Formation of hydroxyapatite layer on graphite sheet immersed in calcium phosphate solution by microwave heating

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The conditions for the formation of hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ layer on the graphite sheet were examined via the processes of (i) the acid treatment of conc. nitric and sulfuric acids at 50°C for 24 h for the formation of carboxyl group, and (ii) the external heating (90°C, 1 h) and/or microwave heating (100°C, 5 min) of the acid-treated graphite sheet in the “apatite-dissolved solution” prepared by bubbling $\text{CO}_2$ gas into the HAp-dispersed water. The combination of external and microwave heating was effective in the formation of large amount of calcium phosphates, because the seed crystals of apatite formed by the evaporation of solvent from the apatite-dissolved solution (i.e., external heating) contributed to enhancing the formation of calcium phosphate layer through the microwave heating. The calcium phosphate layer formed by stacking the plate-like particles possessed the typical thickness of 4.9 µm, and was identified as HAp with Ca/P ratio of 1.72 or carbonate-containing HAp. Based upon the graphite sheet with HAp layer implanted into femur and tibia of the Japanese white rabbit for 4 months, the formation of calcified bone at the interfaces was found to show the excellent biocompatibility.

Key-words : Hydroxyapatite layer, Graphite sheet, Calcium phosphate solution, Microwave heating

1. Introduction

A graphite, which possesses the graphene structure with sp² bonding, has many excellent properties, such as light weight (compared to the metal), antithrombogenicity, wear resistance and biocompatibility. The graphite has essentially bioinert or chemically-inactive properties, and the problem that has to overcome remains with respect to the advanced improvement of biocompatibility. Relating to the biocompatibility of carbon, Tarvainen et al.,¹) reported that the tissue ingrowth was most distinctive in pores where the glassy carbon was in close contact with cortical bone or trabeculae of the cancellous bone. On the other hand, Kwok et al.,²) reported that the doping of calcium and phosphorus to the diamond-like carbon through plasma immersion ion implantation/deposition may enhance the surface blood compatibility.

The present authors³) have reported that the carboxyl group on the surface of vapor-grown carbon nanoﬁber (VGCF) may be formed by the acid treatment, using conc. nitric acid and sulfuric acid, and that the dispersibility of such surface-modified VGCF in the presence of polar solvent appears to be notably enhanced. Since the surface properties of VGCF could be changed from hydrophobic to hydrophilic nature, the biocompatible hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ layer was considered to form on the surface of acid-treated graphite sheet. One of present authors further paid attention to the preparation of apatite-dissolved solution by bubbling the $\text{CO}_2$ into the HAp-suspended solution,³) and the particle morphology of HAp powder may be controlled by a microwave heating.⁴) from the viewpoints of (i) homogenous heating throughout the system, (ii) the efficient energy transfer to the reaction system, and (iii) shorter reaction time compared to the case of external heating. The combination of acid treatment to form the carboxyl group on the graphite sheet (i.e., the modification of surface properties from hydrophobic to hydrophilic nature) and subsequent microwave heating in the apatite-dissolved solution is expected to contribute to forming the HAp layer on the graphite sheet.

On the basis of such background, we examined the optimized preparation conditions of HAp layer on the acid-treated or surface-modiﬁed graphite sheet through the combination of external and microwave heating.

2. Experimental procedure

2.1 Preparation of calcium phosphate layer on the graphite sheet

Commercially available graphite sheets (0.1 g; thickness, 25, 70 and 100 µm) were acid-treated using the mixture of 69% nitric acid (25 cm³) and 98% sulfuric acid (75 cm³) at 50°C for 24 h. The acid-treated graphite sheets were washed with de-ionized water and dried at 50°C for 24 h. Then they were immersed in the “apatite-dissolved solution” prepared by the dissolution of commercially available high-purity HAp powder (HAp-100: Taihei chemical Industrial Co., Ltd., 1.0 g) into the de-ionized water (1 dm³) with bubbling of $\text{CO}_2$ gas for 1 h. The solvent was removed by the evaporation at 90°C for 1 h (defined as external heating) and/or by microwave heating (output power 300 W, frequency 2450 MHz) in order to form the calcium phosphate layer on the graphite sheet. The microwave heating was conducted for 5 min, after the temperature of apatite-dissolved solution increased from room temperature up to approximately 100°C within...
2 min. The calcium phosphate layer formed by the external and/or microwave heating was finally dried at 50°C for 24 h.

