

Enhancement of Apatite Precipitation on an Alkaline Hydrolyzed Poly (Lactic Acid- ϵ -Caprolactone) Film in Simulated Body Fluid

(アルカリ加水分解処理したポリ(乳酸- ϵ -カプロラクトン)共重合体フィルム of 擬似体液中でのアパタイト形成)

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Abstract

Biodegradable materials, such as poly (lactic acid) (PLA), have been applied to regenerate bone. However, pure PLA is brittle, so it can be modified by blending ϵ -caprolactone (PCL). These biodegradable materials have no functional activity. In the present study, a copolymer of PLA/PCL (PLCL) film was fabricated by dissolving the materials in chloroform. Carboxylic acid groups (COOH) of PLCL were chemically introduced onto the PLCL film surface by 0.5N NaOH hydrolysis. Apatite formation was evaluated on an alkaline hydrolyzed PLCL film (PLCL-COOH) after immersion in Hanks' balanced salt solution (HBSS, pH7.4) without organic species. Water adsorption PLCL-COOH in phosphate buffered saline (PBS) was also monitored by comparing PLCL. PLCL-COOH provided a greater degree of apatite precipitation than PLCL after 3 and 7-day immersion. Weight loss was significantly different between PLCL-COOH and PLCL films from during experimental period of immersion in PBS ($p < 0.05$). The contact angle of the PLCL-COOH surface with respect to double distilled water significantly decreased compared with PLCL ($p < 0.05$). Thus, it was predicted that PLCL-COOH would show a better biological response and be applicable in new types of dental materials in dental treatment.

Key words: Biomaterials, Carboxylic acid groups, Hydroxyapatite, Poly (lactic acid- ϵ -caprolactone) film, Simulated body fluid

Introduction

Biodegradable materials such as poly (lactic acid) (PLA) and its copolymer with poly (glycolic acid) (PGA), are promising materials in regenerative medicine¹⁻³). These polymers are widely used for tissue fixation devices (e.g., bone pins, bone screws, bone plates, drug delivery systems, and scaffolds)^{4,5}).

In the dental field, PLA, PGA and/or poly (lactic acid-co-glycolic acid) (PLGA) have been widely utilized as guided tissue regeneration (GTR) and guided bone regeneration (GBR) membranes^{6,7}). However, these membranes did not have any biological functions such as bone compatibility or cell affinity.

Kitahara⁸) introduced carboxylic acid group (COOH) onto the surface of PLA films by using NaOH hydrolysis and found progress of apatite deposition onto the hydrolyzed PLA surface after the immersion in simulated body fluid. Simulated body fluid (SBF) is generally used as a step prior to animal testing and is one valuable method. Fujibayashi et al.⁹) reported that in the vivo bioactivity of ceramics is correctly mirrored by their in vitro apatite forming ability in an SBF.

Kokubo et al.¹⁰) reported a number of studies related to SBF immersion experiments for bone adaptability of biomaterials. They developed their own SBF, in which the ion concentration and composition was almost the same as human blood plasma and found apatite formation on biomaterials after immersion in this SBF¹¹⁻¹⁴). SBF is standard method to investigate the bioactivity of biomaterials. Hanawa et al.^{15,16}) also found that apatite formed on a titanium surface after 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.¹⁷) demonstrated apatite formation on a thin Ca coated titanium fiber mesh surface after immersion in HBSS by a thin Ca coating using the molecular precursor method.

Thus, previous results reported by Kitahara et al.⁸) suggested that hydrolyzed PLA will be effective for bone formation because of the progression of apatite precipitation. However, PLA film is hard and brittle¹⁸) and its characteristics are limited. On the contrary, poly (ϵ -caprolactone) (PCL) is miscible with a variety of polymers. PCL is known for lower toxicity and permeability¹⁹). Adding PCL can change its original properties, such as stickiness, flexibility and elongation²⁰). Copolymers with PCL are now being developed as novel biodegradable materials.

In the present study, we aimed to investigate the efficacy of alkaline hydrolysis of a PLA/PCL (PLCL) copolymer for apatite precipitation after immersion in SBF and for degradation after immersion in phosphate buffered saline (PBS) solution.

