Formation and in Vivo Evaluation of Carbonate Apatite and Carbonate Apatite/CaCO₃ Composite Films Using the Thermal Substrate Method in Aqueous Solution

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We have studied the formation and carried out an in vivo evaluation of carbonate apatite (CO₃²⁻-Ap) and CO₃²⁻-Ap/CaCO₃ composite coatings on titanium substrates using the thermal substrate method. The coatings were formed on commercial pure titanium rods (diameter = 2 mm, length = 5 mm) and plates (thickness = 0.3 mm) by the thermal substrate method in an aqueous solution that contained CaH₂PO₄, CaCl₂, and NaHCO₃. The coating experiments were conducted at 40–140°C and pH = 8 for periods of 15 or 30 min. The coating temperature and NaHCO₃ of the solution had a significant influence on the surface morphology (net-like, plate-like, needle-like, or sphere-like), the phase (single phase of CO₃²⁻-Ap or binary phase of CO₃²⁻-Ap and CaCO₃), and the carbonate content in the precipitated films. A subsequent autoclave treatment also had an effect on the films. A coated rod was implanted in a 10 weeks old male rat’s tibia with a non-coated titanium rod being used as a control. The constructs were retrieved after a period of 14 d postimplantation and examined for new bone formation and for tissue response in the cancellous and cortical bone parts, respectively. Single-phase sphere-like CO₃²⁻-Ap had high osteoconductivity in the cortical bone region, and this increased with increasing carbonate content in the films. However, the osteoconductivity of the CO₃²⁻-Ap/CaCO₃ composite coatings decreased with increasing total carbonate content. [doi:10.2320/matertrans.MRA2007330]

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1. Introduction

Calcium phosphates, and specifically hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂, HAp), are of interest in bone-interfacing implant applications because of their demonstrated osteoconductive properties.³¹ Hydroxyapatite (HAp) is considered to be a bioactive material and is used in the fabrication of medical and dental implants, usually in the form of a coating on a metallic substrate to compensate for its poor intrinsic mechanical properties. However, biological apatite in natural bone is not in the form of pure HAp, and it contains a considerable amount of carbonate ions² (about 7.4 mass% with respect to total bone and 11.4 mass% with respect to the inorganic component in natural bone³⁴). Carbonate apatite (CO₃²⁻-Ap), which replaces PO₄³⁻- and/or OH⁻ ions with CO₃²⁻ ions, is similar to the inorganic component of bone, and it seems to be a more promising bioactive material than stoichiometric HAp is, resulting from the greater solubility of CO₃²⁻-Ap than pure HAp.² On the other hand, it has been reported that CaCO₃ has bioactivity such as cell compatibility and hard tissue compatibility;⁴,⁵ that is to say, it is expected the CO₃²⁻ influences biological reactivity and osteoconductive properties.

Many methods of forming HAp, CO₃²⁻-Ap, and other calcium phosphate coatings on metallic substrates have been reported. These can be classified into two categories: synthesis in aqueous solutions (hydro-process) or synthesis in a high-temperature atmosphere (pyro-process). We have proposed a new hydro-coating method for calcium phosphate, the thermal substrate method, which overcomes several weaknesses in the traditional hydro-processes.⁵⁰ In other papers on the hydro-process,⁷–¹⁴ several factors in the solution affecting the HAp coating have been extensively examined, such as the solute concentration, the pH, the type of ion, its concentration, and additives. In addition, CaCO₃ coatings on organic substrates (alternative soaking process) have been reported and its controlled release has been discussed.¹⁵,¹⁶ We have already reported that HAp coatings on titanium substrates using the thermal substrate method is experimentally feasible and have confirmed experimentally that the ion source, the molar ratio of Ca/P in the solution, and the surface roughness and topography of the substrate affect the precipitate.⁶,¹⁷,¹⁸ In addition, use of the thermal substrate method in an aqueous solution initiates an HAp covering on the substrate forming a complicated surface morphology.¹⁹ Moreover, the osteoconductive properties strongly depend on the surface morphology of HAp.²⁰,²¹

In this work, a single-phase coating of CO₃²⁻-Ap and a composite coating of CO₃²⁻-Ap/CaCO₃ with different surface morphologies and total carbonate content were formed on the titanium substrates using a single method (the thermal substrate method) or a combined process using an autoclave. Furthermore, samples were implanted in a rat’s tibia for a period of 14 d to evaluate the extent of new bone formation.

