Effect of Surface Hydrolysis of PLA Film on Apatite Formation in Vitro

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SYNOPSIS
In the present study, the author prepared surface hydrolyzed poly(lactic acid) (PLA) films (PLA-COOH), i.e., carboxylic acid introduction, by NaOH hydrolysis and investigated apatite formation on PLA-COOH films after immersion in Hanks’ balanced salt solution (HBSS, pH 7.4) without organic species. In addition, the degradation behavior was monitored in buffered solution with or without surface carboxylic acid groups. HBSS was used as simulated body fluid for evaluating in vitro biocompatibility.

PLA-COOH was easily prepared by NaOH hydrolysis for 3h. PLA-COOH showed a greater degree of degradation after immersion in phosphate buffered saline solution (pH 7.4) compared with native PLA. The weight loss of PLA and PLA-COOH films was approximately 3.2% and 4.9% after 115 days immersion, respectively.

Scanning electron microscopy observation revealed that PLA-COOH film showed a quicker and greater amount of precipitation on the film compared with original unmodified PLA after immersion in HBSS. Analysis of the precipitates by X-ray diffraction and Fourier-transformed infrared spectroscopy confirmed that their main-component was carbonate-containing apatite.

In conclusion, it is expected that PLA-COOH is a good candidate for a scaffold material to enhance bone formation in vivo and will have a potential for dental clinical use.

Key words: PLA film, biomaterials, hydroxyapatite, simulated body fluid, carboxylic acid groups

INTRODUCTION
Nowadays, biodegradable polymers, e.g., polylactic acid (PLA), poly(glycolic acid)(PLGA), and poly (lactic acid- glycolic acid)(PLGA), are used as scaffolds in tissue engineering 

bone regeneration (GBR) for reconstruction of gingival recession or bone defects. However, these biodegradable materials have no biological activity such as cell attachment or bone compatibility.

To assess the biocompatibility or stability of PLA or PLGA films, cell culture assays or animal implantation experiments have been performed. In ad-
dition, some preliminary experiments were performed before biological experiments to screen the materials. One useful method is immersion in simulated body fluid (SBF). Kokubo and colleagues published numerous studies related to SBF immersion experiments for bone adaptability of biomaterials. They developed their own SBF, in which the ion concentration and composition were almost equal to human blood plasma and found apatite formation on biomaterials after immersion in this SBF.

Hanawa et al. also found that apatite formed on a titanium surface after the immersion in Hank’s balanced salt solution (HBSS) without organic species at pH 7.4 and reported on the efficacy of HBSS as an SBF. Hayakawa et al. reported that apatite deposition on a phosphorylated bonding agent and Takahashi et al. demonstrated apatite formation on a thin-carbonate apatite coated titanium surface after immersion in HBSS. Ozawa et al. evaluated apatite formation on commercially available dental biodegradable products after immersion in HBSS and found differences in apatite formation.

Some reported the improvement of biological activity of PLA. Yamaoka et al. reacted gelatin and PLA with NaOH solution, and then immobilized gelatin on the PLA surface. NaOH solution produced carboxylic acid group on the PLA surface, and the amino groups of gelatin then interacted with the carboxylic acid groups of PLA. On the contrary, Cai et al. and Cui et al. modified the PLA surface with silk fibroin or gelatin after NaOH surface hydrolysis of PLA film using water-soluble carbodiimide. The proteins were immobilized by a condensation reaction between the carboxylic acid groups on the alkali hydrolyzed PLA film and the amino groups of the proteins. Cell activities such as adhesion and proliferation were improved by surface modification of PLA film with proteins.

Previously, Nagai et al. immobilized fibronectin, which is a cell adhesive protein, on a PLA surface by NaOH surface hydrolysis and condensation reactions using water-soluble carbodiimide according to the method reported by Cai et al. NaOH hydrolysis is a simple and easy method for introducing carboxylic acid groups onto the surface of PLA. The author hypothesized that the carboxylic groups on the PLA surface will enhance apatite formation after immersion in SBF.

