# The *in vitro* antibiotic release from anti-washout apatite cement using chitosan

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The *in vitro* antibiotic release from anti-washout apatite cement using chitosan (aw-AC(chi)) was investigated in a preliminary evaluation. Flomoxef sodium was employed as the antibiotic and was incorporated into the powder phase aw-AC(chi) at up to 10%. The setting times were measured for aw-AC(chi) containing various amounts of flomoxef sodium. X-ray diffraction (XRD) analysis was also conducted for the identification of products. To evaluate the drug release profile, set aw-AC was immersed in saline and the released flomoxef sodium was determined at regular intervals. The setting time was prolonged slightly with the addition of flomoxef sodium. The difference at 10% flomoxef sodium (0% vs. 10%) was not significant ($p > 0.05$), and can be negligible in clinic. The XRD analysis revealed that formation of hydroxyapatite (HAP) from aw-AC(chi) was reduced, even after 24 h, when the aw-AC(chi) contained flomoxef sodium at 8% or more. The flomoxef sodium release from aw-AC(chi) showed the typical profile observed in skeleton type drug delivery system (DDS). Changing the concentration of chitosan can control the rate of drug release from aw-AC. Therefore, we conclude that aw-AC(chi) is a good candidate for potential use as a DDS carrier that may be useful in surgical operations.


## 1. Introduction

Apatite cement (AC) is a bioactive cement which transforms to hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) when moistured [1–11]. Due to the transformation of HAP, it shows excellent tissue response and good osteoconductivity. Anti-washout type fast-setting AC (aw-AC; formerly called non-decay type FSCPC or nd-FSCPC) is one of the ACs [12–23]. The setting behavior of aw-AC and its transformation to HAP are similar to conventional AC (c-AC) [1–11] and fast-setting AC (fs-AC) [24–27] and it is thought to be especially useful in surgical applications such as orthopedic, plastic and reconstructive, and oral and maxillofacial surgery. Although all AC, c-AC, fs-AC and aw-AC, are stable in the liquid phase once they have set, c-AC and fs-AC can be washed out completely upon exposure to liquid phase before they have set. In contrast, aw-AC cannot be washed out but sets within approximately 5–7 min even if the paste is immersed in distilled water or serum immediately after mixing. aw-AC is made by controlling two independent processes which occur when the paste is in contact with the liquid phase. One process is the formation of HAP that is tightened with the setting reaction of the cement. Another is the penetration of liquid into the cement paste that induces washing out of the cement. Formation of HAP is accelerated by employing fs-AC as a base cement, and penetration of liquid into the cement paste is reduced by the addition of chitosan which forms a water-insoluble gel. As thus prepared, aw-AC is thought to be more useful in surgical procedures where complete hemolysis is sometimes very difficult. Also, chitosan is known to have additional pharmacological benefits for bone formation. For example, chitosan induces osteoconductivity, endochondral ossification and direct membranous osteoinduction [28–33]. Therefore, we proposed aw-AC using chitosan; aw-AC(chi) [14, 19, 23]. aw-AC(chi) is expected to be a useful bioactive cement due to its fast setting behavior, transformation to HAP, non-crumbling property and pharmacological effect beneficial to osteoconductivity [14]. aw-AC(chi) showed excellent tissue response when the paste was implanted subcutaneously in rats immediately after mixing [19]. On the other hand, when the paste was implanted in the rat tibia, in the case of aw-AC(chi) no inflammatory response was observed in the surrounding areas of bone and the bone defect was covered

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with new bone [23]. Thus, aw-AC(chi) has shown excellent tissue response to soft and hard tissues along with good osteoconductivity.

At the time of the use of osteoconductive materials, prevention of bacterial infection is one of the key factors in the success of surgical operation that aims for reconstruction of bone defect. Therefore, postsurgical patients are often administered antibiotics systematically either orally or intravenously. Infection, in particular, is a serious problem when using porous materials [34]. In the case of HAP sintered porous block, it is immersed in antibiotic solution before its use in surgical procedures [35]. In contrast, AC cannot be immersed in antibiotic solution since it is applied in the defect of hard tissue before it has set. Therefore, antibiotic addition to the AC is important as a supply of antibiotic inside the set AC for clinical use.

Several studies have demonstrated that calcium phosphate ceramics, cements including c-AC, have potential value as drug carriers in drug delivery systems [36–45]. However, no study has been conducted, to date, on the release of drugs from aw-AC(chi). Moreover, nothing is known about the effects of antibiotics incorporation on the basic properties of aw-AC(chi).