2. Coating Theory and Carbonate Apatite and Carbonate Apatite/CaCO₃ Composite

The thermal substrate method⁶ involves passing an alternating current through a metallic sample immersed in an aqueous solution. The immersed sample can heat up to a temperature >100°C by Joule heating. It is well known that the solubility of HAp and CaCO₃ in an aqueous solution decreases with increasing temper-
The heating of a substrate in an aqueous solution containing Ca\(^{2+}\) and PO\(_{4}^{3-}\) ions thus results in a precipitation of HAp on the substrates. In the solution, when CO\(_{3}^{2-}\) ions are added, CO\(_{3}\)-Ap or CO\(_{3}\)-Ap/CaCO\(_{3}\) composite films will be obtained on a substrate instead of pure HAp.

### 3. Materials and Methods

#### 3.1 CO\(_{3}\)-Ap and CO\(_{3}\)-Ap/CaCO\(_{3}\) composite coatings

Because the experimental apparatus and procedure used to fabricate for the CO\(_{3}\)-Ap coatings was almost the same as discussed in a previous report on HAp coatings,\(^6,20,21\) it will only be described in brief here. The aqueous solution used consisted of 0.3 mM Ca(H\(_2\)PO\(_4\))\(_2\), 0.7 mM CaCl\(_2\) and 20 mM NaHCO\(_3\), where the unit “M” denotes mol dm\(^{-3}\). NaHCO\(_3\) was added to the solution as a source of carbonate ions for the formation of CO\(_3\)-Ap or CaCO\(_3\) and was selected due to its high resolvability in aqueous solution. The pH of the solution was adjusted to pH = 8 by the addition of aqueous NaOH solution.\(^{17,18}\) Commercial pure titanium rods (\(\phi = 2\) mm, \(l = 5\) mm) and plates (thickness = 0.3 mm) were polished with SiC emery paper (#400 grid) before the coating experiments were carried out. The samples were connected between copper electrodes, immersed in 0.2 dm\(^3\) of aqueous solution, and shaken to release any air bubbles that may have adhered to their surface. An alternating current (up to 50 A) was passed through the samples via the copper electrodes, which heated the samples. The temperature of the sample was measured using a calibrated thermocouple, which was in contact with the surface of the samples. The temperature was maintained at 40, 60, or 140°C for a period of 15 or 30 min. using the current amplitude, and the coating process was terminated by switching the power off. In the low-temperature experiments carried out at 40 and 60°C, HAp did not precipitate easily on the substrates using the above solution. Therefore, ethanol (1 M), which decreased the solubility of HAp in the aqueous solution, was added to the solution;\(^24\) during Joule heating, a 100 kHz ultrasonic wave, which accelerated the nucleation of HAp, was applied to the sample using the ultrasonic cleaning equipment\(^25\) and the coating period was extended by a period of 30 min. During the coating process, the solution was water-cooled to prevent any increase in temperature, and the solution temperature was kept at 15°C. After the coating process, some of the samples were autoclaved (121°C, 20 min.). The experimental conditions are summarized in Table 1. The coated samples were studied using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM).

In addition, it was confirmed that the coated films did not contain any Na\(^+\) ions using ICP-AES analysis, although the solution had Na\(^+\) ions from the addition of NaHCO\(_3\) as the source of carbonate ions and NaOH used to adjust the pH of the solution.

