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In situ investigations on enzymatic degradation of poly(ε-caprolactone)

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Abstract

The enzymatic degradation of poly(ε-caprolactone) (PCL) has been investigated by using quartz crystal microbalance with dissipation (QCM-D) and surface plasmon resonance (SPR) in situ. The frequency and dissipation responses of QCM-D and the SPR signal responses can well describe the weight loss and the morphological changes of a PCL film. Our experiments reveal that the degradation has much dependence on the crystallinity and morphology of PCL. PCL with lower crystallinity (45%) and small spherulites degrades in a way of layer-by-layer. Both QCM-D and SPR measurements demonstrate that the degradation is of first-order. However, PCL with higher crystallinity (55%) and larger spherulites exhibits a more complex degradation behavior, namely, the degradation in crystalline region lags behind than that in amorphous region, leading to a microporous structure.

Keywords: Quartz crystal microbalance; Poly(ε-caprolactone); Degradation

1. Introduction

Biodegradable polyesters such as poly(ε-caprolactone) (PCL), poly(L-lactide) (PLLA), and poly(hydroxybutyrate) (PHB) have been used in drug delivery, sutures and environmentally friendly materials [1–3]. Because of the importance given to their applications, their degradability and stability have received much attention, especially the enzymatic biodegradation either in vivo or in vitro [4–24]. Both polymer morphology and degradation conditions can affect the biodegradability and stability. For a crystalline polymer, the biodegradation usually starts from the amorphous region where the degradation rate is much higher than that in the crystalline region, and the crystallinity, crystal order and the interaction between the crystals have great effect on the degradation [25]. However, the exact mechanism of the biodegradation is not clear yet.

The kinetics of polymer biodegradation has been described using the so-called Michael–Menten model where both the substrate and the enzyme are soluble [26]. Alternatively, a heterogeneous kinetics was also suggested, where the enzyme is assumed to be only accessible to the polymer surface. Factors that influence degradation rate include substrate morphology, surface structure, and enzyme concentration [27–29]. So far, most studies deal with the macroscopic degradation. Investigations on microscale level degradation are still limited [30–36]. To understand biodegradation mechanism, it is necessary to develop and use new methods to detect the degradation in situ at molecular or nanoscale level.

Quartz crystal microbalance with dissipation (QCM-D) and surface plasmon resonance (SPR) can provide the information about the changes in mass and structure of a polymer layer in nanogram scale [37–52]. In the present study, we have prepared PCL films with different crystallinities and morphologies by controlling the crystallization conditions [53–55]. By using QCM-D and SPR, we have investigated the degradation of the PCL thin film catalyzed by Lipase

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Pseudomonas (PS) in real time. The mass loss and morphology change of the film have been detected in situ. Our aim is to understand the degradation mechanism of semi-crystalline polymers.

2. Experimental section

2.1. Materials

PCL purchased from Aldrich was purified by dissolving in methylene chloride and precipitating in an excess of methanol. The number-average molecular weight ($M_n = 7.7 \times 10^4$ g/mol) and polydispersity ($M_w/M_n = 1.4$) were estimated by size exclusion chromatography on a Waters 1515 using a series of monodisperse polystyrenes as standard and tetrahydrofuran (THF) as the fluent with a flow rate of 1.0 mL/min. Lipase PS purchased from Aldrich was purified by filtration and freeze-drying before use [30].

PCL thin film on a QCM-D or SPR sensor surface was prepared by spin-casting of PCL solution in THF solution (0.1 wt%) on a spin-coater (CHEMAT, KW-400) at 3000 rpm in air. The thickness of the film was controlled by PCL concentration. The resonator with the film was heated at 120 °C for 1 min in an oven so that PCL was melted. The crystallinity and the crystal size depend on the crystallization temperature and time. Typically, a quick quenching of the film to 0 °C led to a PCL film (PCL-45) with lower crystallinity (45.4%). In contrast, the melted PCL film stood at 40 °C for 7 h to yield sample (PCL-55) with higher crystallinity (55%) [56]. The crystallinity of the PCL films was determined from the wide angle X-ray diffraction patterns. The spherulite morphologies were observed by a Leica DMLP microscope (Germany) with a CCD record system. The thickness (100 ± 10 nm) of the film was measured by atomic force microscopy (Nanoscope IIIa, D.I.) with a silicon cantilever (FESP, D.I.) in air.