The objective of the present investigation was therefore to evaluate the effect of antibiotic addition on the basic properties of aw-AC(chi) and the basic drug releasing characteristics, as a preliminary step in the evaluation of aw-AC(chi) containing antibiotics.

## 2. Materials and methods

### 2.1. Preparation of aw-FSCPC containing antibiotics

The powder phase of AC was made by mixing equimolar amounts of TTCP and DCPA as reported previously [4, 10, 11, 25]. Neutral sodium hydrogen phosphate ($Na_xH_{3-x}PO_4$) solutions were prepared by mixing equivalent concentrations of disodium hydrogen phosphate ($0.2\,mol\,l^{-1}$ $NaH_2PO_4$) and sodium dihydrogen phosphate ($0.2\,mol\,l^{-1}$ $NaHPO_4$) so that the pH of the solution became pH 7.4 at 37 °C. The resultant solution had a chemical formula of approximately $Na_{1.8}H_{1.2}PO_4$. In the liquid phase of aw-AC(chi), 0.5% chitosan (PC-100, Ajinomoto, Tokyo, Japan) was further dissolved in $0.2\,mol\,l^{-1}$ $Na_{1.8}H_{1.2}PO_4$ solution so that the concentration of chitosan became 0.5% and 1.0% in the liquid phase. Cement pastes were prepared by mixing antibiotics (flumarin®; flomoxef sodium, Shionogi, Osaka, Japan) and liquid phase, then AC powder with a spatula at a ratio of AC powder to liquid phase (AC/L ratio) of 3.5, i.e., the amount of antibiotics was not counted in the AC/L ratio since the cement paste became too wet when we made cement paste with (AC + antibiotics)/L = 3.5. Cement pastes thus prepared were packed in cylindrical plastic split molds (6 mm diameter × 3 mm heights) under applied pressure of approximately 0.9 MPa. Both ends of the mold were then covered by glass plates, clamped, and the paste set by storing in an incubator for 24 h at 37 °C and 100% humidity.

### 2.2 Setting time of the cement

The setting time of the cement was measured essentially according to the international standard ISO 1566 for dental zinc phosphate cements. With this method, the cement is considered set when a 400 g weight loaded onto a Vicat needle with a tip diameter of 1 mm fails to make a perceptible circular indentation on the surface of the cement. The standard requires that the cement be maintained at a temperature of 37 °C and relative humidity of at least 37%. In the present investigation, the temperature was 37 °C and the relative humidity 100%. The setting times used were the average value of at least five samples.

### 2.3 X-ray diffraction analysis

The compositions of cements after being kept at 37 °C and the relative humidity of 100% were identified by means of X-ray diffraction (XRD) analysis. The specimens were removed after 24 h, ground into fine powders, and characterized by XRD. The XRD patterns of the specimens were recorded with a vertically mounted diffractometer system (ADG-301, Toshiba, Tokyo, Japan) using Ni-filtered CuKα radiation ($\lambda = 0.1540\,nm$) generated at 30 kV and 10 mA. The samples were first scanned from 3 to 60° in 2θ (where θ is the Bragg angle) to determine the reaction products in continuous mode ($1.0\,°2\theta\,min^{-1}$, time constant 2 s) on a strip-chart recorder.

### 2.4 Antibiotic release from the cement

Each disk-shaped (6 mm diameter × 3 mm heights) specimen removed from the mold after being kept in an incubator for 10 min was immersed in 100 mL of saline. During the experiment, the liquids were stirred continuously in water-circulated (37 °C) double-walled Pyrex vessels. Solution (2.0 mL) was withdrawn at regular intervals and subjected to the determination of antibiotic concentration released into the saline using UV spectrophotometer (Hitachi Doublebeam 200-20, Hitachi, Tokyo, Japan). The solution was replaced into the vessel after measurement. Since flomoxef sodium does not show a distinct $\lambda_{max}$ at wave lengths > 200 nm, the shoulder of the absorption at 265 nm was used for the quantitative analysis. The $\varepsilon_{265}$ was $24.0\,mL\,mg^{-1}\,cm^{-1}$. The same antibiotic release study was repeated three times and the averaged value was used for the concentration of antibiotic. To simulate the condition under which the cement is in apposition to fresh body fluid, saline was replenished every 24 h; thus, the specimen was immersed in fresh saline every 24 h.

## 3. Results

Table I summarizes the effect of added flomoxef sodium (0–10%) on the setting times of aw-AC(chi). Although the setting time was prolonged slightly by the addition of flomoxef sodium, aw-AC(chi) set within approximately 7 min regardless of the amount of added flomoxef sodium. We found no significant difference ($p > 0.05$) between 0% and 10% flomoxef sodium. Thus, clinically there seemed to be no problem.