#### 3.2 Quantitative analysis of CO\(_3\) in the coatings

The CO\(_3\) content in the films was analyzed using automatic coulometric method (Coulomatic “C”).\(^{26}\) The coated sample
(i.e., titanium substrate with coated film) was placed on a mullite boat and decomposed at 900 °C under an O₂ atmosphere. Evolved CO₂ was absorbed into the aqueous solution which contained barium perchlorate, and the amount of CO₂ absorbed was analyzed coulometrically, and the CO₂ content in the coating was calculated from the carbon content. In this paper, the carbonate content is expressed as the CO₃ content. Pure CaCO₃ (GR, Nacalai Tesque, Inc) was used as a reference material. On using pure CaCO₃ and titanium plate used for the substrate, the analytical error limit was confirmed to be <5%. It was considered that pure CaCO₃ and CO₂-Ap were decomposed completely at this temperature, and the amount of solved carbon in the titanium substrate could be ignored. Accordingly, the above analytical conditions led to the total CO₃ content in the CO₂-Ap and CO₂-Ap/CaCO₃ composite films. However, we could not separately obtain the CO₃ content of the CO₂-Ap and CO₂-Ap/CaCO₃ composites by changing the extraction temperature and time.

3.3 In vivo study

Because the experimental procedure for our in vivo study was almost the same as described in previous reports, it is also only described in brief here. Before surgery, all the implants were cleaned in distilled water and immersed in a chlorhexidine gluconate solution. Ten week old male Sprague-Dawley rats (Charles River Japan, Inc.) were used in our experimental procedure. The samples were implanted in the tibial metaphysis of the rats. A slightly oversized hole, which did not pass through to the backside of the bone, was created using a low speed rotary drill. Subsequently, the implants were inserted in these holes, and then the subcutaneous tissue and skin were closed and sterilized.

The rats were sacrificed after a period of 14 d, and the implants with their surrounding tissue were retrieved. The samples were fixed in a 10% neutral buffered formalin solution, dehydrated in a graded series of ethanol, and embedded in methylmethacrylate. Following polymerization, each implant block was sectioned into slices 20 μm thick. These sections were then stained with toluidine blue.

The sum of the linear bone contact with the implant surface was measured and was expressed as a percentage over the entire implant length (the bone-implant contact ratio, Rᵢ) in the cancellous bone and the cortical bone parts. Significant differences in the bone-implant contact ratio were analyzed statistically using the Tukey-Kramer method. Differences were considered statistically significant at p < 0.05.

4. Results and Discussion

4.1 CO₃-Ap and CO₂-Ap/CaCO₃ composite coatings

Typical SEM photographs of the surface of the samples are shown in Figs. 1 and 2, coated at 40–140 °C for a period of 15 or 30 min. before, and after the steam autoclaving treatment. Figures 3 and 4 show the XRD patterns and FT-IR spectra of the coatings. Figure 5 shows the variation in total carbonate content in the films. The addition of carbonate to the solution decreased the amount of precipitate on the titanium substrates slightly, compared to the coatings in the solutions without carbonate. In the 140 °C coating, a white precipitate covered the entire surface of the samples after a period of 15 min. As the substrate was not completely covered with precipitate even after a period of 30 min. at 40 and 60 °C, 100 kHz ultrasonic waves were applied during the coating procedure in a solution that had had 1 M ethanol added. However, in the case where more than 10 mM of NaHCO₃ was added at 60 °C and where more than 5 mM of NaHCO₃ was added at 40 °C, the substrate was not completely covered with the precipitate using the above coating conditions. The precipitates coated from a solution with <0.5 mM NaHCO₃ added, at all temperature, were identified as single-phase HAp by XRD analysis. In the case where the NaHCO₃ content was above this value, the films contained HAp and also CaCO₃, and the XRD spectra showed a mixture of calcite, vaterite, and aragonite. Many crystals of calcite were found in the SEM observation of the coatings from a solution where 20 mM of NaHCO₃ were added at 140 °C (indicated by the arrows in Fig. 1(e-1)). The surface morphology of HAp strongly depended on the coating temperature and the amount of the NaHCO₃ added. The low-temperature coatings (40 °C) had a net-like precipitate, the high-temperature coatings (140 °C) had a needle-like precipitate, and the mid-temperature range (60 °C) coatings had a plate-like precipitate; after much addition of NaHCO₃ (>5 mM) at 140 °C, this changed from a needle-like to a sphere-like precipitate.