2.2. QCM-D measurements

QCM-D and the AT-cut quartz crystal were from Q-sense AB [57]. The quartz crystal with a fundamental resonant frequency of 5 MHz and a diameter of 14 mm was mounted in a fluid cell with one side exposed to the solution. The constant (C) of the crystal used was 17.7 ng/cm^2 Hz. The measurable frequency shift was within ± 1 Hz in aqueous medium. The effects of surface roughness were minimized by using highly polished crystals with a root-mean-square roughness less than 3 nm [58].

When a quartz crystal is excited to oscillate in the thickness shear mode at its fundamental resonant frequency (f_0) by applying a RF voltage across the electrodes near the resonant frequency, the addition of a small layer to the electrodes leads to a decrease in resonant frequency (Δf) which is proportional to the mass (Δm) of the layer. In vacuum or air, if the layer is rigid, evenly distributed and much thinner than the crystal, the Δf is related to Δm and the overtone number ($n = 1, 3, 5, \ldots$) by the Sauerbrey equation [59],

$$\Delta m = -\frac{\rho_q l_q}{f_0}\frac{\Delta f}{n} \tag{1}$$

where f_0 is the fundamental frequency, ρ_q and l_q are the specific density and thickness of the quartz crystal, respectively. The dissipation factor is defined by:

$$\Delta D = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}} \tag{2}$$

where $E_{\text{dissipated}}$ is the energy dissipated during one oscillation and E_{stored} is the energy stored in the oscillating system. The measurement of ΔD is based on the fact that the voltage over the crystal decays exponentially as a damped sinusoidal when the driving power of a piezoelectric oscillator is switched off. By switching the driving voltage on and off periodically, we can simultaneously obtain a series of the changes of the resonant frequency and the dissipation factor.

Each PCL film has been measured by QCM-D in air before and after spin-casting to evaluate the degradation. A measurement of the degradation was initiated by switching the liquid exposed to the gold-coated quartz resonator from a 0.1 M phosphate buffer solution (PBS, pH 7.0) to an enzyme solution. The total volume used for replacement was about 2 mL. Δf and ΔD values from the fundamental were discarded because they were usually noisy due to insufficient energy trapping and discarded [60]. All the experiments were performed at 25 °C.

2.3. SPR measurements

SPR measurements were carried out on Biacore X at 25 °C. The gold-coated glass plate was attached to a glass prism ($n \approx 1.65$) with a silicone opto-interface between them so that they match in refractive index [61,62]. The response is linear to the added mass of the layer with 1000 RU $\approx$ 1 ng/mm^2, where RU is the response unit [4]. Light from a near-infrared light-emitting diode (LED) is focused through the prism onto the sensor surface in a wedge-shaped beam to give a fixed range of incident light angles. Light reflected from the sensor is monitored by a linear array of light-sensitive diodes with a resolution corresponding to approximately 0.1°. The enzyme solution was applied to the sensor surface at a flow rate of 5 μL/min [51].

3. Results and discussion

Fig. 1 shows either PCL-45 or PCL-55 consisting of lamellar crystals with amorphous region between them. PCL spherulites formed at 40 °C (PCL-55) have much larger size than those formed at 0 °C (PCL-45). This is due to the difficulty in nucleation at high temperatures [54,55].