TABLE I Setting time of the anti-washout type fast-setting calcium phosphate cements containing various amounts of flomoxef sodium. The cement was mixed at a powder to liquid ratio of 3.5 and kept at 37 °C and relative humidity of 100%

| Flomoxef sodium (%) | Setting time (min)[a] |
|---|---|
| 0 | 5.7 ± 0.4 |
| 2 | 5.7 ± 0.5 |
| 4 | 5.9 ± 0.4 |
| 6 | 6.1 ± 0.3 |
| 8 | 6.2 ± 0.3 |
| 10 | 6.3 ± 0.4 |

Fig. 1 shows the XRD patterns of aw-AC(chi) with and without flomoxef sodium after being kept in an incubator at 37 °C and 100% relative humidity for 24 h. The powder phase of aw-AC, an equimolar mixture of TTCP and DCPA, and poorly crystallized HAP are also shown for comparison. Formation of HAP was confirmed in AC regardless of the presence or absence of flomoxef sodium. No difference between aw-AC(chi) without flomoxef sodium and aw-AC(chi) containing 6% flomoxef sodium. However, some DCPA still remained unreacted even after being kept in the incubator for 24 h when the aw-AC(chi) contained 10% flomoxef sodium, whereas no unreacted DCPA was found in aw-AC(chi) without containing flomoxef sodium. Also, the amount of remaining TTCP in aw-AC(chi) containing 10% flomoxef sodium was greater than that in aw-AC(chi) containing no flomoxef sodium. Fig. 2 shows the drug release profiles of flomoxef sodium from aw-AC(chi) added various amounts (2%, 4%, and 6%) of flomoxef sodium. The flomoxef sodium was released rapidly from aw-AC(chi) in the initial stage, and then tapered off. Larger amounts of flomoxef sodium were released from aw-AC(chi) containing larger amount of flomoxef sodium. The amounts of flomoxef sodium released from aw-AC(chi) in 24 h were 24%, 34%, and 35% for aw-FSCPC containing 2%, 4%, and 6% flomoxef sodium, respectively. Fig. 3 shows the drug release profile of the flomoxef sodium from aw-AC made with different concentrations of chitosan (0%, 0.5%, and 1.0%). In this experiment, the content of flomoxef sodium was kept constant at 4% and saline (100 mL) was replaced every 24 h. The amount of flomoxef sodium released was larger from aw-AC containing smaller amount of chitosan in the initial 24 h. When the saline was replenished after 24 h, the rate of flomoxef sodium release increased once again. Interestingly, a larger amount of flomoxef sodium was released from aw-AC made with a larger amount of chitosan. At 48 h, the saline was replenished once again. In this stage as well, a larger amount of flomoxef sodium was released from aw-AC made with a larger amount of chitosan, similar to the pattern from 24 to 48 h. However, the differences of the flomoxef sodium concentration released from aw-AC were diminished when the aw-AC was made with different amounts of chitosan: The total amounts of flomoxef sodium released from aw-AC within 72 h were 47%, 48%, and 49% ($p > 0.05$) for aw-AC made with 0%, 0.5%, and 1.0% chitosan, respectively.