2.2 Evaluation

The phases present in the precipitate were examined using an X-ray diffractometer (XRD; Model RINT2100V/P, Rigaku, Tokyo) with monochromatric CuKα radiation generated at 40 kV and 40 mA and using an attenuated total reflection Fourier-transform infrared spectrometer (Model 8600PC, Shimadzu, Kyoto). XRD patterns were indentified using Joint Committee on Powder Diffraction Standards-International Center for Diffraction Data (JCPDS-ICDD) cards. Raman scattering was measured in air at room temperature with a backscattering geometry. The spectra were excited with the 532 nm line of a diode-pumped solid-state laser with single longitudinal mode operation (Showa Optronics J300GS) and recorded with a triple-grating monochromator (Jobin-Yvon T64000).

The particle morphology was studied using a field emission scanning electron microscope (FE-SEM; Model SU-8000, Hitachi, Tokyo); the energy-dispersive X-ray microanalyzer (EDX) attached to the FE-SEM was used in order to determine the Ca and P contents.

The bonding strength of the calcium phosphate layer to graphite sheet was examined using a tape test. A piece of Scotch tape (#810) was adhered to the graphite sheet with calcium phosphate layer and then was removed from it. The evaluation of the bonding strength was conducted qualitatively from the viewpoint of whether the films remained on the tape-side or not.

The graphite sheet with HAp layer, which had been sterilized by the ethylene oxide gas, was implanted for 4 months in the femur and tibia of Japanese white rabbits. The degree of bone regeneration was observed on the basis of the non-decalcified specimens prepared with a Villanueva bone stain.

3. Results and discussion

3.1 Formation of calcium phosphate layer on graphite sheet

This section describes (i) the acid treatment of graphite sheets in order to change the surface properties of graphite sheet from hydrophobic to hydrophilic nature, and (ii) the formation of HAp layer on the acid-treated graphite sheet in the apatite-dissolved solution through the external and/or microwave heating.

3.1.1 Acid treatment of graphite sheet

Commercially available graphite sheets with the thickness of 25, 70 and 100 μm were acid-treated using the mixture of 69% nitric acid and 98% sulfuric acid at 50°C for 24 h. The crystalline phase of acid-treated graphite sheets examined using an XRD are shown in Fig. 1, together with a typical FE-SEM micrograph. XRD patterns of acid-treated graphite sheets showed that no crystalline phase was detected from the graphite sheets, except for carbon. Reflecting the XRD results, FE-SEM micrograph showed the concave parts but no appreciable changes in surface microstructure of graphite sheet.

Since no appreciable difference in XRD pattern was found before and after the acid treatment, the chemical reaction was further examined by Raman spectroscopy. Raman spectroscopy is effective in the structural characterization of graphitic materials, e.g., pyrolytic graphite, carbon fibers, glassy carbon, fullerenes and carbon nanotubes. Raman spectra of acid-treated graphite sheets with the thickness of 25, 70 and 100 μm are shown in Fig. 2. Raman spectrum of graphite sheet with no acid treatment possessed a peak at 1380 cm⁻¹ [Fig. 2(a)]. On the other hand, Raman spectra of acid-treated graphite sheets with the thickness of 25, 70 and 100 μm possessed additional peaks at 1350 and 1625 cm⁻¹, together with the peak at 1582 cm⁻¹ [Figs. 2(b)–2(d)].

The prominent features in the Raman spectra of graphitic materials are known for “G band” appearing at 1582 cm⁻¹, “D band” at 1350 cm⁻¹ and “D’ band” at 1625 cm⁻¹. Among these bands, “G band” at 1582 cm⁻¹ is attributed to the bulk mode of the graphite structure, whereas the “D’ band” at 1350 cm⁻¹ seems to be ascribed to the defects on surface induced by the acid treatment, meaning that the higher the intensity of D band is, the larger the amount of lattice defects exists. The integrated intensity ratio of I_D/I_G for the D and G bands is: 0.91 (the thickness of graphite sheet: 25 μm) < 1.17 (70 μm) < 1.34 (100 μm), which
indicates that the amount of lattice defects becomes larger with increasing thickness of graphite sheet. The sites of lattice defects are considered to those where –COOH group is formed. Based upon the following information from the manufacturer, the density of graphite sheet may be varied, according to the thickness of graphite sheet, i.e., 1.90 g·cm\(^{-3}\) (the thickness of graphite sheet: 25 \(\mu\)m) > 1.21 g·cm\(^{-3}\) (70 \(\mu\)m) > 0.85 g·cm\(^{-3}\) (100 \(\mu\)m).\(^{11}\)

These facts indicate that lower density of graphite sheet, i.e., higher surface area, may be effective in the formation of lattice defects and –COOH group.

