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Effects of poly(L-lactic acid) hydrolysis on attachment of barnacle cypris larvae

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ABSTRACT

Poly(L-lactic acid) (PLLA) applied to immersed solid surfaces in seawater inhibited colonization by barnacles due to the slow-release property of lactic acid. The effect of molecular weight of PLLA on anti-macrofouling activity was confirmed for the first time, with the lowest molecular weight PLLA producing the lowest attaching ratio of cypris larvae of *Balanus amphitrite*. From the direct addition of lactic acid into a culture of cypris larvae, it was found that the anti-barnacle settlement effect was due to the action of slow-released lactic acid to cypris larvae. The anti-macrofouling function of low molecular weight PLLA was also confirmed in a natural sea environment.

1. Introduction

Marine biological fouling, usually termed marine biofouling, is the undesirable accumulation of microorganisms, plants, and animals on immersed surfaces. Such accumulations can have dramatic economic and environmental costs, through increased fuel consumption to shipping, frequent dry-docking and painting for hull maintenance, the hindering of seaweed farming, etc. [1]. In preventing marine biofouling, effective tributyltin (TBT) self-polishing copolymer paints have been the most successful [2]. Following the banning of TBT as an antifoulant, several organic booster biocides have been used in conjunction with copper compounds in antifouling paints as alternative treatments [3,4].

Recently, environmentally friendly alternative methods of anti-fouling have been actively researched. These include treatments using natural products [5], fouling release coatings [6], antifouling topographies [7,8], and electrical antifouling systems [9].

To prevent the development of marine biofouling, especially macrofouling, by the undesirable accumulation of organisms such as barnacles, mussels, algae, etc., many kinds of systems for slow-releasing antifoulants have been developed [2,10]. These slow-release systems have been designed using surface-fragmenting/self-polishing matrices, in which the matrix polymers are hydrolyzed at ester groups of main and side chains. This allows antifoulants such as an agricultural herbicide: Diuron, cuprous oxide, and an antibacterial molecule: chlorhexidine, to be gradually released during immersion time in seawater. Recently, some biodegradable polymers [11] such as poly(3-hydroxyalkanoate)s [12], poly(ε-caprolactone) copolymers [13], poly(ester-anhydride) [14], poly(lactic acid) [15], graft copolymers containing poly(lactic acid) side chains [16], and poly(ε-caprolactone-co-lactide) [17] have been applied as environmentally benign surface-fragmenting/self-polishing matrices to controllably release the antifoulants. Langlois et al. indicated that the rate of biocide release was controlled by the amount of oligo(D,L-lactic acid) units in the graft copolymers [16]. On the other hand, Faÿ et al. reported that the lower the molecular weight of poly(ε-caprolactone-co-lactide), the faster the release of biocide from antifouling paints, resulting in the decrease of the antifouling activity [17]. However, there has been no discussion about the direct contribution of the biodegradable polymers to the overall antifouling effect.

Barnacles and mussels are typical macrofouling organisms found in marine environments and, because their settlement involves the attachment of larvae on immersed solid surfaces, research has focused on processes necessary to their settlement, which are secretion [18–20], network formation [21,22], and cross-linking reactions of cement proteins [23]. Cypris larvae are the final

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larval stage of barnacles and are highly specialized to their role of locating and attaching to suitable surfaces for adult growth. The antennules of cypris larvae are their primary sensory tool. Each antennule is divided into four segments, and the third segment bears the antennular attachment disc, which measures approximately 25–30 μm (long axis) by approximately 15 μm (width) [8] and secretes cement proteins from the unicellular antennule glands [24].

In the case of a 20 kDa barnacle cement protein as one of the key materials for settlement, it was reported that its functions of self-assembly and network formation for the cementation were triggered by a salt concentration higher than that of seawater (0.55 M) and induced at pH 8–10 [22]. The cement protein was characterized by an abundance of cysteine (Cys) residues and charged amino acids. The Cys residues formed intermolecular disulphide-bonds to maintain the topology of the charged amino acids on the molecular surface, and the charged amino acids interacted with a variety of surface metals on substrata [20].

In this paper, the ability of biodegradable polyester poly(L-lactic acid) (PLLA) [25] to function as an anti-barnacle molecule was investigated. Of particular, interest was PLLA's slow-release of lactic acid [26], which, due to its acidity ($pK_a = 3.86$) in a similar way to acetic acid [27], may inhibit the network formation [21,22]/cross-linking reactions [20] of cement proteins at the surface layers of PLLA moldings. Effects of the molecular weight of PLLA were also examined.

2. Materials and methods

2.1. Materials

PLLA (TERRAMAC TE-2000C, $M_n = 3.06 \times 10^4$, $M_w = 6.71 \times 10^4$, *L*-unit 98.5%, T_m = 175 °C) and linear low-density polyethylene (LLDPE, NOVATEC UF840, MFR = 1.5 g·10 min^{-1}, T_m 125 °C) pellets were purchased from UNITIKA Ltd. and Japan Polyethylene Corporation, respectively. Standard D/L-lactic acid (50/50 wt/wt, 90% aqueous solution) and L-lactic acid (90% aqueous solution) were purchased from Wako Pure Chemical Industries, Ltd. and Musashino Chemical Laboratory, Ltd., respectively.

