Carbonate apatite artificial bone

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ABSTRACT
Bone apatite is not hydroxyapatite (HAp), it is carbonate apatite (CO₃Ap), which contains 6–9 mass% carbonate in an apatitic structure. The CO₃Ap block cannot be fabricated by sintering because of its thermal decomposition at the sintering temperature. Chemically pure (100%) CO₃Ap artificial bone was recently fabricated through a dissolution–precipitation reaction in an aqueous solution using a precursor, such as a calcium carbonate block. In this paper, methods of fabricating CO₃Ap artificial bone are reviewed along with their clinical and animal results. CO₃Ap artificial bone is resorbed by osteoclasts and upregulates the differentiation of osteoblasts. As a result, CO₃Ap demonstrates much higher osteoconductivity than HAp and is replaced by new bone via bone remodeling. Granular-type CO₃Ap artificial bone was approved for clinical use in Japan in 2017. Honeycomb-type CO₃Ap artificial bone is fabricated using an extruder and a CaCO₃ honeycomb block as a precursor. Honeycomb CO₃Ap artificial bone allows vertical bone augmentation. A CO₃Ap-coated titanium plate has also been fabricated using a CaCO₃-coated titanium plate as a precursor. The adhesive strength is as high as 76.8 MPa, with excellent tissue response and high osteoconductivity.
1. Bone apatite

Animals with a skeleton are classified as invertebrates or vertebrates. The skeleton of invertebrates is composed of calcium carbonate (CaCO₃) and is likely derived from elements found in seawater. In contrast, vertebrates, including humans, have a skeleton of carbonate apatite [Ca₅(PO₄)₃O(OH)] instead of CaCO₃ [1-5].

A key difference between CaCO₃ and Ca₅(PO₄)₃O(OH) skeletons is phosphorous. Phosphorous or phosphate is important in energy metabolism in the process of generating energy or adenosine triphosphate (ATP). Invertebrates can use phosphates present in seawater, even though their concentrations are low (1.3 μmol/L). However, vertebrates living on the land need to store phosphorous in the body. During evolution from invertebrates to vertebrates, bone became the storage organ of phosphorous in vertebrates. In other words, Ca₅(PO₄)₃O(OH) was chosen as bone apatite as a result of evolution from invertebrates to vertebrates.

Ca₅(PO₄)₃O(OH) or bone apatite should be amenable for use as artificial bone. However, sintered hydroxyapatite (HAp) instead of Ca₅(PO₄)₃O(OH) has been used as a typical artificial bone since the 1970s. This is because the thermal decomposition of Ca₅(PO₄)₃O(OH) begins at approximately 400°C, which prevents the fabrication of sintered Ca₅(PO₄)₃O(OH). Recently, 100% chemically pure Ca₅(PO₄)₃O(OH) blocks were fabricated through a dissolution–precipitation reaction in an aqueous solution using a CaCO₃ block as a precursor.

2. Ca₅(PO₄)₃O(OH) fabrication though dissolution–precipitation reaction using a precursor

There are three key requirements for compositional transformation through the dissolution–precipitation reaction. First, the solubility of the precursor should be higher than that of the final product. Second, any component that is lacking must be supplied from the aqueous solution. Third, precipitates or crystals of the final product should have the ability to interlock with one another to maintain the shape of the block.

Ca₅(PO₄)₃O(OH) is a thermodynamically stable phase under physiological conditions. It is not soluble under physiological conditions. Moreover, Ca₅(PO₄)₃O(OH) crystals can interlock. Many compounds are more soluble than Ca₅(PO₄)₃O(OH), and thus can be precursors for the fabrication of Ca₅(PO₄)₃O(OH) through dissolution–precipitation reactions including CaCO₃ [6-18], α-tricalcium phosphate [19-24], dicalcium phosphate dihydrate [25,26], and CaSO₄ [27-30]. Moreover, a component that is lacking can be supplied from aqueous solution.