In the present study, the influence of carboxylic acid groups on the PLA surface was examined. Namely, the author prepared surface hydrolyzed PLA films, i.e., carboxylic acid introduction, and immersed them into HBSS. Apatite formation on the PLA surface was monitored using scanning electron microscopy, X-ray diffraction and Fourier transformed infrared reflection-absorption spectroscopy. The degradation behavior of PLA films with or without surface carboxylic acid groups in buffered solution was also evaluated.

### MATERIALS AND METHODS

#### Preparation of PLA films and surface hydrolysis

The scheme for surface hydrolysis, i.e., introducing carboxylic acid groups onto the PLA surface, is shown in Fig. 1. The surface hydrolysis of PLA films was performed according to the method...
described in our previous report\textsuperscript{26}. Approximately 1 g of PLA (Purasorb\textregistered, inherent viscosity: 2.53 g/dl, PURAC Biochem, Gorinchem, Netherlands) was dissolved in 20 ml chloroform. This solution was transferred to a 15 mm-diameter glass plate (Matsunami, Glass Inc, Ltd. Osaka, Japan) and evaporated to form a film. PLA films were delaminated from the glass plate and immersed in 0.5 N NaOH solution at 37°C for 3 h for surface hydrolysis. Afterwards, surface hydrolyzed PLA films were rinsed successively with double distilled water, 0.1 N HCl, and double distilled water, dried, and stored in a desiccator.

The data were analyzed by analysis of variance and Scheffe’s test for multiple comparisons among the means. A p value of less than 0.05 was regarded as significant.

**Weight loss in PBS**

Original unmodified PLA films and PLA films hydrolyzed with NaOH (PLA-COOH) were immersed in 20 ml phosphate-buffered saline (PBS) solution (pH 7.4) at 37°C for 115 days. The solution was exchanged every day with the same volume of PBS. The weight of the specimens before and after immersion in PBS was measured to obtain the weight change of each sample during degradation. After wiping the sample surface with a paper towel (Kimwipe, S-200, Nippon Paper Crecia Co., Ltd. Tokyo, Japan) to remove surface water, each weight was measured immediately. Three specimens for same kind of sample were examined separately. Weight loss was calculated as 100 x (weight difference before and after immersion)/weight before immersion.

**SBF immersion**

Original unmodified PLA films and PLA-COOH were immersed in 20 ml HBSS without organic species, as described by Hanawa et al.\textsuperscript{16}, for 28 days in a sealed polystyrene bottle at 37°C. HBSS was prepared in our laboratory, and the ion concentrations of HBSS (mol/l) without organic species are showed in Table 1. The solutions and bottle were exchanged every day to maintain the specimens in fresh solution. After 3, 7, 14, and 28 days immersion, the specimens were rinsed with distilled water to remove HBSS and then immediately dried in a desiccator.

The morphology of the precipitates on the PLA and PLA-COOH films was examined using a scanning electron microscope (SEM, S-2700, Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV. The specimens were coated with gold and palladium before SEM observation.

The cross-sectional view of the precipitates on the PLA or PLA-COOH film was also observed to monitor the

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mol/l)</th>
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<tbody>
<tr>
<td>Na\textsuperscript{+}</td>
<td>1.42×10\textsuperscript{-1}</td>
</tr>
<tr>
<td>K\textsuperscript{+}</td>
<td>5.81×10\textsuperscript{-3}</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>8.11×10\textsuperscript{-4}</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>1.26×10\textsuperscript{-3}</td>
</tr>
<tr>
<td>Cl\textsuperscript{-}</td>
<td>1.45×10\textsuperscript{-1}</td>
</tr>
<tr>
<td>HPO\textsubscript{4}\textsuperscript{2-}</td>
<td>7.78×10\textsuperscript{-4}</td>
</tr>
<tr>
<td>SO\textsubscript{4}\textsuperscript{2-}</td>
<td>8.11×10\textsuperscript{-4}</td>
</tr>
<tr>
<td>HCO\textsubscript{3}\textsuperscript{-}</td>
<td>4.17×10\textsuperscript{-3}</td>
</tr>
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difference in the precipitated amounts on the film. The specimens collected after 28 days immersion in HBSS were embedded in epoxy resin (Strus®, Marumoto Strus Inc, Ltd. Tokyo, Japan). After curing the resin, the samples were cut vertically so that cross-sectional samples of the precipitates/film could be obtained. The cut samples were ground and then polished with diamond paste down to 0.25 μm. After ultrasonic cleaning, the samples were observed using an SEM at an accelerating voltage of 20 kV. The specimens were coated with gold and palladium before SEM observation.