Materials and Methods

Preparation of PLCL Films

Poly (L-lactide-co- ϵ -caprolactone) (PLCL: PLA/CL=75:25) (LCL75:25[®], BMG Inc, Kyoto, Japan) was used. Surface hydrolysis of PLCL films was performed according to a previous method²¹). The simple scheme is shown in Fig. 1. First, approximately 1 g of PLCL was dissolved in 20 ml of chloroform. This solution was transferred to a 15 mm-diameter glass plate (Matsunami, Glass Inc, Ltd. Osaka, Japan) and evaporated to form a film. PLCL films were delaminated from the glass plate and immersed in 0.5 N NaOH solution at 37 °C for 3 hours for surface hydrolysis. Afterwards, surface hydrolyzed PLCL (PLCL-COOH) films were rinsed successively with double distilled water, 0.1 N HCl, and double distilled water, dried, and stored in a desiccator.

The contact angles of PLCL films and PLCL-COOH films

Contact angles of PLCL films before and after NaOH hydrolysis with respect to the double distilled water were measured using a contact angle meter (Face CA-P, Kyowa Kaimenkagaku Co., Ltd. Tokyo, Japan). Three specimens were measured for each surface. The experiment was conducted at the same temperature (25±2 °C) and humidity (50 %). All data were analyzed by Student's t-test. A p value of less than 0.05 was regarded as significant.

SBF immersion

PLCL and PLCL-COOH films were immersed in 20 ml HBSS without organic species for 3, 7, 14 and 28 days in a sealed polystyrene bottle at 37 °C. HBSS was prepared in our laboratory, and the concentrations of electrolytes in HBSS (mmol/l) without organic species are summarized in Table 1. The solutions and bottle were exchanged every day to prevent alterations in the concentrations of ions. After 3, 7, 14, and 28 days' immersion, the specimens were rinsed with distilled water to remove excess HBSS and then immediately dried in a desiccator.

The morphologies of the precipitates on the PLCL and PLCL-COOH films were observed by scanning electron microscope (SEM, S-3400N, Hitachi, Tokyo, Japan) at an accelerating voltage of 10-20 kV. The specimens were coated with palladium before SEM observation.

A cross-sectional view of the precipitates on the PLCL or PLCL-COOH film was also monitored to detect differences in the precipitated amounts on the film. Specimens 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 μ m. The samples were observed using an SEM at an accelerating voltage of 20 kV. The specimens were coated with palladium before SEM observation.

Weight changes of PLCL and PLCL-COOH films in phosphate-buffered saline (PBS)

Unmodified PLCL and PLCL-COOH were immersed in 20 ml of PBS (pH=7.4) at 37 °C for 18 weeks. The solution was exchanged every three days with the same volume of PBS. The weight of the specimens before and after immersion in PBS was measured at 1, 2, 3, 4, and 5 days, and 1, 2, 5, 7, 10 and 18 weeks to obtain the weight change of each sample. After wiping the sample surface with a paper towel (Kimwipe, S-200, Nippon Paper Crexia Co., Ltd. Tokyo, Japan) to remove surface water and drying specimens at room temperature for 5 minutes, the weight of each sample was measured. Four specimens for the same kind of sample were examined separately. Weight loss was calculated as $100 \times (\text{weight difference before and after immersion}) / \text{weight before immersion}$.

Results

Surface characterization of PLCL films and PLCL-COOH films

Table 2 lists the contact angles of PLCL films before and after NaOH hydrolysis with respect to double distilled water. The contact angle of PLCL-COOH films decreased compared to PLCL films ($p < 0.05$).

Apatite precipitation on the surface of PLCL films and PLCL-COOH films

Fig. 2 shows the SEM views of the surface of PLCL and PLCL-COOH films after immersion in HBSS for 3, 7, 14 and 28 days, respectively.

Apatite precipitation was observed on both PLCL and PLCL-COOH film surfaces. In the early stage, tiny grain-like precipitates were observed and larger globules were piled up on the initial precipitated layer. At 3 days' immersion, the PLCL-COOH film was covered with a greater amount of early precipitation, tiny grain-like precipitates, compared with PLCL. At 7 days' immersion, the PLCL-COOH film was completely covered with apatite precipitates, but PLCL films were not yet completely covered.