One of the ideal precursors is CaCO₃. It has low solubility in aqueous solution at neutral pH and contains both calcium and carbonate. Chemically pure CaCO₃ blocks can be easily fabricated by simply exposing calcium hydroxide [Ca(OH)₂] compact to carbon dioxide (CO₂) [eq. (1)].

\[
\text{Ca(OH)}_2 + \text{CO}_2 \rightarrow \text{CaCO}_3 + \text{H}_2\text{O} \quad (1)
\]

The compositional transformation of Ca₅(PO₄)₃O(OH) from CaCO₃ requires phosphate. The CaCO₃ block needs to be immersed in a phosphate salt solution for this compositional transformation.

When CaCO₃ is immersed in an aqueous solution, it dissolves and supplies Ca²⁺ and CO₃²⁻ [eq. (2)]. If other ions are absent, the water becomes saturated with CaCO₃. However, the situation is different when water contains PO₄³⁻. In that case, the phosphate salt aqueous solution can be supersaturated with respect to Ca₅(PO₄)₃O(OH) when both Ca²⁺ and PO₄³⁻ are supplied by the dissolution of CaCO₃. Thus, Ca²⁺, PO₄³⁻ and CO₃²⁻ precipitate as Ca₅(PO₄)₃O(OH) [eq (3)], and the precipitated Ca₅(PO₄)₃O(OH) crystals interlock with one another. Continuous dissolution–precipitation reactions lead to the compositional transformation from CaCO₃ to Ca₅(PO₄)₃O(OH), maintaining the macroscopic structure of the precursor.

\[
\text{CaCO}_3 \rightarrow \text{Ca}^{2+} + \text{CO}_3^{2-} \quad (2)
\]

\[
\text{Ca}^{2+} + \text{PO}_4^{3-} + \text{CO}_3^{2-} \rightarrow \text{Ca}_{10-\delta}(\text{PO}_4)_{6-\delta}(\text{CO}_3)_\delta \quad (3)
\]

Other useful precursors are CaSO₄ · 2H₂O, α-tricalcium phosphate [α-TCP: Ca₃(PO₄)₂], and dicalcium phosphate dihydrate [DCPD: CaHPO₄ · 2H₂O]. However, sulfate ions tend to remain in the apatitic structure when CaSO₄ · 2H₂O is used as a precursor. Ca₅(PO₄)₃O(OH) containing a small amount of HAp tends to form when α-TCP or DCPD are used as precursors because of the competitive reaction to form Ca₅(PO₄)₃O(OH) and HAp. Therefore, CaCO₃ appears to be an ideal precursor. Fabrication of Ca₅(PO₄)₃O(OH) based on compositional transformation through a dissolution–precipitation reaction using CaCO₃ as a precursor may have arisen during the evolution of vertebrates from invertebrates.

3. Ca₅(PO₄)₃O(OH) and bone remodeling

3.1. Bone remodeling process

Bone remodeling involves the replacement of old bone and autograft by new bone. Osteoclasts resorb the old bone or autograft, followed by the formation of new bone by osteoblasts (Figure 1). Apatite is osteoconductive. Therefore, osteoblasts are active on their surfaces, although the degree of activity can differ depending on the type of apatite. The activity of osteoclasts also differs according to the type of apatite. Figure 2 displays representative scanning electron microscopy (SEM) images of osteoclasts incubated on the surfaces of bone, HAp, and Ca₅(PO₄)₃O(OH). The absence of osteoclastic resorption with HAp [6] is evidence that HAp cannot be replaced with new bone because osteoclast resorption does not occur. In
contrast, osteoclastic resorption occurs for bone and CO$_3$Ap.

Osteoclasts form Howship’s lacunae and decrease the pH inside the lacunae to pH 3–5, leading to the dissolution of bone apatite. Thus, osteoclasts resorb apatite by dissolving it using a weak acid (Figure 3).

Figure 4 summarizes the solubilities of HAp and CO$_3$Ap as a function of pH. At physiological pH or pH 7.4, CO$_3$Ap is thermodynamically the most stable phase. This may explain why bone apatite is CO$_3$Ap. However, under weakly acidic conditions produced by osteoclasts or at a pH of 3–5, the solubility of CO$_3$Ap is higher than that of HAp. Therefore, CO$_3$Ap dissolves in weakly acidic conditions and is resorbed by osteoclasts, whereas HAp does not appreciably dissolve under weak acidic conditions and is not resorbed by osteoclasts.