Fresh seawater was drawn at the Sea of Hibiki in Japan and filtrated through a membrane filter (Millipore Corporation, Millex-HP, polyethersulfone type, pore size 0.45 μm). Artificial seawater was prepared by dissolving a Marine Art SF-1 (Tomita Pharmaceutical Co., Ltd., NaCl 22.1 g/L, $MgCl_2 \cdot 6H_2O$ 9.9 g/L, $CaCl_2 \cdot 2H_2O$ 1.5 g/L, Na_2SO_4 3.9 g/L, KCl 0.61 g/L, $NaHCO_3$ 0.19 g/L, KBr 96 mg/L, $Na_2B_4O_7 \cdot 10H_2O$ 78 mg/L, $SrCl_2$ 13 mg/L, NaF 3 mg/L, LiCl 3 mg/L, KI 81 μg/L, $MnCl_2 \cdot 4H_2O$ 0.6 μg/L, $CoCl_2 \cdot 6H_2O$ 2 μg/L, $AlCl_3 \cdot 6H_2O$ 8 μg/L, $FeCl_3 \cdot 6H_2O$ 5 μg/L, $Na_2WO_4 \cdot 2H_2O$ 2 μg/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 18 μg/L) in distilled water.

2.2. Cypris larvae

In this investigation, the cypris larvae of *Balanus amphitrite* were used to estimate the anti-macrofouling function of PLLA. The reproduction of *B. amphitrite* is confined to the summer in natural environments. Therefore, to obtain and use the cypris larvae of *B. amphitrite* throughout the year, adult barnacles were collected from the Ariake Sea in Japan and fed in the laboratory with *Artemia salina* nauplii, every 24 h, in a filtered seawater at 25 °C according to the method of Kitamura [28]. The barnacles were exposed to the sunlight for 6 h per day and the seawater was changed to fresh twice a week. The barnacles began to release nauplii larvae after 1 day of rearing. The nauplii of *B. amphitrite* were reared with *Chaetoceros gracilis* feed in filtrated fresh seawater at 25 °C [29]. Forty to 50% of nauplii reached the cypris stage within 6 days. Cypris larvae were separated from nauplii larvae using a Pasteur pipette with the aid of an optical microscope [30].

Cypris larvae were kept in filtrated fresh seawater, which was oxygenated to saturation by aeration through bubbling with oxygen in the dark at 5 °C in a refrigerator for 3 days before being used for the adhesion test on PLLA sheet samples. While being maintained at 5 °C, the seawater was replaced with fresh seawater everyday [31].

2.3. Preparation of PLLA oligomers by hydrolysis

The hydrolysis of PLLA pellets under high-pressure steam was achieved using an autoclave (Tomy autoclave model SS-325, unobstructed capacity 55 L) at 120 °C (0.202 MPa) for 120, 180, and 240 min (Table 1). The internal temperature of the autoclave was thermostated to within ±0.5 °C. After autoclaving, the PLLA samples were dried in vacuo at room temperature for 1 night and stored in a refrigerator at 4 °C until used in the sheet preparation.

2.4. Preparation of sheet samples from PLLA and LLDPE

PLLA and LLDPE pellets in Table 1 were dried at 40 °C for 2 h at ca. 0.1 kPa in a vacuum oven just before melt-processing. Transparent sheet samples (thickness 400 ± 30 μm) of PLLA and LLDPE were prepared by compression molding of the pellets between metal plates having mirror finished surface at 155–170 °C with a heat-pressing machine IMC-180C (Imoto Machinery Co., Ltd. (Kyoto, Japan)) at prescribed conditions listed in Table 1. Number and weight-average molecular weight values of the sheet samples were measured with a size-exclusion chromatograph (SEC) as listed in Table 1. No significant difference in the values was detected between the pellets and the subsequently prepared sheet samples.

Test samples were prepared by cutting out squares (50 × 50 mm^2) from the sheet samples for the lactic acid elusion and cypris larvae adhesion tests.

2.5. Lactic acid elusion tests

Elution tests of lactic acid from the pellet samples (10 g) were carried out in flasks containing artificial seawater and distilled water (200 mL). The flasks were stirred at 100 rpm on a shaker in an oven thermostated at 25 °C. Changes in pH value of the aqueous media were monitored with a pH meter (HORIBA model F-21). The test media of 4 mL volume were sampled after prescribed periods and filtered with a membrane filter (Millipore Corporation, Millex-HP, polyethersulfone type, pore size 0.45 μm) for the elution volume analysis of lactic acid.

The elution volume of lactic acid into the medium from the sample was measured with a SHIMAZU high-pressure liquid chromatograph LC-10AT system equipped with a conductivity

Table 1
Steam hydrolysis and sheet preparation conditions of PLLA samples.

Sample	Steam hydrolysis[a] time (min)	Compression molding[b]		M_n[c]	M_w[c]
		Temp. (°C)	Press. (MPa)		
PLLA-31[d]	–	160	12.4	30,600	67,100
PLLA-7	120	155	6.2	7200	13,100
PLLA-3	180	155	6.2	2700	9600
PLLA-1	240	155	6.2	1100	3700
LLDPE	–	170	7.2	59,500	228,000

[a] At 120 °C.
[b] Melting (2–5 min) + pressing (4–5 min) + cooling (5 min).
[c] Universal calibration method.
[d] Original PLLA.