Fig. 3 shows the SEM of the cross-sectional views of the PLCL and PLCL-COOH films after immersion in HBSS at 28 days. A layer of precipitates is indicated by the arrow in the pictures. The thickness of the precipitate layer on the PLCL film was approximately 10-20 μm and that on PLCL-COOH was approximately 20-40 μm , which was thicker than that on the PLCL film.

Weight changes of PLCL and PLCL-COOH films in PBS

Fig. 4 shows the weight changes of PLCL and PLCL-COOH films after immersion in PBS. The weight of PLCL films did not decrease at 3 days. On the other hand, weight loss of PLCL-COOH films was recognized at 3 days. After 7 days, no weight loss of PLCL or PLCL-COOH films was observed and almost no great weight change was monitored up to 124 days. Comparing PLCL and PLCL-COOH films, the PLCL-COOH film showed a

significantly greater degree of weight loss than the PLCL film ($p < 0.05$).

Discussion

In the present study, we introduced a carboxylic acid group onto the surface of a PLCL film by using a NaOH hydrolysis method and evaluated the apatite precipitation on the PLCL-COOH surface after immersion in HBSS. The degradation behavior was also monitored by PBS immersion.

Hydrolyzed PLCL (PLCL-COOH) surfaces were verified by contact angle measurement with respect to double-distilled water. Contact angle of PLCL-COOH was significantly lower than that of PLCL. This was due to the introduction of a carboxylic group on the PLCL surface via alkaline hydrolysis. This revealed that the hydrophilicity of PLCL-COOH was increased by NaOH treatment. NaOH treatment is a simple technique for modifying degradable polymeric materials such as PLA and PLGA. Nagai et al.²¹⁾ evaluated the hydrolysis time of PLA for surface hydrolysis. They reported that 3 hours of NaOH treatment produces the lowest contact angle with respect to double-distilled water.

After immersion in HBSS for 3, 7, 14 and 28 days, both PLCL and PLCL-COOH films showed apatite precipitation. The PLCL-COOH film showed a greater amount of precipitation than the PLCL film. Thus, it is presumed that the surface carboxylic group introduced by NaOH treatment accelerated apatite formation. The obtained result was consistent with previous studies by Kitahara⁸⁾. Fan et al.²²⁾ reported that apatite crystal nuclei adsorbed ions from their own surrounding environment and developed via a growth process on a stainless steel composite surface in HBSS. Hanawa¹⁵⁾ also reported the mechanism of apatite formation; positively charged calcium ions first adsorbed onto a titanium surface covered with hydroxyl groups and then negatively charged phosphate ions adsorbed afterwards. As a result, an apatite layer was formed.

Calcium ions bind easily to the PLCL-COOH surface through the interaction of calcium ions and carboxylic groups during the early stage. As a result, carboxylic acid groups affect the speed of apatite layer formation in the early stage.

Many studies have evaluated apatite formation after materials were immersed in SBF. Ozawa et al.²³⁾ found apatite formation on various dental biodegradable materials after immersion in HBSS. Fujibayashi et al.⁹⁾ reported that the *in vivo* bioactivity of biomaterials such as ceramics were definitely mirrored by their *in vitro* apatite-forming ability in SBF. From the research results above, it is suggested that a PLCL-COOH film will be a biocompatible scaffold for bone formation.

NaOH treatment has the potential to control the degradation rate of PLCL. Controlling the degradation rate of biodegradable polymers is very important in terms of tissue regeneration scaffolds. Significant differences in weight changes in PBS were confirmed between PLCL and PLCL-COOH films ($p < 0.05$). PLCL-COOH showed a higher degree of weight loss, namely, material degradation, during PBS immersion. The introduction

of carboxylic groups caused a greater degree of PLCL degradation. The degradation of PLCL was caused by cleavage of its ester bonds. Pitt et al.²⁴⁾ reported that PLA with a terminal carboxylic group caused rapid degradation because of autocatalysis by the carboxylic group.

On the contrary, Kitahara⁸⁾ reported that PLA films with a carboxylic group (PLA-COOH) showed rapid weight loss due to rapid degradation after immersion in PBS. The degradation behavior in PBS of PLCL-COOH was different from PLA-COOH, and a slower degradation rate of PLCL-COOH was identified. This was due to the blending of poly- ϵ -caprolactone into PLA. This suggested that rapid degradation of PLCL-COOH was inhibited by blending with poly- ϵ -caprolactone.