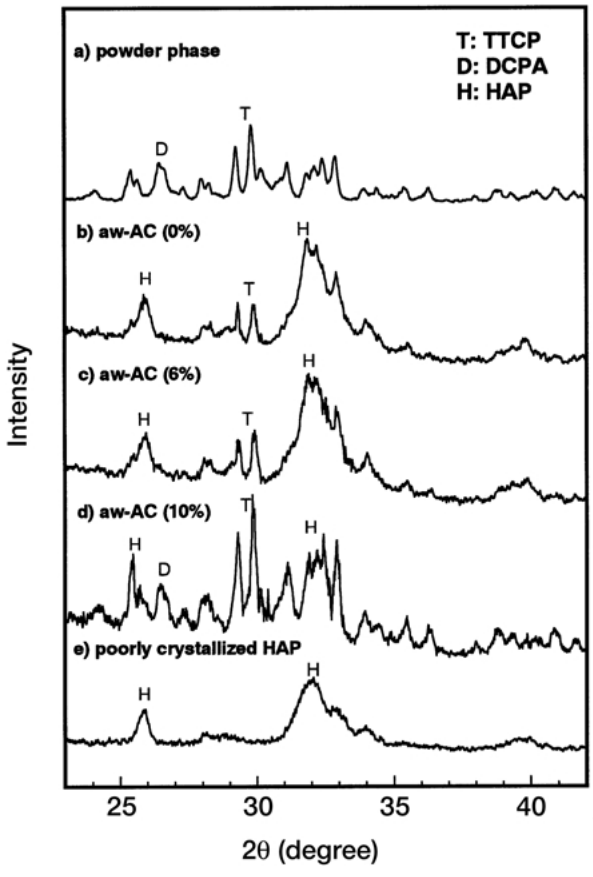


*Figure 1* Powder XRD patterns of the aw-AC containing 0%, 6%, and 10% flomoxef sodium kept in an incubator at 37 °C for 24 h. The powder phases of calcium phosphate cement and poorly crystallized HAP are shown for comparison (TTCP = tetracalcium phosphate; DCPA = dicalcium phosphate anhydrous; HAP = hydroxyapatite).

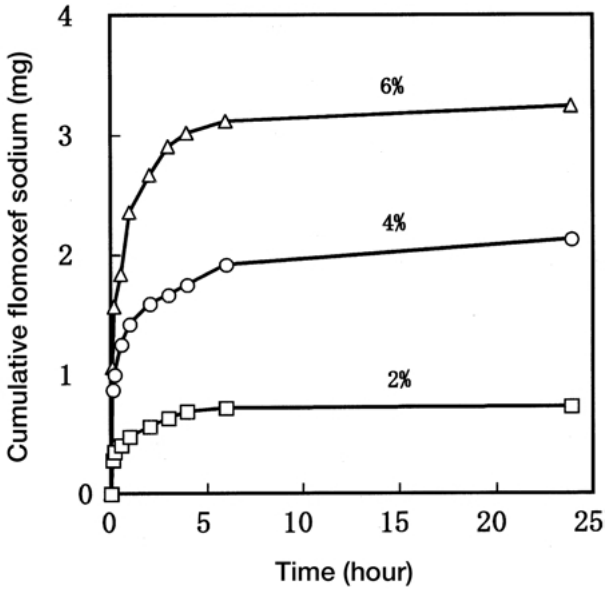


*Figure 2* Time course of the concentration of flomoxef sodium released from aw-AC containing various amounts (2%, 4%, and 6%) of flomoxef sodium. : □ aw-AC containing 2% or 3.1 mg flomoxef sodium ; ○, aw-AC containing 4% or 6.2 mg flomoxef sodium. ; Δ, aw-AC containing 6% or 9.3 mg flomoxef sodium.

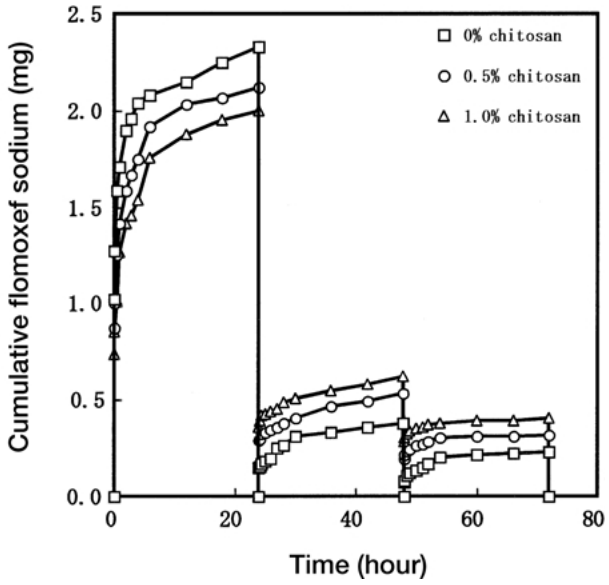


*Figure 3* Time course of the concentration of flomoxef sodium released from aw-AC made with various amounts (0%, 0.5%, and 1.0%) of chitosan. All aw-AC specimens contain 4% flomoxef sodium. The aw-AC specimen (0.2 g) was immersed in 100 mL of saline and the saline was replenished every 24 h. : □, aw-AC made with 0% chitosan; ○, aw-AC made with 0.5% chitosan; Δ, aw-AC made with 1.0% chitosan.

## 4. Discussion

In treating infections of bones with antibiotics, there are two routes of administration; parenteral and local. It is often difficult to maintain an effective concentration of antibiotics in a local region. Therefore, we evaluated the effect of antibiotic addition on the basic properties of aw-AC(chi), and investigated whether or not aw-AC(chi) has potential value as a drug carrier in a DDS or not, as a preliminary step in the evaluation of aw-AC(chi) containing antibiotics.