The crystallographic structure of the precipitate on the PLA and PLA-COOH films after immersion in HBSS was analyzed by means of micro-X-ray diffraction (micro-XRD, θ-2θ diffractometer, RINT 2000, Rigaku, Tokyo), which has X-ray source of CuKα and a power of 50 kV and 200 mA. Fourier-transformed infrared (FT-IR) spectra measurements were also performed to identify the chemical structure of the precipitates. FT-IR spectra were measured in transmission mode for KBr diluted discs at a constant resolution of 4cm⁻¹ on a FT-210 spectrophotometer (Horiba, Ltd., Kyoto, Japan).

RESULTS

Fig. 2 shows the weight loss of PLA and PLA-COOH films after immersion in PBS. Both showed weight loss after immersion in PBS, and until 14 days a rapid decrease in weight was observed. Afterwards, the weight gradually decreased until 115 days. Generally, PLA-COOH tended to decrease greater degree of weight loss after immersion in PBS than PLA. At 115 days immersion, weight loss of the PLA film was approximately 3.2% but that of PLA-COOH was approximately 4.9%.

Fig. 3 shows the SEM pictures of the surface of PLA films after immersion in HBSS. At 3 days, only a small amount of tiny precipitates was observed on the PLA surface. At 7 days, precipitates with a globular shape formed and completely covered the PLA surface. At 14 and 28 days, greater amounts of globular precipitates were observed, and new globule crystals were piled up on the earlier precipitates.
Fig. 4 shows the SEM pictures of the surface of PLA-COOH films after immersion in HBSS. There were distinct differences at 3 days between PLA and PLA-COOH film. At 3 days, the PLA-COOH surface was already completely covered with precipitates and large globules were deposited on the earlier precipitates. At 7, 14, and 28 days, greater amounts of globular precipitates were observed, and new globule crystals were piled up on the earlier precipitates.

Fig. 3 Surface appearance of PLA films after immersion in Hanks’ balanced salt solution
At 3 days, only a small amount of tiny precipitates was observed on the PLA surface. At 7 days, precipitates with a globular shape formed and completely covered the PLA surface. At 14 and 28 days, greater amounts of globular precipitates were observed, and new globule crystals were piled up on the earlier precipitates.
Precipitates were observed, and new globule crystals were also piled up on the earlier precipitates. However, it was difficult to compare the difference in the precipitated amounts between the PLA and PLA-COOH film by surface observation. So the author decided to observed the cross-sectional views.

Fig. 5 shows the cross-sectional view of PLA and PLA-COOH films after 28 days immersion in Hanks’ balanced salt solution. A layer of precipitates was observed and is indicated by the arrow in the pictures. PLA-COOH produced a thicker and denser precipitate layer. The thickness of the precipitate layer on original unmodified PLA film was approximately 15-30 μm and that on PLA-COOH film was approximately 40-55 μm.

Micro-XRD spectra demonstrated hydroxyapatite formation on the PLA and PLA-COOH films in HBSS after 7 and 28 days as shown Figs. 6 and 7. PLA and PLA-COOH showed similar XRD patterns, and no distinct differences in XRD patterns existed between 7 and 28 days immersion. Major peaks in the spectra could be assigned as 210 (θ =28°), 211, 112, and 300 (θ =32°), 222 (θ =46°) of peaks of hydroxyapatite.

FT-IR spectra of PLA films and PLA-COOH films in HBSS after 7 and 28 days are shown in Figs. 8 and 9. PLA and PLA-COOH showed similar FT-IR spectra, and no clear differences in FT-IR were observed between 7 and 28 days immersion. There were two clusters of peaks around 500-600 cm⁻¹ and 900-1300 cm⁻¹. The former cluster was...
Fig. 6 X-ray diffraction patterns of the white precipitate on PLA film after immersion in Hanks’ balanced salt solution.