Fig. 2 shows the time dependence of frequency changes (Δf) of the quartz resonator coated with PCL-45 and PCL-55 at different overtones after lipase PS was introduced, where the concentration of lipase PS (C_e) is 0.5 mg/mL. For the sake of comparison, Δf at each overtone is divided by n. The

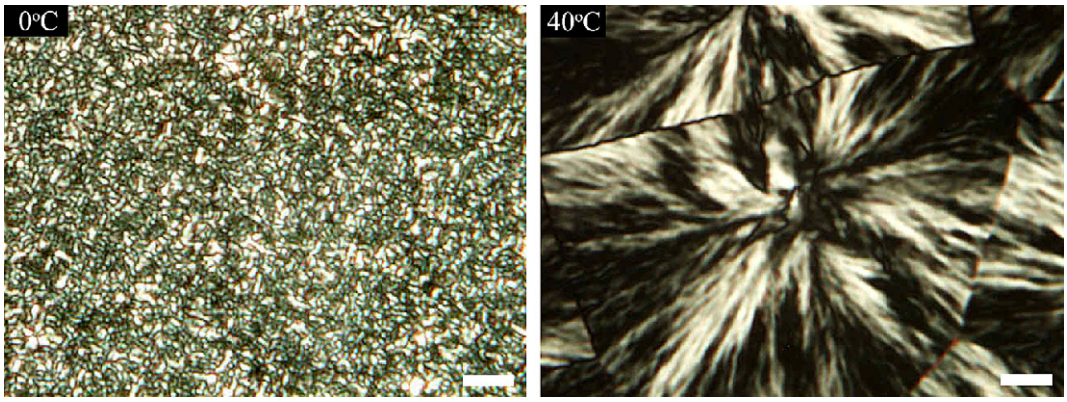

Fig. 1. The polarized optical micrographs of PCL spherulites after melt-crystallizing at 0 °C and 40 °C. The scale bar is 10 μm.

frequency response of a viscoelastic polymer layer is dependent on the thickness, density, storage modulus, and loss modulus of the layer. Since the polymer has a density close to the buffer solution, the density slightly fluctuates. Moreover, the PCL film is much thinner than the sensor crystal. Thus, the frequency shift can be attributed to the mass or thickness change of PCL [63]. For either PCL-45 or PCL-55, a slight negative frequency shift can be observed after the buffer in QCM cell was replaced with lipase PS due to the fluctuation of concentration and temperature. The following sharp increase in Δf clearly indicates the mass loss of the PCL film. Namely, some PCL chains degrade into small molecules which disperse into the solution. Finally, Δf tends to be a constant (Δf_c), indicating the completion of the degradation. Before the degradation experiments, we have also measured the frequency shift (Δf_a) induced by the dry PCL film in air. For PCL-45, Δf_c is close to Δf_a at each overtone, e.g., Δf_c and Δf_a at $n = 3$ are about 1074 Hz. This indicates that PCL-45 is completely degraded. For PCL-55, $\Delta f_c < \Delta f_a$ indicates that PCL-55 does not degrade completely. Fig. 2 also shows that PCL-55 degrades much slower than PCL-45. Such a phenomenon has been observed before [34,35]. Note that the difference in crystallinity between PCL-45 and PCL-55 is only ~10 wt%. Such a difference may not be significant enough to make a huge difference in their degradation. Actually, the crystalline morphology, crystallite size, and amorphous PCL distribution can also influence the degradation. As shown Fig. 1, PCL-45 has small crystallites which are randomly distributed in amorphous PCL matrix. As a result, it degrades in a way of layer-by-layer with the decrease in thickness, and the film can be completely degraded. In contrast, PCL-55 has big crystalline lamellae which are difficult to contact with lipase PS. Consequently, PCL-55 degrades slower than PCL-45, and some of the crystalline lamellae do not degrade in the last stage [64]. On the basis of Eq. (1), we estimate that the undegraded PCL is ~14 wt%.