3.2. Differentiation of osteoblasts

Osteoblastic activity is the counterpart of osteoclastic activity in bone remodeling (Figure 3). One of the parameters of osteoblastic activity is differentiation. Osteoblastic differentiation markers include type I collagen, alkaline phosphatase, osteopontin, and osteocalcin (Figure 5) [31]. Human bone marrow cells (hBMCs) incubated on CO$_3$Ap demonstrated much higher expression than HAp. Upregulation of osteoblast differentiation is likely one of the causes of the higher osteoconductivity of CO$_3$Ap artificial bone, in addition to the activation of osteoblasts through cell-cell interactions with osteoclasts.

3.3. Histological findings

Figure 6 summarizes the representative results of Villanueva Goldner (VG) histologic staining comparison of CO$_3$Ap and sintered HAp (Neobone*) used for reconstruction of mandibular bone defects adjacent to dental implants in beagle dogs 4 weeks after reconstruction surgery [32]. In VG staining, mature bone is stained green. Reconstruction of a defect with HAp involves the formation of only a limited amount of bone at the defect site, on the surface of the bone defect, and adjacent to the dental implant. This is one reason why no artificial bones have been approved
for implant-related bone defect reconstruction surgeries in Japan. In contrast, more bone forms even at the center of bone defects reconstructed using $\text{CO}_3\text{Ap}$. The surfaces of bone defects and dental implants become covered with bone. The documented bonding that results between bone, $\text{CO}_3\text{Ap}$, and dental implant clearly indicating the usefulness of $\text{CO}_3\text{Ap}$ as an artificial bone for dental implants.

### 3.4. Clinical trials

The first human clinical trial was performed at three university hospitals in patients requiring sinus floor augmentation [33,34]. Figure 7 illustrates the procedure for two-stage sinus floor augmentation [34]. After the sinus floor membrane was elevated with a mucosal elevator, $\text{CO}_3\text{Ap}$ granules were placed in the elevated space (Figure 7(b)). Implant placement was planned for $8 \pm 2$ months after the augmentation. Prior to implantation, a bone biopsy can be performed using a trephine bur (Figure 7(c)).

Figure 8 summarizes the micro-computed tomography ($\mu$-CT) images and the appearance of biopsy tissue stained with hematoxylin and eosin (H-E) or VG. $\text{CO}_3\text{Ap}$ granules were replaced with new bone, even though a small amount of $\text{CO}_3\text{Ap}$ granules remain at this stage [34]. The presence of both mature bone and osteoids indicated active bone remodeling. Few inflammatory cells or foreign-body giant cells were observed in the biopsy specimens. The mean preoperative residual bone height of $3.4 \pm 1.3$ mm was increased to $13.0 \pm 1.9$ mm by the sinus floor augmentation using the $\text{CO}_3\text{Ap}$ granules. Since the height after sinus floor augmentation is sufficient for dental implants, all patients received dental implants without any problems [33,34]. Based on the trial results, $\text{CO}_3\text{Ap}$ granules were approved for clinical use as Cytrans Granules (GC Co, Tokyo, Japan) by the Pharmaceuticals and Medical Devices Agency (PMDA) in 2017. The chemically pure $\text{CO}_3\text{Ap}$ granules are the first to be commercially available globally and the first artificial bone that can be used for bone reconstruction aimed at dental implantation in Japan.

### 4. $\text{CO}_3\text{Ap}$ honeycomb artificial bone

Not only composition but also architecture play important roles in governing the ability of artificial bone. In other words, regulation of architecture may be one of the important keys for artificial bone to demonstrate the similar osteoconductivity of autograft. One of the attractive architectures is the honeycomb. Fabrication of the $\text{CO}_3\text{Ap}$ honeycomb by the dissolution–precipitation reaction requires a precursor. In general, a honeycomb is fabricated by extruding a raw material through a honeycomb die.
Figure 5. Relative gene expression levels of type I collagen, alkaline phosphatase, osteopontin, and osteocalcin on the surface of plastic well, CO$_3$Ap, and sintered HAp [31].