Fig. 7 X-ray diffraction patterns of the white precipitate on PL-CONH film after immersion in Hanks’ balanced salt solution.
Fig. 8 FT-IR spectra of the white precipitate on PLA film after immersion in Hanks’ balanced salt solution

Fig. 9 FT-IR spectra of the white precipitate on PLA-COOH film after immersion in Hanks’ balanced salt solution
assigned as P-O bending modes, and the latter was assigned as P-O stretching modes. These are attributed to the P-O bonds of inorganic calcium phosphate. The peaks around 1635 and 3450 cm$^{-1}$ were assigned as the hydroxyl group of hydroxyapatite. Peaks derived from carbonate groups were also observed around 870 and 1420 cm$^{-1}$. The obtained spectra indicated that the precipitates on PLA and PLA-COOH after immersion in HBSS were hydroxyapatite containing carbonate groups in its structure.

**DISCUSSION**

In the present study, the author investigated the weight loss in PBS and apatite formation on PLA-COOH surfaces in HBSS compared with original unmodified PLA.

PLA-COOH was easily prepared by NaOH treatment. Nagai *et al.* evaluated the influence of NaOH hydrolysis time of PLA for surface hydrolysis. They reported that 3h hydrolysis provided the lowest contact angle with respect to double-distilled water. Surface characterization by X-ray photoelectron spectroscopy confirmed the introduction of COOH groups on the PLA surface after NaOH hydrolysis. Thus, in the present study, the author performed NaOH hydrolysis of PLA for 3h.

Weight loss of PLA in PBS consisted of degradation of the PLA film. The introduction of carboxylic groups caused a greater degree of degradation of PLA. The degradation of PLA was caused by the cleavage of the ester bonds of PLA. It has been reported that PLA with a terminal carboxylic acid group caused rapid degradation because of autocatalysis by the carboxylic group. Nagai reported that PLA degradation in PBS was accelerated by block polymer formation with copoly (ethyl sebacate/hexamethylene sebacate) because the terminal carboxylic group was locally concentrated in the block polymer. The present results were in agreement with these previous reports. The greater degree of PLA-COOH degradation was caused by autocatalysis of the surface carboxylic groups of PLA-COOH.

The control of degradation rate of PLA will be very important for clinical use. In the present study, NaOH surface hydrolysis will have a potential to control the degradation rate of PLA.

For the evaluation of *in vitro* biocompatibility, many studies have reported the formation of an apatite layer after materials were immersed in SBF. It was reported that *in vivo* bioactivity (osteocompatibility) of biomaterials such as ceramics is precisely mirrored by their *in vitro* apatite-forming ability in SBF. In the present study, HBSS was employed as a simulated body fluid. Hanawa *et al.* reported that an apatite layer was formed on a titanium surface after immersion in HBSS. Tanimoto *et al.* reported that *in vivo* bone formation of sintered tricalcium sheets confirmed the results of the *in vitro* SBF immersion experiment, namely more apatite formation corresponded with better bone formation. Based on the above reports, it is expected that PLA-COOH will be a good candidate as a scaffold material to enhance bone formation *in vivo*.

As for the mechanism of apatite formation on titanium in HBSS, Hanawa suggested that positively charged calcium ions first adsorbed onto the titanium surface covered with hydroxyl groups and then the negatively charged phosphate ions adsorbed afterwards. Eventually an apatite layer was formed by repetition of this process. It is presumed that calcium ions will bind easily to the PLA-COOH surface through the interaction of calcium ions and carboxylic groups. Thus, PLA-COOH showed a greater amount of apatite formation. The present study
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revealed that the presence of carboxylic acid groups was favorable for the precipitation of an apatite layer.

The carbonate ions were incorporated into the apatite and thus carbonate-containing apatite formed on the PLA and PLA-COOH surface because of the high levels of carbonate ions in HBSS. The deposition of carbonate-containing apatite on PLA-COOH is interesting because of its chemical resemblance to bone mineral.

Further in vitro cell culture and in vivo animal experiment will be needed to confirm the effectiveness of PLA-COOH.

CONCLUSION
Surface hydrolyzed PLA film by NaOH hydrolysis, i.e., PLA-COOH, showed a greater degree of degradation after immersion in PBS and also showed greater amount of apatite formation on the surface after immersion in HBSS. The present study suggested that PLA-COOH may be applied for dental clinical use, e.g., GTR membrane, GBR membrane and dental implant.

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REFERENCES


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