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Enzymatic degradation of polycaprolactone–gelatin blend

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Abstract

Blends of polycaprolactone (PCL), a synthetic polymer and gelatin, natural polymer offer a optimal combination of strength, water wettability and cytocompatibility for use as a resorbable biomaterial. The enzymatic degradation of PCL, gelatin and PCL–gelatin blended films was studied in the presence of lipase (Novozym 435, immobilized) and lysozyme. Novozym 435 degraded the PCL films whereas lysozyme degraded the gelatin. Though Novozym 435 and lysozyme individually could degrade PCL–gelatin blended films, the combination of these enzymes showed the highest degradation of these blended films. Moreover, the enzymatic degradation was much faster when fresh enzymes were added at regular intervals. The changes in physico-chemical properties of polymer films due to degradation were studied by scanning electron microscopy, Fourier transform infrared spectroscopy and differential scanning calorimetry. These results have important implications for designing resorbable biomedical implants.

1. Introduction

In recent times, resorbable polymeric materials in different forms such as films, nanofibers, hydrogel and scaffolds have found potential applications in a variety of biomedical needs including resorbable sutures and fracture fixation devices, drug delivery and regenerative medicine among others [1]. The main purpose of these polymeric matrices is to support cell growth and even provide mechano-chemical cues for the desired biological functions. In this regard, synthetic polymers such as polycaprolactone (PCL) have been studied extensively as potential biomaterials [2]. However, the lack of specific cell binding sites and hydrophobicity pose a critical challenge for using PCL-based materials in tissue engineering. Gelatin, a natural polymer derived from partial hydrolysis of collagen, has been explored as a potential biomaterial due to its excellent biocompatibility and presence of cell binding motifs. However, the lack of mechanical strength and fast biodegradation inhibits its application [3, 4]. Unlike gelatin, degradation of PCL is slow and exhibits lower hydrophilicity [4–8]. The blending of gelatin and PCL can be useful to provide mechanical strength with increased cytocompatibility. Recent studies have reported different techniques for preparing blended-polymer matrix optimal for particular tissue engineering requirements [9, 10].

The degradation characteristics of polymeric matrices depend upon their structure, conformation, porosity and degree of inter-structure network [11]. In order to design polymer supports for tissue engineering applications, it is of utmost importance to understand their degradation characteristics and also tune their properties. There are many studies on application of PCL–gelatin matrix for tissue engineering and biomedical applications [12, 13]. In addition to the hydrolysis of PCL in physiological buffers, fungi-mediated and enzymatic degradation of PCL is well documented [13–23]. Lysozyme is one of the important enzymes present in serum and plays a critical role in the enzymatic degradation of biomaterials [24]. On the other hand, lipase is present in the macrophages that are responsible for recognizing foreign bodies and subsequent clearance of these bodies. Lipases have been reported to catalyze the hydrolysis of ester bonds present in PCL [18]. Gelatin, being derived from collagen, can generally be degraded by various proteinases. Falconi *et al* have studied enzymatic degradation of cross-linked gelatin scaffolds by lysozyme [25].

Though the degradation of PCL and gelatin has been studied separately, reports on the enzymatic degradation characteristics of PCL–gelatin blended polymeric matrices are not available. In this study, enzymatic degradation of neat PCL, neat gelatin and PCL–gelatin blend films along with the degradation kinetics has been studied in the presence of Novozym 435 (immobilized lipase) and lysozyme and their combination.

2. Materials and methods

2.1. Materials

PCL (average molecular weight, $M_n = 80\,000$), gelatin (from porcine skin) and Novozym 435 were purchased from Sigma-Aldrich Chemicals. Lysozyme was procured from Himedia (India). All solvents and salts were purchased from S. D. Fine Chemicals (India).

2.2. Film preparation

PCL, gelatin and PCL–gelatin blended films were prepared by solvent casting method. For pure films, a 8% (w/v) solution of PCL or gelatin in tri-fluoroethanol (TFE) was prepared by stirring for 2 h at room temperature. 1.5 ml of the solution was pipetted onto polypropylene petri-dish (60 mm internal diameter) and left for 12 h for solvent evaporation. To ensure complete solvent evaporation, samples were then kept in vacuum desiccator for another 24 h. For PCL–gelatin blends, individual polymer solutions were prepared and then mixed at 1:1 ratio under constant stirring. The PCL–gelatin blended films were prepared as described above. 50 μl of 0.5% glutaraldehyde was added in 1.5 ml solvent solution (TFE) to crosslink the gelatin in case of pure and PCL-blended gelatin films. Thickness of all prepared films was measured to be 0.07 ± 0.002 mm.

