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In vitro evaluation of biodegradation of poly(lactic-*co*-glycolic acid) sponges

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ABSTRACT

Evaluation of the degradability of porous scaffolds is very important for tissue engineering. A protocol in which the condition is close to the *in vivo* pH environment was established for *in vitro* evaluation of biodegradable porous scaffolds. Degradation of PLGA sponges in phosphate-buffered solution (PBS) was evaluated with the protocol. The PLGA sponges degraded with incubation time. For the first 12 weeks, the weight loss increased gradually and then remarkably after 12 weeks. In contrast, the number-average molecular weight (M_n) decreased dramatically for the first 12 weeks and then less markedly after 12 weeks. Thermal analysis showed that the glass transition temperatures (T_g) decreased rapidly for the first 12 weeks, and the change became less evident after 12 weeks. These results suggest that the degradation mechanism of PLGA sponges was dominated by autocatalyzed bulk degradation for the first 12 weeks and then by surface degradation after 12 weeks. Physical aging was observed during incubation at 37 °C. The heterogeneous structure caused by physical aging might be one of the driving forces that induced autocatalyzed bulk degradation. The degradation mechanism was further supported by the data of pH change and the morphology of the degraded PLGA sponges. The autocatalyzed acidic products flooded out after 8 weeks, the pH dropped, and the walls of the sponges became more porous. The increase of the pore surface area facilitated surface degradation after 12 weeks. The pH was in the range between 7.43 and 7.24 during the entire incubation time. The protocol suppressed extreme changes of the pH and will be useful in the biodegradation evaluation of porous scaffolds for tissue engineering.

1. Introduction

Porous scaffolds play an important role in tissue engineering as templates to accommodate cells and guide new tissue formation [1–3]. At first, the scaffolds serve as platforms to support cell adhesion, promote cell proliferation and differentiation, facilitate the transport of nutrients and metabolic wastes, and to hold the integrated cells and extracellular matrices. They also provide the initial mechanical strength necessary to protect the new tissues or organs from the suppression or tension of the surrounding environment. After the cells and extracellular matrices are assembled into functional tissues or organs, the scaffolds are no longer necessary [1–3]. After the new tissues or organs are regenerated, the scaffolds should degrade and eventually disappear. The ideal porous scaffolds should have a degree of biodegradability that matches the formation of the new tissues or organs to allow the templating role of the scaffolding to gradually be replaced by the extracellular matrices. Aliphatic biodegradable polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-*co*-glycolic acid) (PLGA), have been widely used to construct porous scaffolds for the tissue engineering of various tissues or organs such as skin, cartilage, bone, blood vessels, etc. because of their versatile biodegradability and mechanical properties.

The degradability of biodegradable polyesters is affected by both the intrinsic properties of the polymer and the external environment. Polymer properties include monomer structure [4–6], molecular weight [7], copolymer ratio [8], crystallinity [9–11], and shape [12], while the external environment includes pH [9,13], temperature [14], and enzymes [15]. The degradation of biodegradable polyesters *in vivo* commonly proceeds by a chemical hydrolysis reaction of ester bonds in its backbone and then results in producing carboxylic acid end groups that act as a catalyst in the reaction [11]. Evaluation of the degradation of porous scaffolds provides important information for the design and selection of biodegradable polymers for tissue engineering. Many methods have been established by mimicking the *in vivo* environment to study the degradation of biodegradable porous scaffolds. Lu et al. investigated the effects of film thickness on the degradation behavior of PLGA thin films *in vitro* [16]. Wu et al. reported on the *in vitro* degradation of PLGA porous scaffolds with various copolymer ratios, porosities, and pore sizes [17–19]. In these studies, the buffer solution remained unchanged or completely changed, which resulted in an extreme change of pH before and after the change of buffer solution. The physiological pH is 7.4 and its change is very

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mild. A protocol that is close to the *in vivo* pH environment is necessary to evaluate the degradation of porous scaffolds. During the degradation, properties such as the thermal property of the polymer might change. However, the effect of such a change on the degradability of the scaffolds has not been elucidated. In this study, we established a new protocol with a stable pH for *in vitro* degradation evaluation of PLGA sponges. Their degradation mechanism was discussed in detail by analyzing the changes in weight, molecular weight, thermal properties, morphology, and pH.