After steam autoclaving, the CaCO₃ peaks were disappeared in the XRD spectra, and calcite could not be found (Fig. 2(e-2)). It is thought that the CaCO₃ had dissolved in the hot water (or steam). However, the morphology of the films did not change according to the autoclave treatment. From a cross-sectional view of the sample, the thickness of the films formed in the above conditions was <2–3 μm, regardless of the use or not of the autoclaving treatment. The total carbonate content in the as-coated films in the solution that contained 0.5–10 mM NaHCO₃ was about 11–15 mass%, which is close to the carbonate content of natural bone. In the solution with 20 mM of NaHCO₃ added, the carbonate content reached a value of 25 mass%. In the solution with no NaHCO₃ added, the as-coated films had a value of 7–9 mass% of CO₂ at all temperatures. It is thought that this is the reason why the solution used contained carbonate from dissolving the CO₂ contained in air. That is to say, carbonate ions can exist in the solution in ambient air (pCO₂/atm = 0.0003): the concentration of total dissolved carbonate ions reached about 0.1 mM in a solution at 25 °C. Therefore, the as-coated film in an NaHCO₃-free solution had a measurable carbonate content. In consideration of above, we prepared a solution under an Ar atmosphere, and bubbled Ar gas into the solution both before and during coating. This did not eliminate the carbonate in the films because the carbonate in the distilled water used was not removed completely, and carbonate-free films could not be fabricated. Even after autoclaving, the concentration of carbonate in films was 4–6 mass%, and this amount did not depend on the additional NaHCO₃ content in the solution and coating temperature.

The FT-IR spectra of the sample with <0.5 mM NaHCO₃ added showed PO₄³⁻, OH⁻, CO₃²⁻ absorption bands. The CO₂⁻ absorption band, which had been substituted for PO₄³⁻, was clearly detected at 1420 and 1470 cm⁻¹.
(designated as Type B),\textsuperscript{29}) and was similar to biological apatite.\textsuperscript{28}) Since the band occurring at 1545 cm\textsuperscript{-1} was due to substituted CO\textsubscript{3}\textsuperscript{2−} (as Type A)\textsuperscript{29} was very weak, this suggests that Type B CO\textsubscript{3}\textsuperscript{−}-Ap formed in advance of Type A. In the case of the sample with >5 mM added, the OH\textsuperscript{−} band (at 3570 cm\textsuperscript{-1}) disappeared, and an alternative band appeared at 1545 cm\textsuperscript{-1}. In addition, Type B site substitution still occurred. The surface morphology changed markedly from needle-like to sphere-like. Therefore, in the samples with <0.5 mM NaHCO\textsubscript{3} added, Type B CO\textsubscript{3}\textsuperscript{−}-Ap was obtained, and in the samples with >5 mM NaHCO\textsubscript{3} added (i.e., having the binary phase of CO\textsubscript{3}-Ap/CaCO\textsubscript{3}), Type AB CO\textsubscript{3}\textsuperscript{−}-Ap was formed. Autoclaving did not change the FT-IR spectra and the autoclaved films contained carbonate (4~6 mass%).
Fig. 3  XRD patterns of (1) as-coated samples and (2) after steam autoclaving coated in a solution at pH ¼ 8 containing 0.3 mmol dm⁻³ Ca(H₂PO₄)₂ and 0.7 mmol dm⁻³ CaCl₂ at 140 °C. NaHCO₃ content: (a-1) and (a-2) = no addition, (b-1) and (b-2) = 0.5 mM, (c-1) and (c-2) = 5 mM, (d-1) and (d-2) = 10 mM, (e-1) and (e-2) = 20 mM. •: HAp, □: CaCO₃ (aragonite), ◼: CaCO₃ (vaterite), △: CaCO₃ (calcite).