2.3. Characterization

Differential scanning calorimetry (DSC) analysis of the films was performed on TA Instruments Q2000. For this, the films were heated from −50 °C to 100 °C at 10 °C min^{-1}. Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer) was performed on polymer films before and after degradation. The surface morphology of polymer films was investigated by scanning electron microscope (SEM) in an ESEM Quanta 200 instrument with gold-sputter coated samples.

2.4. Enzymatic degradation

The enzymatic degradation of the polymer films was performed at 37 °C in 1 M phosphate buffer (pH 7.4) containing 1 mg ml^{-1} of respective enzymes in a shaker incubator. Sodium azide (0.02%, w/v) was added to the degradation buffer as antibacterial agent. Polymer films having same dimensions were cut, packed into a nylon mesh and immersed in 20 ml of enzyme containing buffer solution in a 50 ml tubes. Degradation experiments were carried out in two different ways. The first set of experiments (Set I) was carried out by adding enzyme only once at the start of the experiment at time $t = 0$ h. In the second set (Set II), fresh enzymes were added every 4 days during the course of entire experiment.

2.5. Weight loss measurements

Samples were weighed before being placed in degradation medium in order to determine the initial weight of the film (M_0). At regular time intervals, the samples were removed, thoroughly washed with DI water, dried in vacuum and weighed again to obtain the dry weight left after degradation. Weight loss was then calculated as $\%\ \text{weight loss} = \frac{M_0 - M_t}{M_0} \times 100$ where M_t is the weight of the film at any time t while M_0 is the initial weight of the film.

3. Results and discussion

3.1. Degradation

Figure 1 shows the overall degradation profile of various polymeric films in terms of % weight loss as a function of degradation time. As evident from figure 1(a), PCL films did not show any significant degradation (<2% weight-loss) in the absence of enzymes. The presence of lysozyme did not result in the degradation of PCL films either. However, the films showed significant weight loss in the presence of Novozym 435. After 23 days of incubation, PCL films lost ~42% weight in presence of Novozym 435 due to lipase-catalyzed hydrolysis of PCL ester bonds [18]. Interestingly, when the PCL films were incubated with Novozym 435 in combination with lysozyme, the total weight loss decreased to 32%. This could be due to the trace protease activity of lysozyme, which likely resulted in the partial inactivation of Novozym 435 in the presence of lysozyme [26]. In Set II, where fresh enzymes were added at regular interval (every 4th day), the degradation profile of PCL films was found to

