood. Although blood is composed of a number of cell types, such as red and white blood cells and platelets, these cells did not affect setting of the CPC pastes. Therefore, because it enables relatively simple conditions to be employed, we selected pure water as the immersion solution for evaluating the fragmentation resistance of CPC pastes.

Table 2. Material properties of resulting CPC pastes and fabricated CPC specimens. Error (±) indicate the standard deviation (n = 4)

| Sample name       | Setting time min | Compressive strength MPa |
|-------------------|------------------|--------------------------|
| IP$_6$/β-TCP(0)-3h| 16.5 ± 1.1       | 1.93 ± 0.17              |
| IP$_6$/β-TCP(3000)-3h | 14.8 ± 0.8     | 2.60 ± 0.69              |

increase.18) The surface charge of IP$_6$/β-TCP(3000)-3h powder was lower than that of IP$_6$/β-TCP(0)-3h powder as given in Table 1. Thus, we consider that the consistency of IP$_6$/β-TCP(3000)-3h powder was higher than that of IP$_6$/β-TCP(0)-3h powder. At a consistency of 2.0 or higher, the CPC paste was injectable by syringe through an 18G needle. Thus, the optimum P/L ratio for the IP$_6$/β-TCP(0)-3h and IP$_6$/β-TCP(3000)-3h pastes was determined to be 1/1.2 and 1/1.1 [g cm$^{-3}$], respectively.

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Figure 4 shows the XRD patterns of the specimens after compressive strength testing. The crystalline phases of the cement specimens were of β-TCP and HAp biphases. Moreover, the HAp phase constituted 70.0 and 54.3% of the IP$_6$/β-TCP(0)-3h and IP$_6$/β-TCP(3000)-3h cement specimens, respectively. Black and white circles represent β-TCP and HAp, respectively.

![Figure 3: Overview after anti-washout test results and washout rate of the prepared β-TCP cement pastes.](image)

![Figure 4: XRD patterns of cement specimens after compressive strength testing.](image)
3.4 In vivo evaluation of the fragmentation resistance of IP6/β-TCP(3000)-3h cement specimens

Figure 7 shows the X-ray μ-CT images of IP6/β-TCP(3000)-3h cement specimens implanted in pig thigh muscle. (A, C) and (B, D) show specimens implanted for 4 and 24 weeks, respectively. Scale bars: 1 mm.

The IP6/β-TCP(3000)-3h cement specimens implanted into cortical bone or the bone marrow cavity area for 4 or 24 weeks also set as a lump (Fig. 8). Because no voids or cracks formed in the internal structure of the IP6/β-TCP(3000)-3h cement specimens, we concluded to be fragmentation resistance.

In the present study, the fragmentation resistance of cement specimens was evaluated independently in vitro and in vivo. The results demonstrated that IP6/β-TCP(3000)-3h cement specimens possessed the fragmentation resistance both in vitro and in vivo. Moreover, the in vitro and in vivo results agreed very closely, suggesting that the method used in the present study to evaluate the fragmentation resistance for the CPCs would be useful in the development of novel CPCs.

4. Conclusion

The properties of resistance to fragmentation of various CPC pastes were evaluated both in vitro and in vivo. The results of in vitro experiments agreed well with those of the in vivo experiments, demonstrating that the fragmentation resistance of injectable CPC pastes can be evaluated in vitro. We also established a method for evaluating fragmentation resistance of injectable CPC pastes. This method is both rapid and simple to perform and involves observation of the internal structure of CPC specimens immersed in pure water for 24 h. If there were no any voids...
and defects in the CPC specimens, it can be judged that the CPC specimen has properties of the resistance to fragmentation. We anticipate that the X-ray μ-CT method described here will become the gold standard for evaluating the fragmentation resistance of injectable CPC pastes.

Acknowledgements  This study was supported in part by the Kanagawa Academy of Science and Technology (KAST).

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