Figure 6. VG-stained histological image of sintered HAp (Neobone®) and CO$_3$Ap 4 weeks after reconstruction surgery of beagle dogs’ mandibular bone defect adjacent to a dental implant [32].
An organic binder is necessary for extrusion. Therefore, the organic binder must be eliminated in the subsequent debinding.

The CaCO$_3$ honeycomb becomes a CO$_3$Ap honeycomb by immersion in a phosphate salt aqueous solution based on compositional transformation through a dissolution–precipitation reaction that maintains the honeycomb structure. Figure 9 shows typical SEM images of CaCO$_3$ and CO$_3$Ap honeycombs. The macroscopic honeycomb structure was maintained during the compositional transformation. However, the microstructure is changed during the dissolution–precipitation reaction, indicating a dependence on the crystal structure of each honeycomb composition.

Figure 10 displays a representative histological image one month after reconstruction of the femoral bone defect using the CO$_3$Ap honeycomb [13]. Tissue penetration into all pores of CO$_3$Ap honeycomb is evident. At higher magnification, the new bone formation at the pore surface of CO$_3$Ap honeycomb is evident. The presence of osteoclasts and osteoblasts on the surface of the newly formed bone indicates active bone remodeling. Osteocytes are also observed within the bone matrix. Interestingly, numerous vascular
endothelial cells and red blood cells are found inside the pores, indicating the formation of blood vessels [12].

Vertical bone augmentation can be performed using the CO$_3$Ap honeycomb. Figure 11 summarizes the results of vertical bone augmentation on rabbit cranium. New bone formation was confirmed 4 weeks after implantation, even at the top of the CO$_3$Ap honeycomb (Figure 11(b)). Magnified images of the top (Figure 11(c)) and bottom (Figure 11(d)) parts of the CO$_3$Ap honeycomb along the pores, cross section of the CO$_3$Ap honeycomb at mid-height (Figure 11(e)), and new bone formation at the pore surface of the CO$_3$Ap honeycomb (Figure 11(f)) are shown. The same histological results were obtained for vertical bone augmentation as for femoral bone defect augmentation. Osteoclasts, osteoblasts, and osteocytes were also observed. In addition, numerous vascular endothelial cells and red blood cells were found inside the pores.

A comparison of the cell and tissue responses among the CO$_3$Ap, HAp, and β-TCP honeycombs has also been reported [35]. Alkaline phosphatase (ALP) activity is an index of osteoblast maturation. ALP activity of MC3T3-E1 cells 7 days following seeding was approximately two-fold higher for the CO$_3$Ap honeycomb than for β-TCP and HAp honeycombs (Figure 12).
Figure 13 summarizes μ-CT and H-E stain histological findings 4 weeks after bone defects made at the distal epiphysis of rabbit femurs were reconstructed with CO$_3$Ap, HAp, and β-TCP honeycombs [35]. In the case of the CO$_3$Ap honeycomb, new mature bone formed along the walls surrounding the macropores. Osteoblasts were detected along the new bone, osteoclasts were detected on the material surface, and blood vessels were detected in the macropores (Figure 13(c), Figure 13(g)). In the HAp honeycomb, immature bone, but not mature bone, was observed in only a small portion of the macropores at 4 weeks post-operation (Figure 13(e), Figure 13(h)). Osteoblasts, osteoclasts, and blood vessels were not observed in any macropores in the HAp honeycomb, whereas these cells and tissues were present in every macropore examined in the CO$_3$Ap honeycomb. Even at 12 weeks after surgery, almost all macropores were occupied by immature bone, and osteoblasts and osteoclasts were
not observed in the macropores. In the case of β-TCP honeycomb 4 weeks after surgery, almost all macropores were filled with mesenchyme, and no osteoblasts or osteoclasts were observed in the macropores (Figure 13f, Figure 13(i)). New mature bone formation was observed within a small portion of macropores. The area of mature bone at 4 and 12 weeks after surgery is summarized in Figure 14 along with the remaining mineral area. The area of mature bone area was significantly larger for CO₃Ap honeycomb compared to that of HAp and β-TCP honeycombs. Remaining materials area was largest for HAp honeycomb followed by CO₃Ap honeycomb and β-TCP honeycomb for both 4 and 12 weeks after surgery.