Nagai et al.²¹⁾ and Fuse et al.²⁵⁾ reported an improvement in the biological activity of PLA, such as cell attachment, biocompatibility, immobilized fibronectin, and cell-adhesive protein, on the surface of a PLA film following NaOH surface hydrolysis and condensation reactions using water-soluble carbodiimide. The result demonstrated that fibronectin immobilization enhanced cell attachment and proliferation on the surface of the PLA film. Thus, NaOH hydrolysis is a simple and easy method for introducing carboxylic acid groups. The modification of PLCL-COOH with cell adhesive protein will be further investigated in the next series of our experiments.

In conclusion, the present research indicated that bioactive bone-like apatite can be formed on a PLCL-COOH surface in SBF. PLCL-COOH could be potentially applied to dental implants, orthopedic implants and drug-controlled carriers. More detailed studies, for example, *in vitro* cell cultures and *in vivo* animal experiments, will be needed to confirm the effectiveness of PLCL-COOH.

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Tables and Figures

Table1 Concentration of electrolytes in Hanks' balanced salt solution

Ion	Concentration (mol/ L)
Na ⁺	1.42×10 ⁻¹
K ⁺	5.81×10 ⁻³
Mg ²⁺	8.11×10 ⁻⁴
Ca ²⁺	1.26×10 ⁻³
Cl ⁻	1.45×10 ⁻¹
HPO ₄ ²⁻	7.78×10 ⁻⁴
SO ₄ ²⁻	8.11×10 ⁻⁴
HCO ₃ ⁻	4.17×10 ⁻³

Table2 Contact angle of PLCL and PLCL-COOH films with respect to double-distilled water

	Contact angle (°)
PLCL	72.4±2.6
PLCL-COOH	53.6±1.7

Mean values of the contact angle of PLCL and PLCL-COOH films after NaOH hydrolysis were ^{M±SD} significantly different at p<0.05.