### 3.1.2 Formation of calcium phosphate layer on acid-treated graphite sheet

Firstly, the acid-treated graphite sheets with different thickness, i.e., 25, 70 and 100 \(\mu\)m, were immersed in the apatite-dissolved solution, and then the solvent was completely evaporated at 90°C for 1 h. XRD patterns of these acid-treated graphite sheets after the evaporation of solvent from the apatite-dissolved solution are shown in Fig. 3, together with typical FE-SEM micrographs. According to the XRD patterns, the crystalline phases of calcium phosphate layer were unknown, and no formation of HAp was found in these cases. On the other hand, FE-SEM observation showed that the agglomerates of irregular-shaped particles were present at the local sites on the surface of graphite sheets. Furthermore, EDX results showed that the Ca/P ratios of these agglomerates were in the range of 1.04 to 2.08 (not shown here). These data indicate that the inhomogeneous heat transfer may have been occurred during the external heating to the apatite-dissolved solution, due to the convection, thereby forming the calcium phosphate layer with different Ca/P ratios of 1.04 to 2.08 at local sites.

The conditions to homogeneously form the calcium phosphate layer on the surface of acid-treated graphite sheets were further examined by changing the heating technique from “external” to “internal (=microwave)” heating. **Figure 4** shows the typical temperature rise due to the microwave heating to the apatite-dissolved solution. The temperature increased with the microwave irradiation time and attained boiling temperature (100°C) within 200 s. As the present data indicate, the rapid temperature rise up to 100°C may be achieved by the microwave heating, compared to the case of external heating. The advantages of microwave heating must be, therefore, not only the rapid temperature rise, but also the homogeneous heating of the whole system.

**Figure 5** is the XRD patterns showing the effects of microwave heating and amount of apatite-dissolved solution on the formation of calcium phosphate, together with typical FE-SEM micrographs. After the microwave heating to the acid-treated graphite sheet immersed in the apatite-dissolved solution, XRD patterns showed that HAp\(^{12}\) was detected from the acid-treated graphite sheets, together with the reflection of graphite,\(^{8}\) and the X-ray intensity of HAp increased with the amount of apatite-dissolved solution from 300 to 600 cm\(^3\). FE-SEM micrograph showed that the plate-like particles were stuck together to form agglomerates with sizes of 1 to 5 \(\mu\)m.

As the above results indicate, the amount of HAp was enhanced by the microwave heating, compared to the case of external heating. The microwave interaction with materials may be occurred by transferring energy and activating complex ions.
through excitation of various rotational modes, thereby forming HAp.

3.1.3 Combined effect of external/microwave heating

As the results previously shown indicate, the amount of HAp layer formed by the microwave heating was larger, compared to the case of external heating. However, the resulting HAp particles formed by the microwave heating were partly adhered to form the agglomerates. In contrast, HAp particles formed by the external heating were present at the local sites, and no marked agglomeration was found. On the basis of such research results, we designed the homogeneous formation of larger amount of HAp layer on the graphite sheet: (i) the formation of seed crystals of HAp due to the external heating, and (ii) the promotion of the increase in amount of HAp layer due to the microwave heating, from the prospect that the seed crystals of apatite formed by the external heating may help increasing the amount of precipitant during the microwave irradiation. The results obtained by such two-step heatings are described in this section.

Figure 6 is the XRD pattern showing the effect of external and microwave heating on the formation of calcium phosphate, together with the typical FE-SEM micrographs. After the external and subsequent microwave heating to the acid-treated graphite sheet immersed in apatite-dissolved solution: 300 cm³, the typical hexagonal plates seem to be formed by the crystallization and subsequent crystal growth via the seed crystals, because no hexagonal plates have never been formed simply by the microwave heating.