2. Materials and methods

2.1. Fabrication of PLGA porous scaffold

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) with a copolymer ratio of 75/25 (lactic acid/glycolic acid) was purchased from Sigma–Aldrich, Inc., (St. Louis, MO). Its weight-average (M_w) and number-average (M_n) molecular weights and polydispersity index (PDI) measured by gel permeation chromatography (GPC) were $109{,}520 \pm 1670$, $47{,}870 \pm 1560$, and 2.3 ± 0.1, respectively. A three-dimensional porous scaffold was fabricated by the particulate-leaching technique [20] using sieved sodium chloride (NaCl) particulates with a diameter ranging from 355 to 425 μm. Briefly, the NaCl particulates were added to a PLGA solution in chloroform at a weight ratio of PLGA/NaCl of 9/1, and mixed well. Chloroform was allowed to evaporate by air drying in a draft for 1 day, followed by 3 days of vacuum drying. To leach out the NaCl particulates, the dried PLGA/NaCl composite was immersed in deionized water that was changed every hour. The washing was continued until the weight of the dried sponge did not change. Finally, the perfect leaching out of NaCl was confirmed by a simple qualitative analysis with a silver nitrate ($AgNO_3$) aqueous solution, by which the change of transmittance caused by silver chloride precipitates of an ionic-reaction-product was detected by measuring light transmission. After drying, the resultant sponges were cut into pieces having a dimension of $13.5 \times 13.5 \times 5$ mm. NaCl, chloroform, and $AgNO_3$ solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deionized water was obtained with a Milli-Q water filter system from Millipore Corporation (Bedford, MA).

The cross-section of PLGA scaffold was observed by a scanning electron microscope (SEM) (JSM-6400Fs, JEOL, Ltd., Tokyo, Japan) operated at a voltage of 20 kV. The PLGA sponge was cut with a razor blade and then coated with platinum using a sputter coater (Sanyu Denshi Co., Tokyo, Japan). The pore size of the PLGA was measured by analyzing the pore size of 10 randomly chosen SEM images of the cross-sections of the PLGA sponges. The porosity of the PLGA sponges was determined by a mercury porosimeter (Autopore IV, Shimadzu, Kyoto, Japan).

2.2. In vitro *degradation test*

An *in vitro* degradation test of the PLGA scaffold was conducted in phosphate buffer solution (PBS) under pH 7.4 at 37 °C with mechanical shaking (60 shakes/min). PBS was prepared by mixing 18.2% (v/v) of 1/15 mol/l KH_2PO_4 aqueous solution and 81.8% (v/v) of 1/15 mol/l KH_2PO_4 aqueous solution; the pH of the mixture solution was adjusted to 7.4. The PBS was autoclaved before use. Before the degradation test, the PLGA sponges were sterilized by 70% ethanol aqueous solution. This treatment served another important role as a pre-wetting treatment, which makes PBS to permeate into all the pores of the sponges. After complete washing with sterile PBS, the sponges were immersed in 20 ml sterile PBS. Disposable sterile polypropylene centrifuge tubes (50 ml) were used as test vessels. To suppress the pH change during the degradation test to a minimum, only the upper three-fourths of the PBS was replaced with fresh PBS every week. The sponges were collected every 4 weeks, washed with deionized water, air-dried for 1 day, and vacuum-dried for another 3 days. The dried samples were used for various evaluations. The pH of the removed PBS was measured by a pH meter (Shimadzu, Co., Kyoto, Japan).

2.3. Evaluation of degraded PLGA scaffolds

The weights of the PLGA sponges were measured with an electrical balance, AG 135 (Mettler-Toledo International Inc., NY, USA). Weight loss in percentage was calculated according to a simple equation:

$$\text{Weight loss } (\%) = \frac{(W_0 - W_t)}{W_0} 100 \quad (1)$$

where W_0 is the initial weight and W_t is the weight at a given time point. Both W_0 and W_t were measured after vacuum drying for 3 days. M_n and PDI of the PLGA sponges before and after degradation were determined by GPC using a high-performance liquid chromatography system, HLC-8220GPC (Tosoh Co., Tokyo, Japan), with two TSK gel columns (GMH_{HR}-M, Tosoh Co., Tokyo, Japan). Chloroform was used as the elution solvent at a flow rate of 1.0 ml/min at 40 °C; TSK polystyrene standards (Tosoh Co., Tokyo, Japan) were used for calibration.