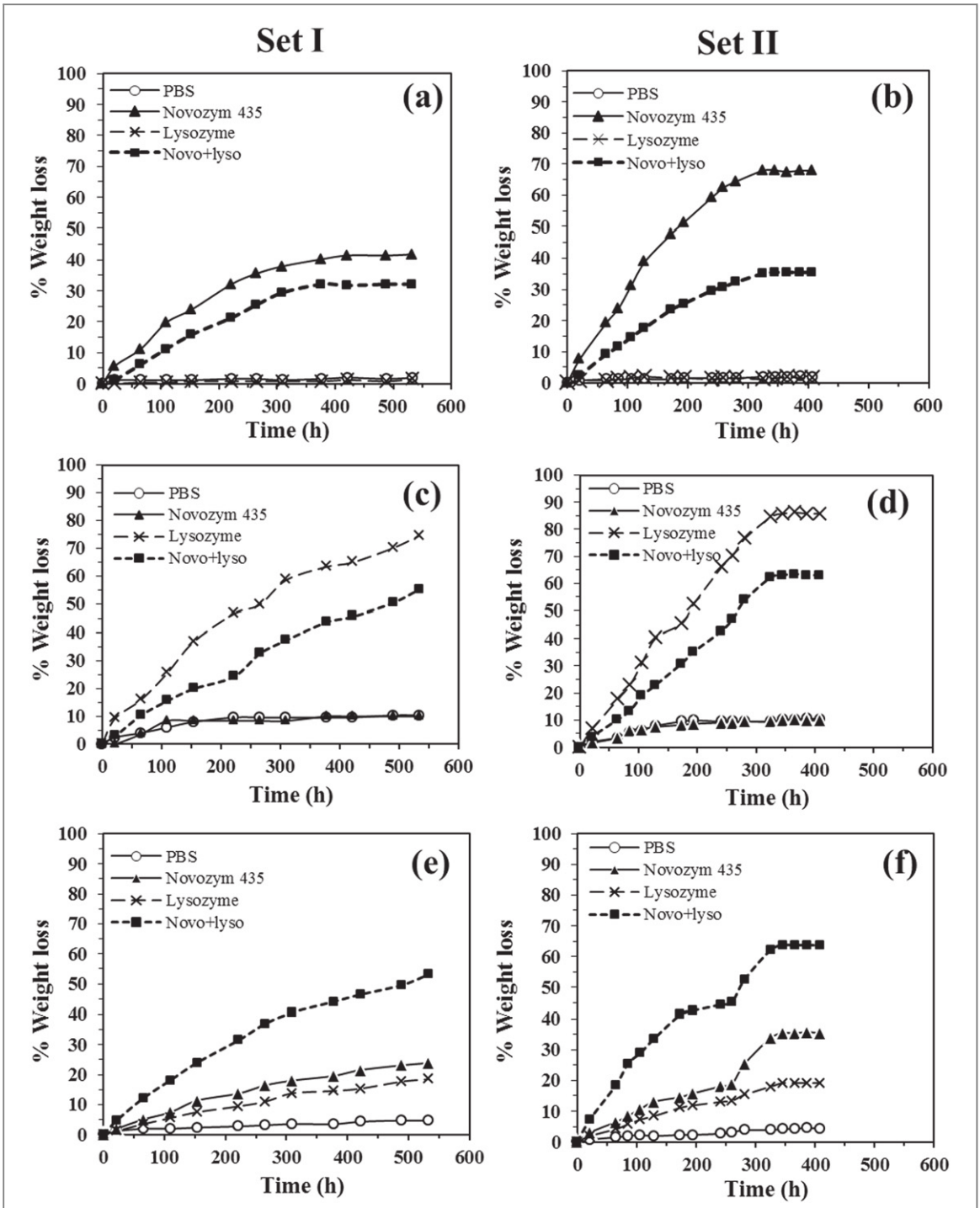

Figure 1. Weight loss of PCL (a) and (b); gelatin (c) and (d) and PCL–gelatin blend (e) and (f) films with time in the presence of enzymes. Corresponding enzymes were added only once at the beginning of experiments in Set I whereas fresh enzymes were added at regular intervals of 85 h in Set II.

be similar as that of Set I. However, the degradation was much faster and weight loss (~65% after 13 days) was much higher in Set II in the presence of Novozym 435. The higher degree of degradation is expected in Set II compared to Set I as the degradation buffer is replenished with fresh Novozym 435 at regular intervals. For example, figures 1(a) and (b) show that Novozym 435 took only 106 h to cause ~30% weight loss in Set II, whereas the duration was 221 h in Set I. The % weight-loss remained almost same (~35%) in Set I and Set II when lysozyme and Novozym 435 were present together.

In the case of gelatin films, as can be seen in figures 1(c) and (d), lysozyme demonstrated significant degradation while Novozym 435 was unable to degrade gelatin. There was a basal-level weight loss (~10%) in gelatin films in PBS, possibly due to dissolution of non-crosslinked gelatin present in these films. Similar to PCL films, gelatin films were also degraded much faster by lysozyme when replenished with fresh enzymes (Set II) as

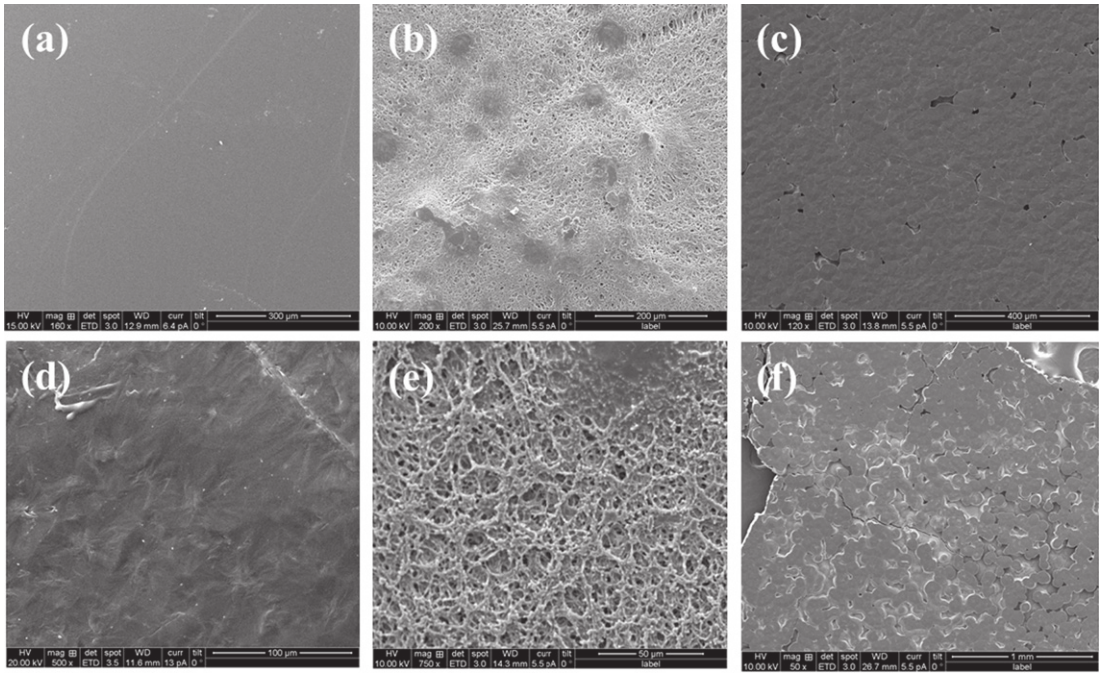

Figure 2. Film morphology before (a)–(c) and after (d)–(f) degradation studies. (a) and (d) Represents PCL films, (b) and (e) shows gelatin and PCL–gelatin blend film shown in (c) and (f).