Fig. 4  FT-IR spectra of (1) as-coated samples and (2) after steam autoclaving coated in a solution at pH ¼ 8 containing 0.3 mmol dm⁻³ Ca(H₂PO₄)₂ and 0.7 mmol dm⁻³ CaCl₂ at 140 °C. NaHCO₃ content: (a-1) and (a-2) = no addition, (b-1) and (b-2) = 0.5 mM, (c-1) and (c-2) = 5 mM, (d-1) and (d-2) = 10 mM, (e-1) and (e-2) = 20 mM.
Despite the disappearance of CaCO₃ peaks in the XRD patterns. The carbonate only remained as a substitutional component in the coatings after autoclaving. However, as mentioned above, as the carbonate content in the autoclaved films decreased below that of as-coated single-phase of CO₃-
Ap, it is thought that the amount of carbonate in the CO₃-
Ap film also decreased.

The samples obtained using a combination of the thermal substrate method coating with a steam autoclaving treatment can be summarized as follows: (1) single-phase films of CO₃-
Ap with four types of morphology, net-like, plate-like, needle-like, and sphere-like, (2) single-phase films of CO₃-
Ap with different carbonate contents with the same surface morphology, (3) composite films of CO₃-
Ap/CaCO₃ with four types of morphology, and (4) composite films of CO₃-
Ap/CaCO₃ with different carbonate contents having a sphere-like coating. The four types of samples above were subjected to in vivo testing.

4.2 In vivo study

Figure 6 shows the relationship between the bone-implant contact ratio, Rₘₐ, of the coated samples at a period of 14 d after implantation along with the carbonate content in the coatings, classified using the following four surface morphologies: (A) needle-like, (B) plate-like, (C) net-like, and (D) sphere-like. These are compared with the control implant ((E) non-coated titanium). In Fig. 6, the samples are distinguished using different colors according to whether ((E) non-coated titanium). In Fig. 6, the samples are distinguished using different colors according to whether

Fig. 5 Relationship between the total carbonate content in a coating and the NaHCO₃ content in the solution. ◦: 140°C, □: 60°C, △: 40°C, ■: as-coated, ●: after autoclaving.

Fig. 6 The bone-implant contact ratio, Rₘₐ, showing the percentage of bone formed on the coated implants with various surface morphologies and carbonate content. ■ CO₃-
Ap (as-coated), □ CO₃-
Ap (after autoclaving), △ CO₃-
Ap/CaCO₃ (as-coated) (1) cortical bone and (2) cancellous bone (A) needle-like, (B) plate-like, (C) net-like, (D) sphere-like coating and (E) control Ti. *p < 0.05.

4.2 CO₃-
Ap coatings

In the cancellous bone part(2) in Fig. 6, the Rₘₐ value of the coatings with single-phase of CO₃-
Ap (black and gray bars) was Rₘₐ ≈ 30% (except for the needle-like CO₃-
Ap,20,21) which agrees well with the ratio of the hard tissue in the natural bone. In our previous papers,20,21 we reported that the needle-like CO₃-
Ap had a higher osteoconductivity than the net- and plate-like CO₃-
Ap in the early implantation stage. However, the reason is not clear yet. On the other hand, in the cortical bone part(1) in Fig. 6, the value of Rₘₐ in the CO₃-
Ap coatings increased with increasing carbonate content in the coating for all surface morphologies, and the sphere-like CO₃-
Ap morphology exhibited the highest value. It is thought that the surface morphology, which determines the formation of a scaffold on the bone in the body, and the carbonate content in the films both influenced the osteoconductivity in the cortical bone part. We could not elucidate which of these factors had the highest effect on the osteoconductivity.