Glass transition temperature (T_g) and enthalpy relaxation (ΔH_g) of the sponges were determined by differential scanning calorimetry (DSC) using a DSC instrument, DSC8240 (Rigaku Co., Tokyo, Japan). Each DSC measurement consisted of the following three steps: (1) heating from −15 to 200 °C; (2) cooling from 200 to −15 °C; (3) heating from −15 to 200 °C. All steps were scanned at a rate of 10 °C/min under a nitrogen gas flow at a rate of 50 ml/min. Only the DSC curves obtained from heating steps 1 and 3 were recorded, which were called as the 1st and 2nd scans, respectively. The instrument was calibrated with indium, tin, and lead.

The morphology of the degraded PLGA sponges was observed by the SEM operated at a voltage of 20 kV.

At each time point 10 samples were used for the weight and pH measurements and 3 samples for GPC. The data were used to calculate the means and standard deviations.

3. Results

3.1. Pore size and porosity of intact scaffolds

The PLGA sponges were prepared by the particulate-leaching method. Fig. 1 shows the typical cross-section of a PLGA sponge. The PLGA sponges had a highly porous structure with the average pore size of 388.3 ± 51.8 μm. The average pore size was highly consistent with that of the NaCl particulates. The porosity of the PLGA sponges was $89.9 \pm 1.1\%$, measured by mercury porosimetry. The value was very near to the theoretical porosity (90%) calculated from the ratio of polymer and NaCl. These results indicate that porous structures of PLGA sponges such as pore size and porosity could be controlled by the size of porogen, and the ratio of porogen and polymer.

3.2. Change of weight and molecular weight

Fig. 2 shows the weight change of the PLGA sponges with incubation time. The PLGA sponges lost weight with an increase in incubation time. The weight loss was slow for the first 8 weeks, moderate between 8 and 12 weeks, and increased rapidly after 12 weeks.

The M_n and PDI of the PLGA sponges were determined by GPC using a high-performance liquid chromatography system. Figs. 3 and 4 show the changes of M_n and PDI with incubation time, respectively. The M_n decreased dramatically for the first 12 weeks and only slightly after 12 weeks. After 24 weeks, the M_n decreased to 3030 ± 480. The PDI increased rapidly for the first 8 weeks and then decreased after 8 weeks. The decrease became less evident after 12 weeks.

Degradation of biodegradable polyesters commonly proceeds by chemical hydrolysis reaction of the ester bonds in its backbone, and

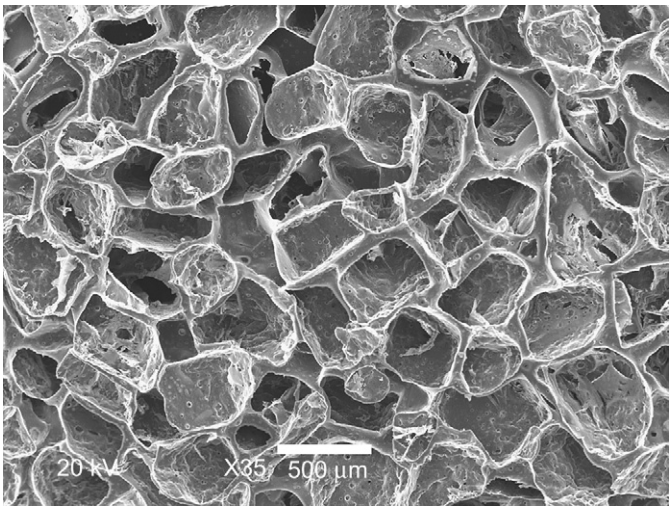

Fig. 1. SEM photomicrograph of cross-section of PLGA sponge. Scale bar indicates 500 μm.