Fig. 2 also shows that the time dependence of frequency response at each overtone exhibits the same trend. However, for PCL-45, it shows $\Delta f_7/7 > \Delta f_5/5 > \Delta f_3/3$ in the initial state. This is probably because the outer layer of the film is rough and the film is more viscoelastic. As discussed above, PCL-45 degrades in a way of layer-by-layer. As more PCL

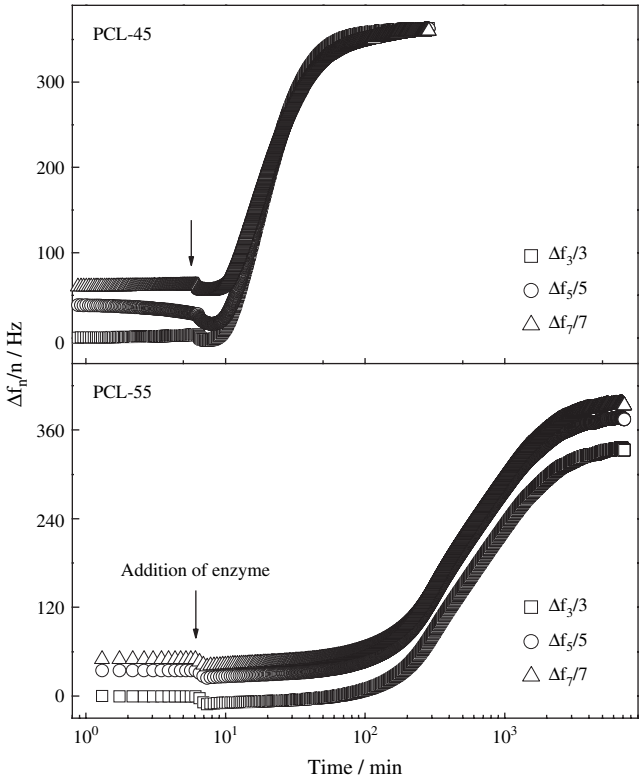

Fig. 2. The frequency shift (Δf) of QCM during enzymatic degradation of PCL-45 and PCL-55 catalyzed by lipase PS, where $C_e = 0.5$ mg/mL and $n = 3, 5, 7$.

degrades, the newly formed outer layer becomes smoother so that the curves combine with each other. For PCL-55, difference between the frequency shifts at different overtones holds until the completion of the degradation. This is because the degradation of PCL crystallites lags behind that of the amorphous PCL, leading to a film consisting of crystalline skeleton and micropores [65–67]. Thus, the viscoelasticity does not decrease with the degradation and the difference between the frequency shifts at different overtones slightly changes.

Fig. 3 shows the time dependence of dissipation changes (ΔD) of the quartz resonator coated with PCL-45 and PCL-55