compared to one-time addition of lysozyme (Set I) (figures 1(c) and (d)). For example, gelatin films lost 84% weight and reached a plateau by 325 h in case of Set II, whereas only 63% weight-loss was observed in Set I by 377 h. However, the weight-loss of gelatin films decreased by ~20% when lysozyme was present along with Novozym 435. It is believed that, due to protease activity of lysozyme as mentioned earlier [26], Novozym 435 can compete with gelatin as a substrate for lysozyme. This could lead to reduced weight-loss of gelatin films by lysozyme in presence of Novozym 435, when compared to that of lysozyme alone.

Figures 1(e) and (f) show the overall weight-loss profile of PCL–gelatin blended films by Novozym 435, lysozyme enzyme and with their combination. The blended films showed similar profile of weight loss in the presence of either lysozyme or Novozym 435 in Set I with the latter being slightly more active in degrading the film. However, when these enzymes were employed together, the weight loss was 53%, almost twice that of either of the enzymes alone, by the end of the degradation period of 575 h. In Set II degradation, the weight-loss of the blended films followed the pattern similar as that of Set I, except the degradation was higher and much faster. In Set I, 18% of polymer degradation occurred within 310 h by Novozym 435 and in 533 h by lysozyme. The same amount of weight-loss was noticed in Set II within a shorter period of time, i.e., 260 h with Novozym 435 and 325 h with lysozyme. Similar to the neat films, blended films showed highest degradation (63% weight-loss by 410 h) in Set II degradation with combined enzyme solution.

3.2. Physico-chemical changes

During the enzymatic degradation, the changes in the morphology of the polymeric films were investigated under SEM (figure 2). It may be mentioned here that SEM images of only PCL films degraded by Novozym 435, gelatin films degraded by lysozyme and PCL–gelatin blended film degraded by Novozym 435—lysozyme combination in Set II are presented here as the corresponding conditions showed highest amount of degradation in those specific types of films. Novozym 435 mediated degradation of PCL films can be seen from the disappearance of the smooth surface texture (figure 2(d)) as compared to nontreated PCL films (figure 2(a)). Figure 2(b) shows that as prepared gelatin films had porous structures and it became more porous due to degradation by lysozyme (figure 2(e)). The morphology of the PCL–gelatin films (figure 2(c)) also changed as a result of enzymatic degradation, as evident from the 'etched out' appearance (figure 2(f)) of the degraded films.

Figure 3 showed the DSC analysis plots of pure PCL and PCL–gelatin blend films before and after enzymatic degradation by Novozym 435 and Novozym 435—lysozyme combination, respectively. A minor shift in the crystallization peak with an increase of melting temperature has been observed for PCL (figure 3(a)) and PCL–gelatin blend sample (figure 3(b)) with enzyme treatment. The increase in melting point is due to continuous formation of thicker and more perfect lamellae due to enzymatic degradation [27, 28]. The melting point of neat

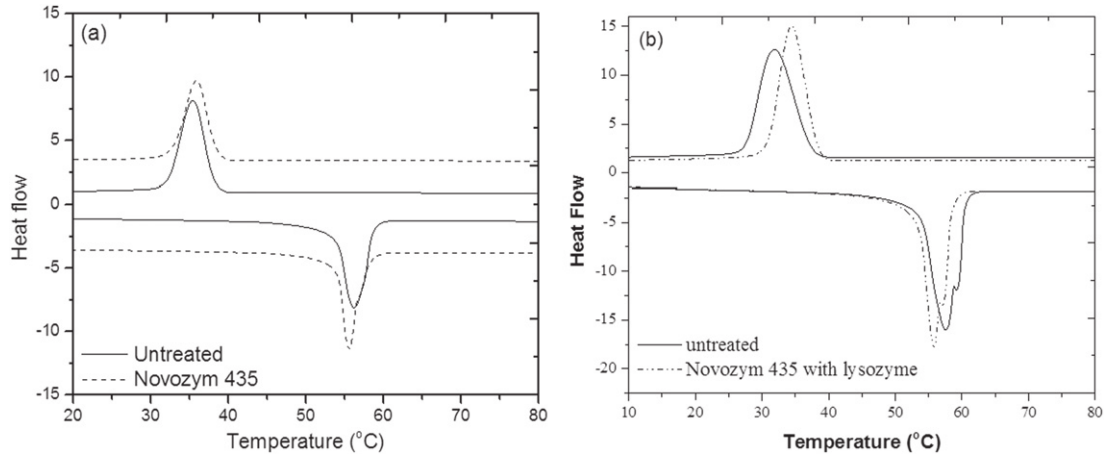

Figure 3. DSC chromatograms of (a) PCL and (b) PCL–gelatin film before and after degradation.