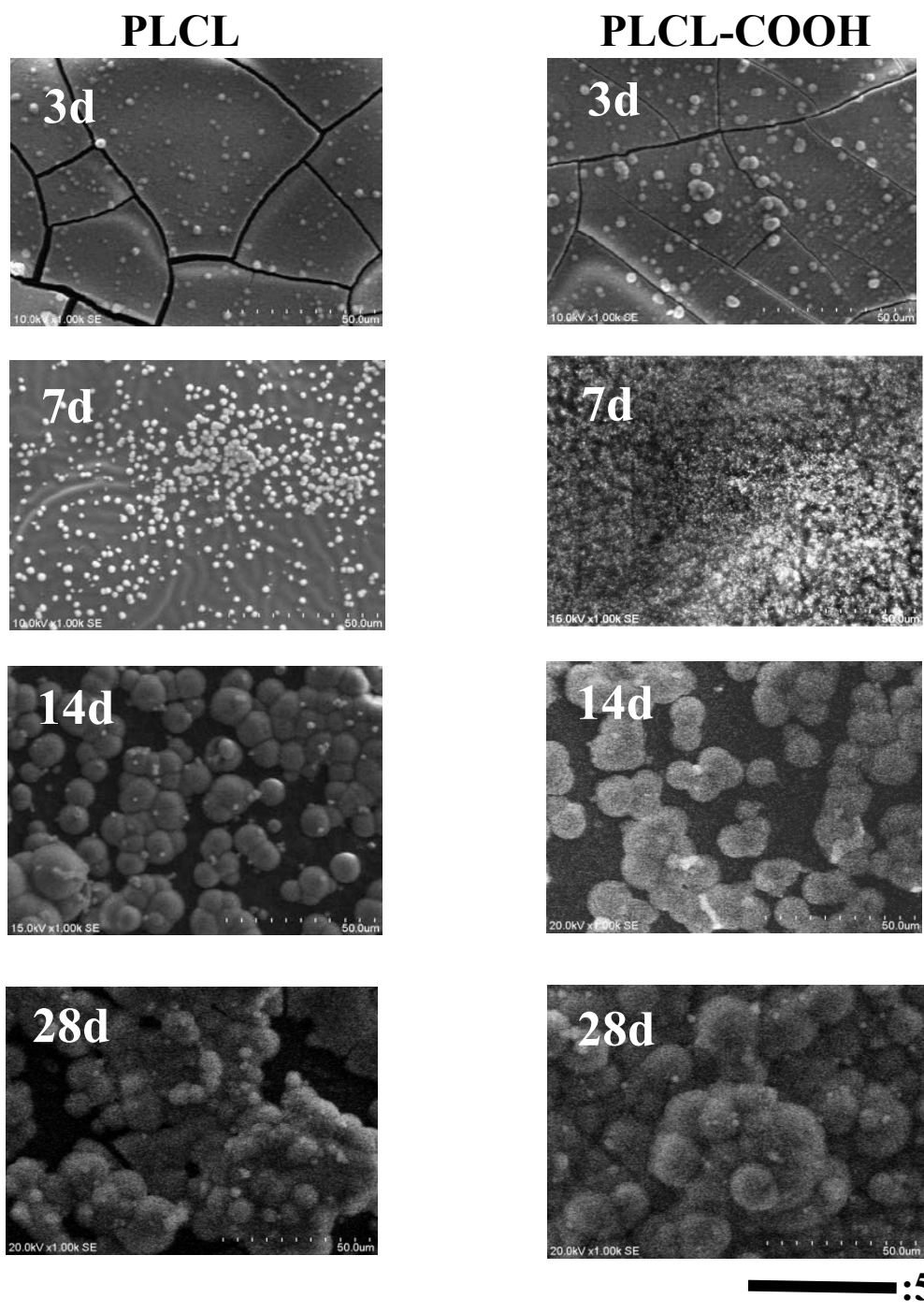


Fig.2 SEM views of surfaces of PLCL and PLCL-COOH films after immersion in HBSS for 3, 7, 14 and 28days.