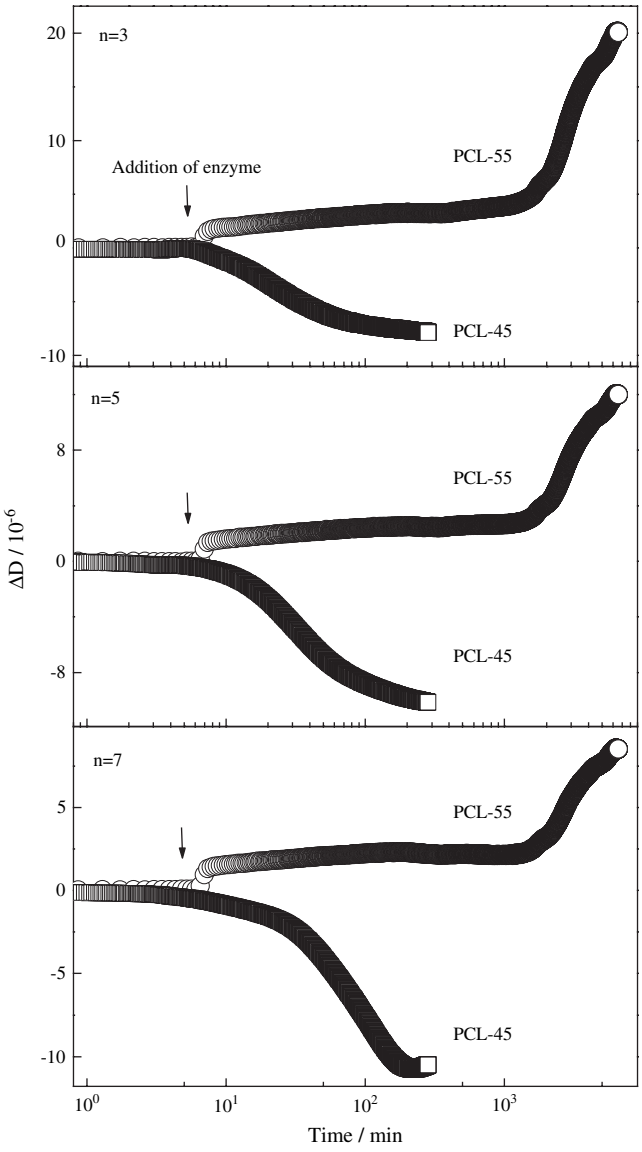

Fig. 3. The dissipation shift (ΔD) of QCM during enzymatic degradation of PCL-45 and PCL-55 catalyzed by lipase PS, where $C_e = 0.5$ mg/mL and $n = 3$, 5, 7.

at different overtones after lipase PS was introduced, where $C_e = 0.5$ mg/mL. It is known that the dissipation of a polymer layer on quartz resonator increases with its thickness and looseness [60]. A dense and rigid structure leads to a small dissipation of energy, while a looser structure results in a larger dissipation. For PCL-45, the decrease in dissipation further suggests that the degradation is in a way of layer-by-layer. In other words, the decrease in ΔD is due to the decrease of the thickness of the PCL film. For PCL-55, the degradation leads PCL-55 to form a loose structure, leading ΔD to increase. At last, there are still some undegraded PCL chains forming the skeleton [54,56], so that the dissipation cannot return to the initial value. This is consistent with the results in Fig. 2.

Fig. 4 shows the relation of ΔD vs Δf. Such a relation has been used to describe the morphology of films [39,52].

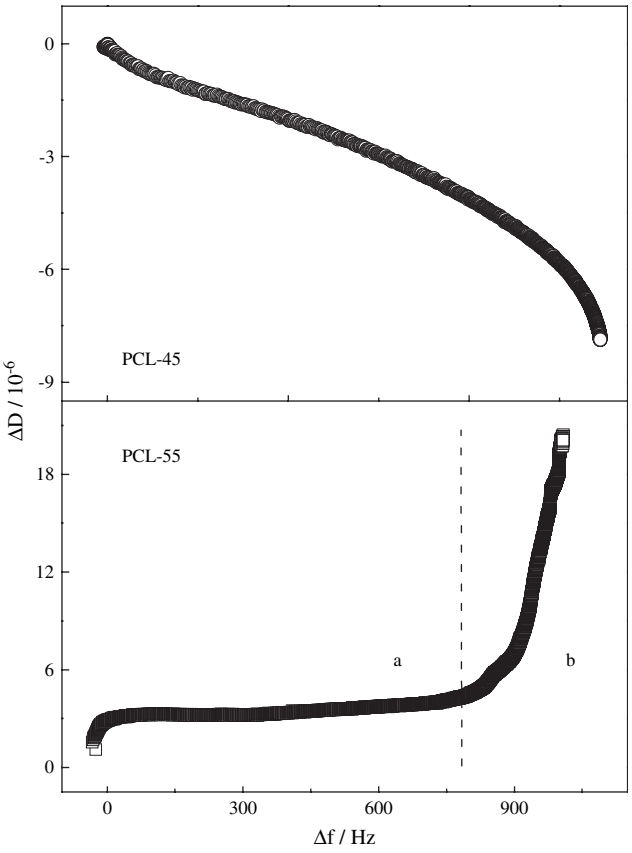

Fig. 4. The relations of ΔD vs Δf for enzymatic degradation of PCL-45 and PCL-55 catalyzed by lipase PS at $n = 3$, where $C_e = 0.5$ mg/mL.

