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Controlled protein release from electrospun biodegradable fiber mesh composed of poly(ε-caprolactone) and poly(ethylene oxide)

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Abstract

A blend mixture of poly(ε-caprolactone) (PCL) and poly(ethylene oxide) (PEO) was electrospun to produce fibrous meshes that could release a protein drug in a controlled manner. Various biodegradable polymers, such as poly(L-lactic acid) (PLLA), poly(ε-caprolactone) (PCL), and poly(D,L-lactic-*co*-glycolic acid) (PLGA) were dissolved, along with PEO and lysozyme, in a mixture of chloroform and dimethylsulfoxide (DMSO). The mixture was electrospun to produce lysozyme loaded fibrous meshes. Among the polymers, the PCL/PEO blend meshes showed good morphological stability upon incubation in the buffer solution, resulting in controlled release of lysozyme over an extended period with reduced initial bursts. With varying the PCL/PEO blending ratio, the release rate of lysozyme from the corresponding meshes could be readily modulated. The lysozyme release was facilitated by increasing the amount of PEO, indicating that entrapped lysozyme was mainly released out by controlled dissolution of PEO from the blend meshes. Lysozyme released from the electrospun fibers retained sufficient catalytic activity.

Keywords: Electro-spinning; Biodegradable; Nanofibers; Protein release; Lysozyme

1. Introduction

Electrospun ultrafine nanofibers have been explored during the past several years as potential biomedical device including tissue engineering scaffolds, wound dressing materials, and drug delivery carriers. Recently, nanofibrous polymeric meshes prepared by an electrospinning technique have gained much interest for delivering various bioactive agents in a sustained manner, such as antibiotics, anti-tumor agents, proteins, and plasmid DNA (Luu et al., 2003; Kim et al., 2004; Casper et al., 2005; Chew et al., 2005; Zeng et al., 2005a). The electrospinning process enables a diverse range of drugs to be directly encapsulated within the bulk phase of nanoscale fibers by dissolving or dispersing them in the organic solvent used for electrospinning. The resultant fibrous mesh possesses a three-dimensional open porous structure with a high specific surface area, providing an ideal condition for controlled drug delivery. It has been shown that drug release patterns from nanofibrous meshes can be tailored by various formulation conditions such as polymer type, polymer concentration, blending of different polymers, surface coating, and the state of drug molecules in an electrospinning medium (e.g. emulsion or suspension, direct dissolution, and coaxial electrospinning) (Zeng et al., 2003; Jiang et al., 2005; Xu et al., 2005; Zeng et al., 2005a). In addition, drug solubility and compatibility with the polymer solution have decisively influenced drug release profiles by altering the drug distribution inside the electrospun nanofibers (Zeng et al., 2005b; Jiang et al., 2006). More recently, electrospun core/shell nanofibrous polymer meshes prepared by coaxial electrospinning were also utilized for delivering protein drugs in a sustained manner. The core-shell structured fibers showed a wide range of protein release profiles by varying the electrospinning parameters. However, most biodegradable nanofibers directly entrapping water soluble drugs exhibited high burst effects with poor controlled release patterns, probably due to incompatibility of drug/polymer/solvent system and slow degradation of biodegradable polymer (Zeng et al., 2003, 2005b).

Biodegradable nanofibrous polymer scaffolds with an open pore structure have been extensively investigated for tissue

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engineering applications, since they have a nanofibrous skeletal structure similar to that of the extracellular matrix (ECM) present in the living tissue. We previously reported a study on the surface immobilization of a cell adhesive Gly-Arg-Gly-Asp-Tyr (GRGDY) peptide on the electrospun PLGA nanofibrous mesh (Kim and Park, 2006). Amine functionalized nanofibers were produced by electrospinning a blend mixture of PLGA and PLGA-b-PEG-NH_2 di-block copolymer, followed by covalent conjugation with the peptide. The cell adhesive peptide functionalized meshes exhibited enhanced cell adhesion, spreading, and proliferation. The ECM mimicking electrospun nanofibrous meshes can be used as attractive scaffold materials for tissue regeneration (Mo et al., 2004; Khil et al., 2005). To further mimic the function of the ECM, growth factor releasing nanofibrous and biodegradable meshes are highly desirable, because they could modulate diverse cellular functions such as differentiation. In particular, nanofibrous meshes releasing growth factors can be potentially used as wound dressing material that provides sustained release of growth factors at the wound site. Many growth factors have been used for wound healing and tissue regeneration due to their potent mitogenic effects (Pandit et al., 2000; Breitbart et al., 2001).