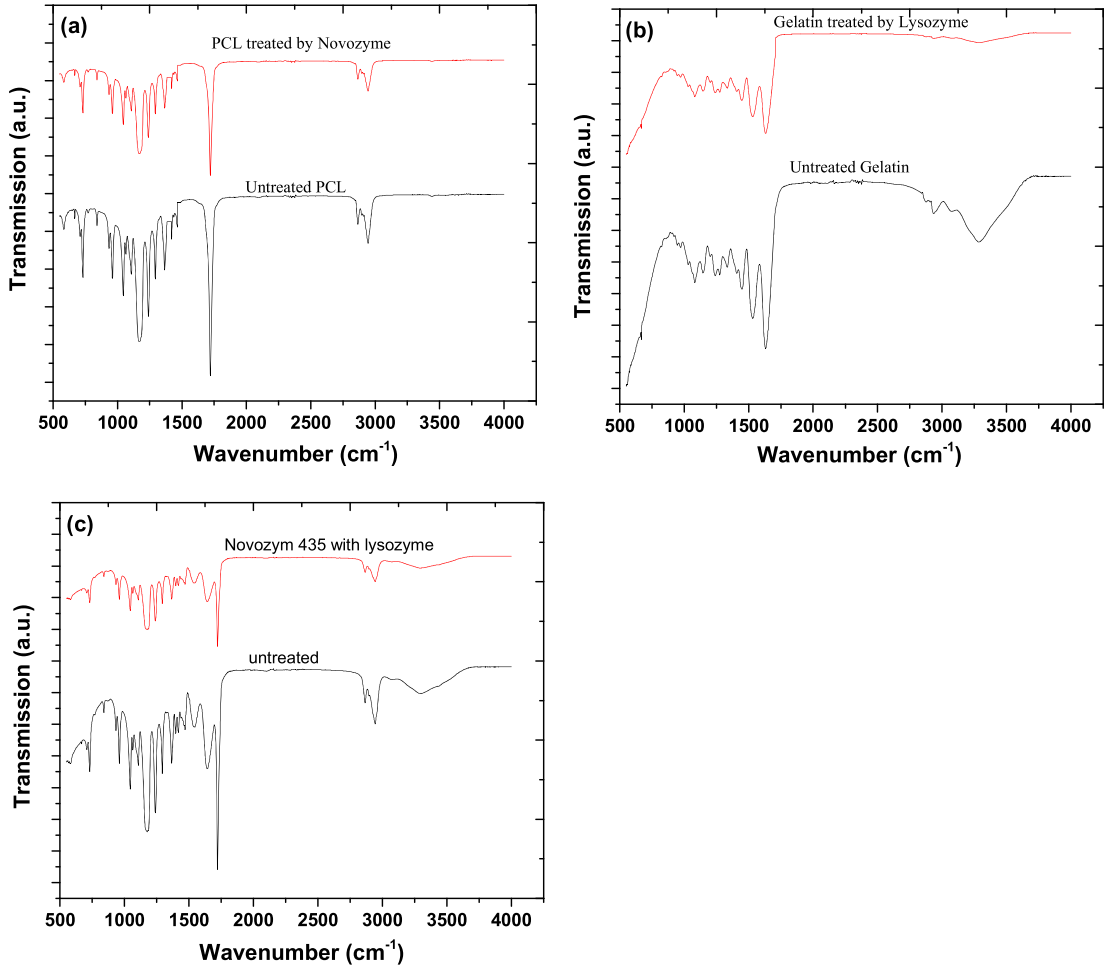

Figure 4. FTIR spectra for (a) PCL, (b) gelatin and (c) PCL–gelatin films before and after degradation.

gelatin film was observed at 90 °C before degradation. However, after degradation, the film loses its configuration and results in a DSC curve without any specific peak (data not shown).

Figure 4 shows the FTIR data for pure polymer and their blends before and after the degradation (Set II). Table 1 summarizes the important IR peaks in PCL and gelatin films. The peak corresponding to PCL ester group (1720 cm^{-1}) was reduced by 38% after Novozym 435 mediated degradation of PCL films (figure 4(a)). It was observed that, after normalization with respect to ester peak, C–O and C–C stretching in crystalline region

Table 1. FTIR peak details.