4.2.2 CO₃-
Ap/CaCO₃ composite coatings

In the case of implantation of the CO₃-
Ap/CaCO₃ composites, the value of Rₘₐ for all surface morphologies (white bar) was lower than that for single phase CO₃-
Ap samples having same morphology in the cortical bone part(1). This tendency was not as clear in the cancellous bone part(2) for the needle-like, plate-like, and net-like coatings, where the value of Rₘₐ of the CO₃-
Ap/CaCO₃ composites remained at Rₘₐ ≈ 30%. However, in the CO₃-
Ap/CaCO₃ composites with a sphere-like morphology (white bar in (D)), the value of Rₘₐ in the cancellous bone part(2) also decreased with increasing carbonate content in the coating, in the same manner as the cortical bone part(1). In addition, this value of Rₘₐ was lower than the value of Rₘₐ in the titanium control sample (E)(1). That is to say, a CaCO₃ content above that of natural bone, has a negative effect on the osteoconductivity. The reason of this effect is as follows. It is thought that CaCO₃ in the CO₃-
Ap/CaCO₃ composites biodegraded in advance of CO₃-
Ap, as the solubility of...
CaCO$_3$ is greater than that of pure HAp and CO$_3$-Ap. In particular, CaCO$_3$ synthesized in an aqueous solution must have a higher rate of degradation than sintered CaCO$_3$ does. Therefore, it is thought that the rapid degradation of CaCO$_3$ disrupts the ion balance near the interface between the implant and the biomedical tissue, as a Ca- and CO$_3$-rich layer remained in the implants coated with the CO$_3$-Ap/ CaCO$_3$ composites. In particular, these ions are retained longer in the cortical bone part, which has less body fluid circulation, and this gives rise to the obvious negative osteoconductivity effects. The amount of CaCO$_3$ in the needle-like, plate-like, and net-like composites was not high enough to decrease the value of $R_{B,1}$ in the cancellous bone(2). The slow biodegradation of CaCO$_3$, and the moderate release rate of Ca$^{2+}$ and CO$_3^{2-}$ ions from the composite film, will improve the osteoconductivity, as investigated by the research group of Akashi et al. However, we did not investigate the effect of the crystal structure of CaCO$_3$ (e.g., calcite, vaterite and aragonite) on the osteoconductivity, because the composite films contained all these crystal structures.

4.2.3 Influence on the osteoconductivity

In all examples of our samples, having different surface morphology, carbonate content, and the presence or absence of CaCO$_3$, it was clear that they did not influence significantly on the osteoconductivity in the cancellous bone part(2). However, the sphere-like CO$_3$-Ap single phase films with high substitutional carbonate content had the highest osteoconductivity in the cortical bone part(1), which was not influenced by a steam autoclaving treatment.

5. Conclusions

(1) All the titanium samples were covered with either single-phase CO$_3$-Ap or binary phase CO$_3$-Ap and CaCO$_3$ with different surface morphologies, such as net-like, plate-like, needle-like, and sphere-like, using the thermal substrate method in an aqueous solution, depending on the NaHCO$_3$ content of the aqueous solution. The total carbonate content was $7\sim9$ mass$\%$ in the as-coated CO$_3$-Ap and $11\sim25$ mass$\%$ in the CO$_3$-Ap/CaCO$_3$ composites. Combining the thermal substrate coating with a steam autoclaving treatment changed the CO$_3$-Ap/CaCO$_3$ composites into CO$_3$-Ap films, in which $4\sim6$ mass$\%$ CO$_3$ remained as a surface morphology.

(2) In the cortical bone parts, the osteoconductivity of the single phase of CO$_3$-Ap coatings decreased with decreasing carbonate content in the coating, and sphere-like CO$_3$-Ap had the highest osteoconductivity. On the other hand, in the CO$_3$-Ap/CaCO$_3$ composite films, the osteoconductivity decreased with increasing carbonate content. The sphere-like CO$_3$-Ap/CaCO$_3$ composite films showed the same tendency in the cancellous bone parts.

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