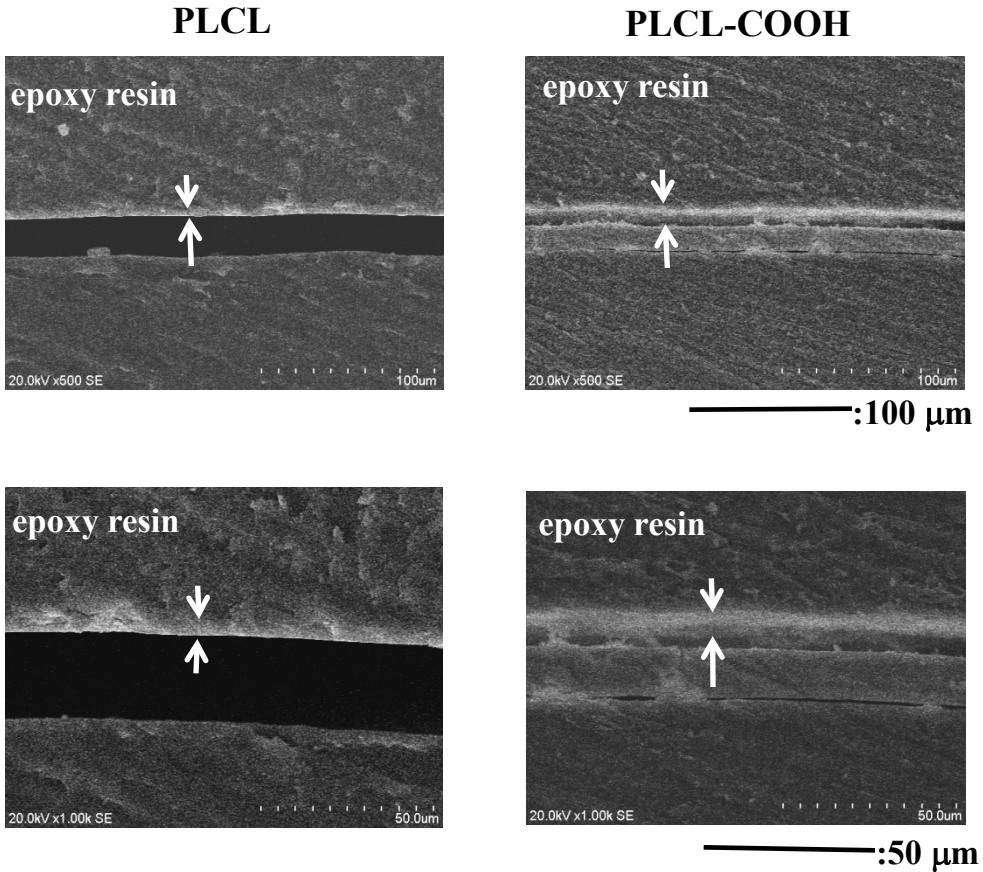


Fig.3 SEM views of cross-sections of PLCL and PLCL-COOH films after immersion in HBSS for 28days. Between two arrows indicates apatite layer

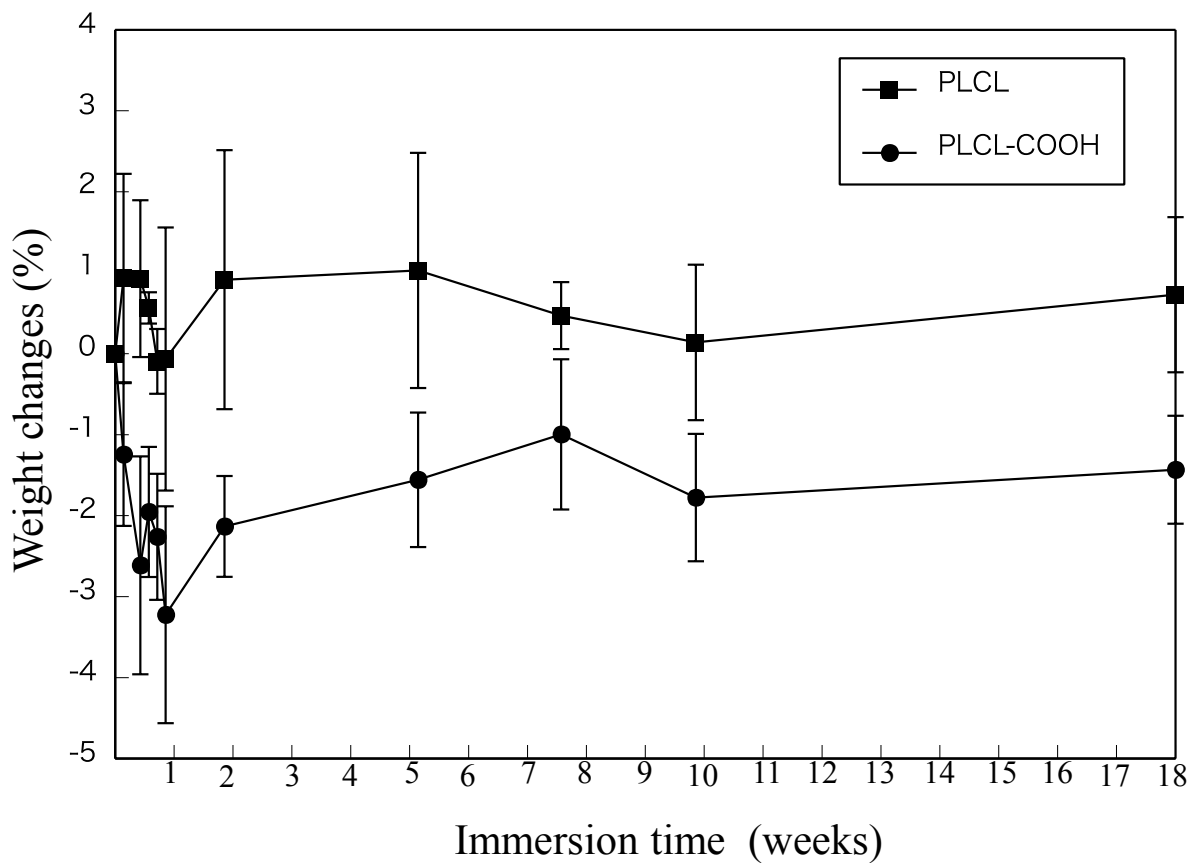


Fig.4 Weight changes in the PLCL and PLCL-COOH films after immersion in PBS