	Peak at	Bonds and functional group
	2948	Asymmetric CH_2 stretching
	2870	Symmetric CH_2 stretching
	1720	Ester group
PCL film	1294	C–O and C–C stretching in crystalline region
	1240	Asymmetric C=O=C stretching
	720	$-(CH_2)_n$- stretching
	3310	N–H stretching for Amide A
Gelatin film	2938	C–H stretching for Amide B
	1650	N–H bond stretching in amide I
	1540	C=O bond in amide II

(1294 cm^{-1}) increased by 9% due to treatment of Novozym 435. With an increase in crystalline region, FTIR spectra showed 8% increment for asymmetric C=O=C stretching (1240 cm^{-1}) by Novozym 435 in PCL films. On the other hand, lysozyme mediated degradation of gelatin films resulted in 28% reduction in peak due to N–H bond stretching (figure 4(b)). Due to lysozyme action on gelatin films, when normalized to N–H stretching peak, the peak corresponding to C=O bond in amide I region was reduced by 31% and the peak at 3310 cm^{-1} (N–H stretching for Amide A) was reduced by 79%. The peaks at 2938 and 3310 cm^{-1} almost disappeared with enzymatic treatment of gelatin films.

FTIR spectra of blended film (figure 4(c)) showed characteristics peaks both due to PCL (2870, 1720, 1294 cm^{-1}) and gelatin (3310, 1650 and 1540 cm^{-1}). The peak due to ester group was reduced by 51% with Novozym 435 and lysozyme. With respect to ester peak, N–H stretching for Amide I increased by 26% in the presence of Novozym 435 and lysozyme. Due to enzymatic degradation, PCL-specific peaks (1294, and 1240 cm^{-1}) increased (when normalized with ester peak) while gelatin specific peaks (at 1540 and 1650 cm^{-1}) decreased with time.

3.3. Degradation kinetics

Enzymatic degradation, according to first order rate kinetics, can be expressed as

$$\ln \frac{M_t}{M_0} = -k_m t. \tag{1}$$

M_t and M_0 represent the mass of polymer at time t and initial ($t = 0$); k_m is the degradation rate constant. The plots of $\ln \frac{M_t}{M_0}$ with respect to time t, from the initial weight loss data of different films in presence of enzymes, showed that the enzymatic degradation of PCL, gelatin and PCL–gelatin blended films followed the first order rate kinetics (figures 5 and 6). Table 2 summarizes the k_m value obtained from the semi-logarithmic plot with time representing equation (1).

One of the most important parameters to measure and quantify degradation is the determination of molecular weight other than the mass loss. Gelatin dissolves in the mixtures of dimethylformamide/water [29] and acetonitrile/water [30]. PCL dissolves in THF [20, 21] and chloroform [31]. However, there is no common solvent for the dissolution of both PCL and gelatin. Thus, the molecular weight of the blends of PCL and gelatin cannot be determined using gel permeation chromatographic techniques. However, the molecular weights of the individual polymers, PCL and gelatin, and its evolution with time have been determined. Madras and coworkers [20, 21] have proposed and experimentally verified the relationship of mass loss with molecular weight. The molecular weight of a polymer in solution is the ratio of the mass concentration to the molar concentration [21]. In case of polymers such as PCL and gelatin that undergo degradation by specific chain scission [21], the molar concentration of the polymer is invariant in time (equation (6) in [21]), while the polymer mass concentration reduces with time. Equation (1) can be thus rewritten as

$$\ln \frac{M_{nt}}{M_{n0}} = -k_m t \tag{2}$$

i.e., $M_{nt} = M_{n0} \exp(-k_m t)$, where M_{nt} is the number average molecular weight at any time, M_{n0} is the initial number average molecular weight and k_m is the rate constant determined from equation (1) based on mass loss. The change in the molecular weight with time has been experimentally verified in this study for the degradation of PCL following the procedure followed using gel permeation chromatography, as mentioned in previous study [21].