5. CO₃Ap coated titanium plate

Titanium (Ti) is used as an implant device because of its high mechanical strength and ease of osseointegration [36,37]. In particular, surface-roughed Ti implants demonstrate greater osseointegration and a higher implantation success rate. However, osteogenesis of Ti, even with a roughened surface, is poorer than that of osteoconductive materials like apatite. Therefore, initial loosening of Ti implants is a problem for early loading [38,39,40].

Ti roughened surface coated with CO₃Ap may be an ideal Ti implant because of its pronounced osteoconductivity and replacement by new bone. CO₃Ap coated Ti (CO₃Ap–Ti) can be fabricated by compositional transformation through a dissolution–precipitation reaction using calcite-coated titanium (CaCO₃–Ti) as a precursor [39–41].

In this process, CaCO₃–Ti is established on the roughened surface Ti by wetting the Ti with an ethanol solution of Ca(NO₃)₂, followed by heating at 550°C in a CO₂ gas atmosphere. This results in the thermal decomposition of Ca(NO₃)₂ and its carbonation. CO₃Ap–Ti is then fabricated through a dissolution–precipitation reaction in an aqueous solution of Na₂HPO₄ using CaCO₃–Ti as a precursor.

The adhesion strengths of CaCO₃–Ti and CO₃Ap–Ti are as high as 56.6 and 76.8 MPa, respectively. Figure 15 summarizes the V5-stained histological results 4 weeks

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**Figure 13.** μ-CT images, (a–c), and HE-stained histological images, (d–i), of bone defects 4 weeks after reconstruction with CO₃Ap (a, d, g), HAp (b, e, h), and β-TCP (c, f, i) honeycombs. ‘OB,’ ‘OC,’ ‘MB,’ ‘IB,’ ‘MC,’ ‘M,’ ‘BV,’ and ‘AC’ indicate osteoblast, osteoclast, new mature bone, immature bone, mesenchyme, material, blood vessel, and adipose cell, respectively [35].
after implantation in the straight defect of the proximal tibia 2 cm away from the knee joint in rabbits. Abundant mineralized bone formed on the surface of CO$_3$Ap–Ti. In contrast, osteoid, rather than mineralized bone, was primarily present on the surface of the rough–Ti surface. The bone-implant contact percentages in CO$_3$Ap–Ti to bone (72.9% ± 6.4%) exceeded those of rough–Ti to bone (48.2 ± 7.8%) and calcite–Ti to bone (59.9 ± 15.4%).

Figure 16 illustrates the detaching test used to measure the adhesion strength of the implant to the bone and the results. The increased bone-implant contact resulted in markedly greater adhesion strengths of CO$_3$Ap–Ti to bone (42.5 ± 14.7 N) than that of rough–Ti to bone (8.7 ± 4.3 N) and calcite–Ti to bone (24.0 ± 8.9 N). Most of the fracture occurred at the interface between Ti plate and calcite for calcite–Ti, and interface between Ti plate and bone for rough-Ti. Very high bonding strength with bone and fracture at bone may be caused, at least in part, to the expansion of CO$_3$Ap in the roughened Ti during the dissolution–precipitation reaction.

The documented excellent tissue response, higher osteoconductivity, and higher adhesion strength guarantee the usefulness of CO$_3$Ap–Ti for dental and orthopedic implants.

6. Conclusion

Chemically pure (100%) CO$_3$Ap artificial bone can be fabricated by compositional transformation through
a dissolution–precipitation reaction using a precursor. Although the results obtained to date are encouraging, little is known about CO₃Ap artificial bone compared with other artificial bones, such as HAp and β-TCP.

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