Figure 7 is the FT-IR spectrum showing the effect of external/microwave heating on the formation of HAp, together with the typical EDX results. FT-IR spectrum showed the presence of absorptions in the range of 560–610, 900–1180, 1380–1580 cm⁻¹. Moreover, Ca/P ratio of the precipitants was 1.72, which was close to the stoichiometric ratio of HAp (≈1.67).

These absorption band in the range of 560–610/900–1180 and 1415–1580 cm⁻¹ may be assigned to PO₄³⁻ and CO₃²⁻, respectively. Thus the crystalline phase of precipitant is found to be carbonate-containing apatite. The formation of carbonate-containing apatite may also be supported by the Ca/P ratio of 1.72, as measured by EDX. On the basis of such information, CO₃²⁻ may be present in the general form of Ca₁₀₋₄ₓ(OH)₂₋₂ₓ(PO₄)₄₋ₓ(CO₃)ₓ(OH)₂₋₂₋ₓ(CO₃)ₓ, since the presence of CO₃²⁻ in the HAp structure enhances the solubility in the aqueous solution. Moreover, the absorption peak at 1380 cm⁻¹ may be assigned to the C–O symmetric vibrations in COO⁻ ions, suggesting that the presence of such COO⁻ group demonstrates the electrostatic bonding with a-plane of positively-charged HAp.

Since the HAp layer could be formed on the graphite sheet, the thickness of HAp layer was measured with the results being shown in Fig. 8, together with the tape test showing the bonding strength of HAp particles to the graphite sheet. The thickness of HAp layer could be arranged as follows: 1.7 μm (the amount of apatite-dissolved solution: 300 cm³) < 2.5 μm (the amount of apatite-dissolved solution: 600 cm³) < 4.9 μm (the amount of apatite-dissolved solution: 300 cm³ for the external heating + 300 cm³ for the microwave heating). The FE-SEM micrographs of HAp layer formed by the combination of external/microwave heating showed that the plate-like particles were closely packed, and that no appreciable changes in microstructure were found before and after the tape test. This fact demonstrates the strong bonding of HAp particles to the graphite sheet via the carboxyl group, as well as the bonding among HAp particles via the repetition of solution-precipitation.
The formation mechanism of HAp particles to the graphite sheet is summarized in Fig. 9. The acid treatment by conc. H$_2$SO$_4$ and HNO$_3$ at 90°C for 1 h forms the carboxyl group on the graphite sheet [Fig. (a) $\rightarrow$ (b)]. When the carboxyl-group formed graphite sheet is microwave-heated in the apatite-dissolved solution, the carbonate-containing HAp layer may be formed on the graphite sheet [Figs. 9(c) $\rightarrow$ 9(d)].

3.2 Biocompatibility of graphite sheet with HAp layer

The graphite sheet with HAp layer was implanted into femur and tibia of the Japanese white rabbit. The microscopic examination of graphite sheet with HAp layer in the femur and tibia of the Japanese white rabbit was conducted using the sectioned specimen (Villanueva bone stain) in order to observe the interfaces between graphite sheet with HAp layer and living bone. The micrographs of sectioned graphite sheet with HAp layer after the implantation for 4 months are shown in Fig. 10. The formation of calcified bone (new bone) at the interfaces was found from this specimen implanted for 4 months (see the arrow marks). No coloring of cytoplasm in violet indicates the absence of macrophage and is proved to be biocompatibility. The marked changes in microstructure at the interfaces indicate that the bone may form at the interfaces after the implantation for 4 months.

4. Conclusions

The conditions for the formation of HAp layer on the graphite sheet were examined via the processes of (i) the acid treatment by conc. H$_2$SO$_4$ and HNO$_3$ at 90°C for 1 h, (c) Reaction of Ca$^{2+}$, HPO$_4^{2-}$ and OH$^-$ with carboxyl group on graphite sheet. (d) Stacking of plate-like HAp particles on the graphite sheet.

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HAp layer through the microwave heating. The layer formed by the stacking of plate-like HAp particles with Ca/P ratio of 1.72 possessed the typical thickness of 4.9 μm or carbonate-containing HAp.

(iii) When the graphite sheet with HAp layer was implanted into femur and tibia of the Japanese white rabbit for 4 months, the calcified bone at the interfaces was found to show the excellent biocompatibility.

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