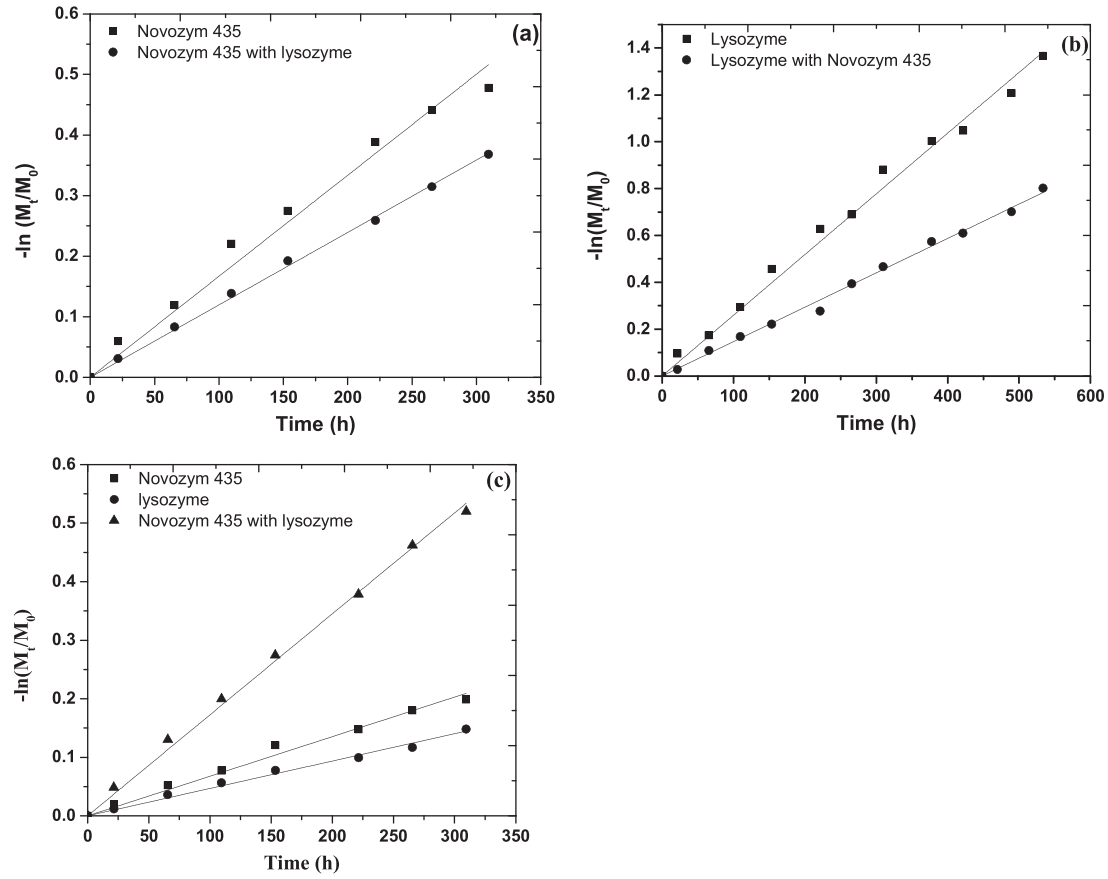

Figure 5. Degradation rate coefficient determination for Set I experiments: (a) PCL with Novozym 435 and combination enzyme effect; (b) gelatin with lysozyme and combination enzyme effect and (c) PCL–gelatin blend with Novozym 435, lysozyme and combination enzyme effect.

In Set I degradation, highest degradation rate coefficient was observed in the case of gelatin films in the presence of lysozyme enzyme, while the lowest was observed with lysozyme enzyme on PCL–gelatin blended films. It is evident from table 2 that the degradation rate of PCL is highest by Novozym 435 whereas degradation of gelatin by lysozyme is fastest. For the PCL–gelatin blend sample, combination of lipase-lysozyme enzyme results in higher degradation than pure enzymes.

Table 2 lists the degradation rate coefficients for PCL, gelatin and blended films in Set II enzymatic degradation. It was observed that degradation rate coefficient of PCL by Novozym 435 was higher for second and third cycle of adding fresh enzyme than the first cycle. A similar trend was noticed for gelatin degradation by lysozyme. This could be due to the exposure of more enzyme binding site along with cleavable bond to fresh enzymes after the first degradation cycle. With the effect of combined enzyme solution on PCL, the degradation rate coefficient did not change for initial two cycles and eventually decreases. With Novozym 435 and lysozyme, the degradation rate coefficient increased with each fresh cycle of enzyme (table 2 and figure 6). For the blend, the fourth (last) cycle of combination enzyme solution gave the highest degradation rate coefficient followed by first cycle and onward (figure 6 and table 2).

4. Conclusions

The enzymatic degradation of synthetic and natural polymer has been studied in the presence of various enzymes. The rate of degradation has been measured in terms of weight loss. SEM, DSC and FTIR have been also used to study the enzymatic effect on polymers. Novozym 435 has a better activity than the combination with lysozyme for the degradation of PCL films. Similarly, the presence of lipase inhibits lysozyme for the degradation of gelatin. By comparing two set of experiments, it can be concluded that adding fresh enzyme in solutions increases the total degradation up to certain extent. From their morphology and mass loss, enzyme degradation mainly followed the surface degradation process.