In this study, biodegradable fibrous meshes that can release a protein drug in a sustained manner were produced by directly dissolving protein molecules in an electrospinning solvent medium. Lysozyme was used as a model protein. Various biodegradable polymers such as PLLA, PCL, and PLGA were blended with PEO in varying ratios, co-dissolved with lysozyme in a mixed solvent of chloroform/DMSO, and electrospun to produce lysozyme loaded fibrous meshes. Hydrophilic PEO was incorporated into the hydrophobic bulk phase of biodegradable fibers in order to facilitate lysozyme release by forming extractable pore channels upon incubation in the buffer medium. Lysozyme release patterns were examined by varying the formulation parameters such as polymer type and blend ratio. Enzyme activity of the released fractions was analyzed.

2. Materials and methods

2.1. Materials

PEO (Polyox WSRN-80, Mw: 200,000) was obtained from Union Carbide Corp. (Danbury, CT). Poly(D,L-lactic-*co*-glycolic acid) (PLGA RG756 LA/GA = 75/25, Mw: 100,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(ε-caprolactone) (PCL, Mw: 65,000) and poly(L-lactic acid) (PLLA, Mw: 50,000) were supplied by Aldrich (Milwaukee, WI) and Polysciences Inc. (Warington, PA), respectively. Lysozyme (from chicken egg white, 50,000 units/mg protein) (E.C. 3.2.1.17, mucopeptide *N*-acetylmuramylhydrolase), Micrococcus lysodeikticus, and fluorescamine were obtained from Sigma (St. Louis, USA). Micro-bicinchoninic acid (BCA) assay kit was from Pierce (Rockford, IL). All other chemicals were of analytical grade.

2.2. Electrospinning

A blend mixture of PEO and PLLA, PCL, or PLGA (7/3 weight ratio) was dissolved in chloroform at a concentration of 15% (w/v). PEO/PCL blends with varying ratios (9/1, 7/3, and 5/5) were dissolved in chloroform at a concentration range of 10–15% (w/v). The final volume of each polymer blend solution was 3 ml. Lysozyme was first dialyzed against distilled deionized water to remove residual salts. After adjusting pH to 3.0 with 1.0 M HCl, the solution was lyophilized. Salt-free dry lysozyme was dissolved in DMSO at a concentration of 30 mg/ml. The lysozyme solution (0.2 ml) was mixed with the polymer blend solution, followed by gentle stirring. The electrospinning apparatus used in the present study was constructed based on our previous study (Kim and Park, 2006). Each polymer/lysozyme solution was added into a 5 ml syringe with a metal blunt needle (22G) and then mounted in a programmable syringe pump (model 210, KD Scientific Inc., USA) operated at 20 μl/min. The positive lead from high voltage power generator (CPS-40 K03VIT, Chungpa EMT Co., Korea) was connected to the needle tip and a DC voltage of 15 kV was applied. Stretched and solidified polymeric fibers were deposited on a rotating mandrel-type collector placed 12 cm away from the needle. All electrospinning processes were carried out under ambient conditions.

2.3. Characterization of electrospun fiber meshes

The morphological observation of each electrospun fiber was carried out with a scanning electron microscope (SEM, Philips 535M, Netherlands) after sputter coating with Au particles. From the SEM images, each fiber diameter was determined by using an image analyzer (Image J, developed by the National Institute of Health, USA). For visual observation of encapsulated lysozyme in the fiber, lysozyme was pre-conjugated with fluorescamine in DMSO, and then the fluorescent lysozyme/polymer composite solution was electrospun onto a slide glass. Distribution of fluorescent lysozyme in the fiber was examined by a laser scanning confocal microscope (LSCM, Carl Zeiss LSM5100, Germany).

2.4. In vitro lysozyme release

The circular pieces of lysozyme loaded fibrous mesh (ca. 20 mg) were placed, in triplicate, in a 12 well tissue culture plate and immersed in 2 ml of 33 mM phosphate buffer saline (PBS, pH 7.4, 0.02% NaN_3) solution at 37 °C in a humidified 5% CO_2 environmental incubator. At pre-determined time intervals, 1 ml of release medium was collected and replaced with an equal volume of fresh buffer medium. The amount of lysozyme in the collected solution was measured by using a micro-BCA protein assay kit.

2.5. Polymer erosion

Blend PEO/PCL electrospun meshes were incubated in 5 ml of PBS solution at 37 °C under static condition. The incubation medium was changed daily. The samples were retrieved after

fixed time intervals, washed three times with deionized water, and freeze-dried. The remaining weight was calculated by comparing initial mass (W_0) with mass obtained at each time point (W_t) as follows.

$$\text{Remaining weight}\,(\%) = \frac{W_0 - W_t}{W_0} \times 100$$

In order to characterize morphological changes of eroding PEO/PCL blend electrospun meshes, differential scanning calorimetry (DSC) (DSC 6100, Seiko Instruments, Japan) was used to trace thermal melting behaviors of PEO and PCL. Each sample was scanned from 10 to 100 °C under a flow of nitrogen gas with a heating rate of 10 °C/min.