Although transformation to HAP was observed in all aw-AC containing flomoxef sodium (up to 10%), unreacted DCPA was observed in aw-AC containing 8% or more flomoxef sodium even after 24 h. In the case of aw-AC containing 6% or less flomoxef sodium, no significant difference was observed in XRD patterns compared with aw-AC containing no flomoxef sodium. It is apparent that this does not mean there is no inhibitory effect when the amount of flomoxef sodium is less than 6%, but rather that the difference could not be detected after 24 h by XRD. Otsuka *et al*. [39–45] evaluated c-AC as a DDS devised for various drugs (approximately 5%) such as cephalexin, norfloxacin, indomethacin, 6-mercaptopurine, and reported that the drugs had no inhibitory effect on the setting reaction or formation of HAP. Although they employed a different cement, TTCP and dicalcium phosphate dihydrate (DCPD: $CaHPO \cdot 2H_2O$) containing 40% HAP as seed crystal, and different drugs, the possibility of an inhibitory effect of drugs on the formation of HAP, and thus the setting reaction of ACs, should be noted.

As shown in this study, the inhibitory effect on HAP formation became more prominent with an increase in the percent flomoxef sodium after 24 h. A small amount of antibiotics could be expected to inhibit the formation of HAP to only a slight extent, and could be negligible in clinical applications.

The quantity of antibiotics that should be incorporated in aw-AC(chi) cannot be determined based solely on the results of this preliminary investigation. Also, we did not evaluate whether aw-AC(chi) containing antibiotics delivered a level of antibiotic that exceeded the minimum inhibitory concentration (MIC). The amount required to prevent bacterial infection differs among various surgical operations, and it also depends on whether the antibiotic is administered orally or intravenously, as well as the kind of antibiotic. In practice therefore the amount of antibiotics should be selected based on the requirements in each case. For the determination of the percentage antibiotics, the results obtained in the present study suggest that microbiological assay may also be important and should be performed.

The release profile of antibiotics from aw-AC(chi) is classified as that of skeleton type DDS [39–45]. A relatively larger amount of antibiotic is released initially, and the rate of release decreases with time. An interesting drug release profile was obtained when the concentration of chitosan was altered. Whereas larger amounts of flomoxef sodium were released from aw-AC containing smaller amounts of chitosan within 24 h, aw-AC containing larger amounts of chitosan released larger amounts of flomoxef sodium after 24 h, when the saline was replenished with fresh saline. The same pattern was seen after 48 h; the saline was once again replenished with new saline after 48 h, but the difference of the concentration of flomoxef sodium was not as marked. These interesting drug release profiles may be due to the release rate of flomoxef sodium from aw-AC. When aw-AC is made with a relatively small amount of chitosan, the release of the drug is rapid due to fewer skeletal components, and thus a smaller proportion of the drug remains after 24 h. In contrast, the drug is released more slowly when the drug carrier has a well-developed skeleton, i.e. aw-AC containing a larger amount of chitosan, and thus a greater proportion of the drug remains after 24 h. When saline was replenished with new saline, the aw-AC made with a large amount of chitosan released a larger amount of drug, since it still contained a large amount of drugs. The release tapered off after 72 h regardless of the content of chitosan in aw-AC. These results indicate that the release rate can be controled easily by adjusting the content of chitosan in aw-AC. At 0%, 0.5%, and 1.0% chitosan, the total amounts of flomoxef sodium released within 72 h were essentially the same; 47%, 48%, and 49%, respectively. These results indicate that some portion of the drugs contained in the aw-AC was not released and may remain in the closed pores of aw-AC. The residual drug may be useful should the surface of the set AC become infected by bacteria. Infection causes the pH to drop, and thus dissolution of AC would occur. As a result, the antibiotics contained within the AC closed pores would be released along with calcium and phosphate. These results suggest that a DDS using aw-AC(chi) may be an effective way to treat localized bone infections with high therapeutic effectiveness.

It seems that aw-AC(chi) could be used as a drug carrier not only of antibiotics but also other drugs. For example, the slow but relatively stable release observed in aw-AC(chi) may be useful for the release of growth

factors [46–51] such as bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), and fibroblast growth factor (FGF), since longer release could activate cells for a longer period. For these growth factors, the remaining growth factor inside the AC pores may be helpful in promoting the actual bone remodeling process. Bone contains various growth factors and these would be released with the resorption of the bone by osteoclasts. The growth factors released from bone could activate osteoblast activity, initiating the bone remodeling process. In this process as well, the pharmacological activity of these factors is important. These growth factors are normally found in bones, but their stability in aw-AC(chi) has not been studied to date.

In conclusion, aw-AC(chi) seems to hold promise as a DDS, since it appears to have good potential for use in surgical procedures. Further *in vivo* evaluation is needed to test the validity of the basic properties as identified in this preliminary investigation.

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