Roughly, the degradation of PCL-45 film involves only one stage. Namely, as Δf increases or the mass decreases, the thickness decreases so that ΔD decreases. As discussed above, crystallites in PCL-45 are small and randomly distributed in amorphous PCL matrix, and the degradation at different sites on the same layer occurs simultaneously. Note that ΔD decreases more sharply at $\Delta f > 1000$ Hz. This is because the disappearance of the PCL layer causes a quicker decrease of the thickness at the final stage of the degradation. In contrast, PCL-55 shows two-stage degradation kinetics. The two stages are attributed to the degradation of amorphous PCL and PCL crystallites, respectively. In the first stage (a), the degradation of amorphous PCL in outer layer leads the film thickness to decrease. At the same time, it leaves the undegraded PCL to form a microporous structure, which increases the dissipation. Consequently, ΔD slightly increases with Δf. In the second stage (b), the degradation has extended inside the film, i.e., the enzyme can be adsorbed not only on the outer layer but also inside the micropores in the film. Namely, the degradation is expected to be multi-dimensional, leading the film to be more porous. Thus, ΔD greatly increases with Δf.

The degradation has also been investigated by SPR. Fig. 5 shows the response shift (ΔRU) of SPR for PCL-45 and PCL-55 after lipase PS was introduced, where $C_e = 0.5$ mg/mL. Obviously, PCL-45 degrades more quickly than PCL-55 because the crystallites in PCL-45 are smaller. Finally, ΔRU tends to be a constant (ΔRU_c), indicating the completion of the

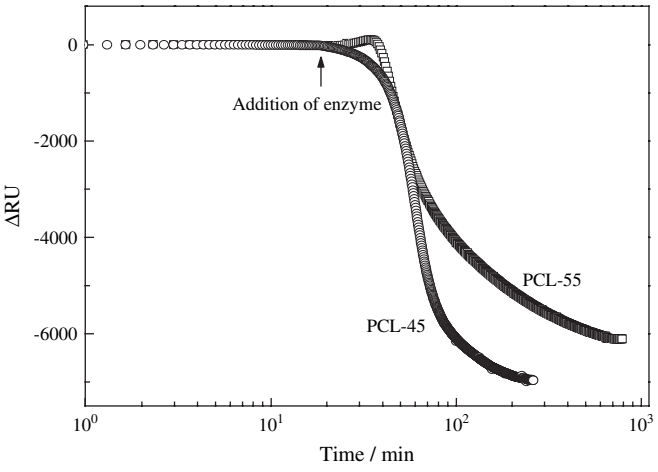

Fig. 5. The signal response shift (ΔRU) of SPR for PCL-45 and PCL-55 after lipase PS was introduced, where $C_e = 0.5$ mg/mL.

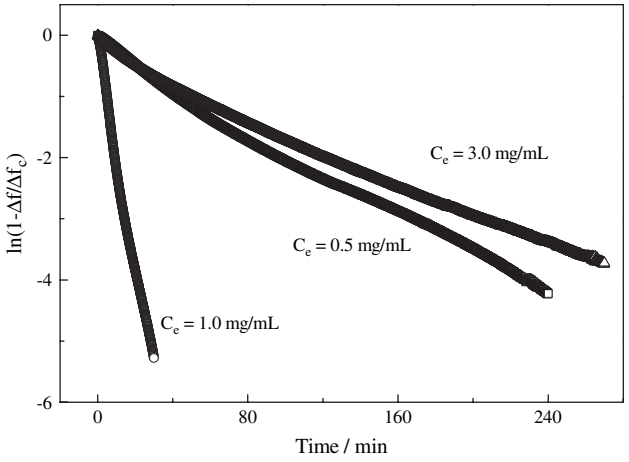

Fig. 6. Time dependence of $\ln(1 - \Delta f/\Delta f_c)$ for the enzymatic degradation of PCL-45 film at $n = 3$.

degradation. Considering that PCL-45 and PCL-55 films have almost the same weights, the smaller ΔRU_c for PCL-55 further indicates the incomplete degradation of PCL-55. From the difference of ΔRU_c between PCL-45 and PCL-55, we can estimate that about 13 wt% of PCL-55 has not degraded. This is in agreement with the QCM-D results.