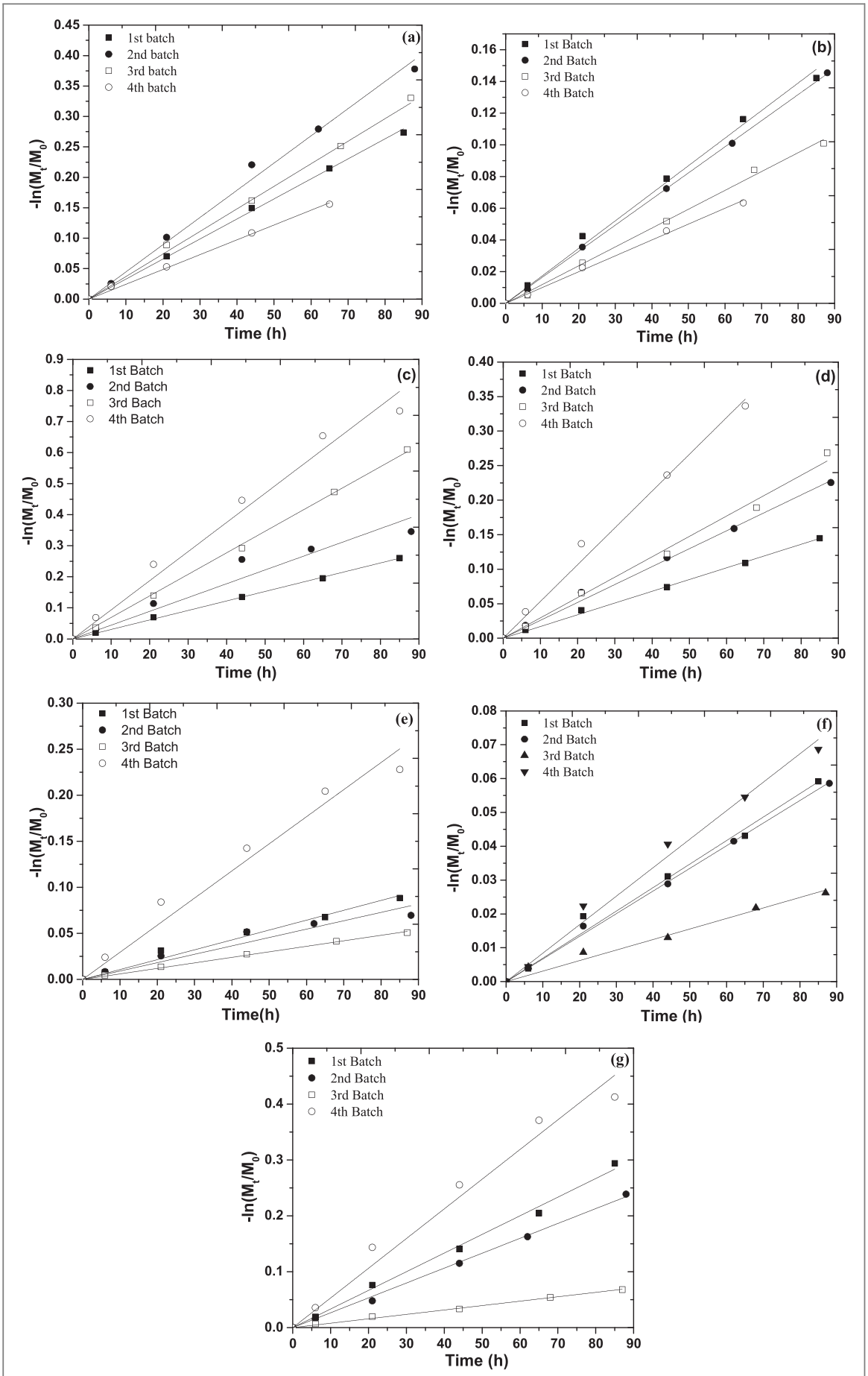

Figure 6. Degradation rate coefficient determination for set II studies: (a) Novozym on PCL films; (b) Novozym 435 and lysozyme enzyme on PCL; (c) lysozyme enzyme on gelatin; (d) lysozyme and Novozym 435 on gelatin; (e) Novozym 435 on PCL–gelatin blend film; (f) lysozyme enzyme on PCL–gelatin blend flim; (g) Novozym 435 and lysozyme on PCL–gelatin blend film.

Table 2. Rate coefficient ($\times 10^{-3}$) for polymer and polymer blend with presence of various enzymes (Set I and Set II).

			k_m		
	Polymer	Enzyme cycle	NV	LS	NV + LS
	PCL	—	1.67	NA	1.20
Set 1	Gelatin	—	NA	2.592	1.47
	PCL–Gelatin	—	0.676	0.468	1.73
		1st	3.29	NA	1.74
		2nd	4.47	NA	1.65
	PCL	3rd	3.7	NA	1.19
		4th	2.43	NA	1.01
Set II		1st	NA	3.06	1.73
		2nd	NA	4.44	2.59
	Gelatin	3rd	NA	6.93	2.95
		4th	NA	9.38	5.33
		1st	1.07	0.69	3.34
		2nd	0.91	0.67	2.66
	PCL–Gelatin	3rd	0.59	0.31	0.79
		4th	2.95	0.84	5.31

NV = Novozym 435; LS = lysozyme; NV + LS = Novozym 435 with lysozyme;

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