Since the mass (Δm) of degraded PCL is proportional to Δf in QCM-D or ΔRU in SPR, PCL degradation kinetics can be described in terms of Δf or ΔRU. Since PCL-55 exhibits complex degradation kinetics, the degradation order cannot be determined. Here, we only present the results about PCL-45. Fig. 6 shows that $\ln(1 - \Delta f/\Delta f_c)$ linearly decreases with time. Since enzyme is much excessive relative to PCL, the enzyme concentration can be taken as a constant. Clearly, the degradation is a pseudo first-order reaction. Fig. 7 shows that $\ln(1 - \Delta RU/\Delta RU_c)$ linearly decreases with time, further indicating the first-order degradation. The previous laser light scattering studies also reveal that enzymatic biodegradation of PCL is of first-order [30–33]. Therefore, QCM-D, SPR and laser light scattering results are consistent.

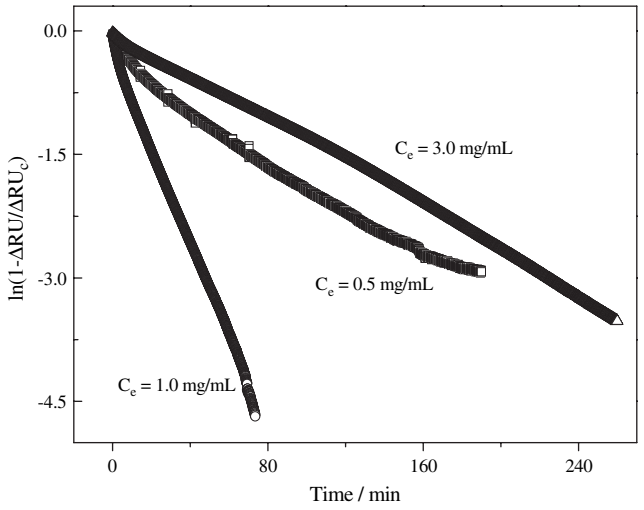

Fig. 7. Time dependence of $\ln(1 - \Delta RU/\Delta RU_c)$ for the enzymatic degradation of PCL-45 film.

The degradation rate constant (k_d) is reflected in the slope. Figs. 6 and 7 also show that k_d increases with enzyme concentration in the range $C_e < 1.0$ mg/mL. Further increasing the enzyme concentration leads k_d to decrease. Namely, as the enzyme concentration increases, the degradation rate (R_d) exhibits a maximum. Similar phenomenon has also been observed in the enzymatic degradation of PHB film [34]. Mukai et al. [28] suggested that the coverage of enzyme on the film surface has quite an effect on the degradation rate. Assuming the adsorbed enzyme has coverage of θ and a local concentration of C_l, we have $R_d \propto C_l(1 - \theta)$ [15,34]. At a low enzyme concentration, both C_l and θ are very small, the enzyme can freely access to the film surface, so the degradation rate increases with the enzyme concentration, i.e., $R_d \propto C_l$. At a high enzyme concentration, most of the film surface is covered by the enzyme molecules and the adsorption of the coming enzyme is hindered by the already adsorbed enzyme so that the degradation rate decreases.

4. Conclusion

The present study leads to the following conclusion. QCM-D and SPR can be used to investigate the enzymatic degradation of a polymer film in real time, where the changes in mass and morphology of the film can be well described by the frequency and dissipation in QCM-D as well as the SPR signal response. PCL with small crystallites degrades in a way of layer-by-layer with pseudo first-order degradation kinetics. The degradation of PCL with crystalline lamellae exhibits two-stage kinetics, which relates to the degradation of amorphous PCL and PCL crystallites, respectively. The former degrades more quickly than the latter, leading the film to be microporous.

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