2.6. Measurement of lysozyme activity

Residual activities of released lysozyme fractions at each time point were measured by using M. lysodeikticus cells as a substrate. The collected sample (0.1 mL) retrieved at a given time point was added to 2.5 ml of 0.015% (w/v) M. lysodeikticus suspension in a 66 mM potassium phosphate buffer solution (pH 6.24), followed by shaking the mixture for 5 min. Decrease in absorbance value at 450 nm was measured using a UV spectrophotometer. The specific activity was then calculated by determining M. lysodeikticus lysis activity divided by amount of lysozyme. The activity of each sample was normalized to the highest value.

3. Results and discussion

Three different biodegradable polymers (PLLA, PCL, and PLGA) were used as skeletal base materials for production of lysozyme loaded fibrous mesh, whereas high Mw PEO (Mw 200,000) was blended, as an extractable self-pore forming polymer additive, to control the release rate of lysozyme from the blend fibrous meshes. PLLA and PCL are semi-crystalline polymers, while PLGA is an amorphous polymer. The three biodegradable polymers employed here have lower molecular weight than that of PEO. PEO is primarily used to improve compatibility with the encapsulated lysozyme because it is a hydrophilic and biocompatible polymer. However, PEO electrospun fiber mesh alone cannot be used for controlled release of lysozyme due to its high water-absorbing capacity and rapid dissolution in aqueous environment. By blending hydrophobic biodegradable polymers with PEO with higher Mw, the two polymers were phase separated to form hydrophilic and hydrophobic domains in the fiber structure. It was expected that entrapped lysozyme molecules were preferentially located within more hydrophilic PEO domains and diffused out through the self-generated pores that were gradually created by slow dissolution of the PEO phase domains upon incubation in the aqueous medium. Meanwhile, phase separated hydrophobic biodegradable polymer domains constituted a porous skeletal structure while maintaining structural integrity of the fibrous mesh during release.

3.1. PEO/PLLA, PCL, and PLGA electrospun fibers for lysozyme release

Various electrospinning parameters including polymer solution property (surface tension, conductivity, and viscosity), spinning voltage, flow rate, motion of collector, and distance between the needle tip and collector were responsible for determining the morphology of electrospun fiber meshes (Doshi and Reneker, 1995; Fridrikh et al., 2003; Theron et al., 2004). Electric potential and polymer concentration are generally considered as the key factors governing fiber diameter, apparent density, and porosity (Zong et al., 2002). Blending different polymers is a straightforward way to create a unique physical and mechanical property that each polymer does not possess (Park et al., 1992; Nijenhuis et al., 1996). Overall morphology, degradation rate, and matrix characteristics of the final products can be tailored by controlling electrospinning parameters and polymer blend composition. In this study, each polymer blend was dissolved in chloroform at different concentrations of 10–15 wt% to produce a stable and regular fibrous structure. To incorporate lysozyme in the bulk phase of the fiber, lysozyme was molecularly dissolved in DMSO after the desalting and freeze drying process, as reported in our previous study (Park et al., 1998). The lysozyme/DMSO solution was then combined with the polymer/chloroform solution for electrospinning. The mixed solution was slightly cloudy, indicating that lysozyme was partially aggregated. The electric voltage used here was fixed at 15 kV. When a higher voltage was applied, a large whipping motion was detected, resulting in uneven deposition on the rotating collector. After electrospinning, the dried electrospun mesh showed mechanical stability good enough to handle. As shown in Fig. 1, average diameters of PEO/PLLA, PEO/PCL, and PEO/PLGA blend fibers were 1.43, 1.02, and 0.99 μm, respectively. They all exhibited typical morphological characters of non-woven electrospun fiber mesh without showing any structural defects. It is of interest to note that lysozyme particles can be seen on the surface of PEO/PLLA and PEO/PCL fibers, but not for PEO/PLGA blend fibers. This was probably due to the semi-crystalline nature of PLLA and PCL that expelled the lysozyme aggregates onto the fiber surface during polymer crystallization under the electrified jet thinning process. It can be visualized that the number of lysozyme particles on the PEO/PLLA fiber surface was greater than that on the PEO/PCL fiber surface. This was most likely caused by the different crystallization behavior between PLLA and PCL during electrospinning. Fig. 2 shows lysozyme release profiles from the three blend fibers. The PEO/PCL mesh shows the smallest extent of burst release (ca. 26%) with a more sustained release pattern during a one week period, whereas the PEO/PLLA and PEO/PLGA meshes show much higher extents of initial burst with less controlled release profiles during the same period. The high initial burst (ca. 45%) observed for the PEO/PLLA mesh was attributed to the presence of unentrapped lysozyme particles on the fiber surface as shown in Fig. 1. Similarly, PEO/PLGA mesh also exhibited high burst release, which was probably caused by the fact that amorphous PLGA is more hydrophilic than semi-crystalline PLLA, thus absorbing water more quickly

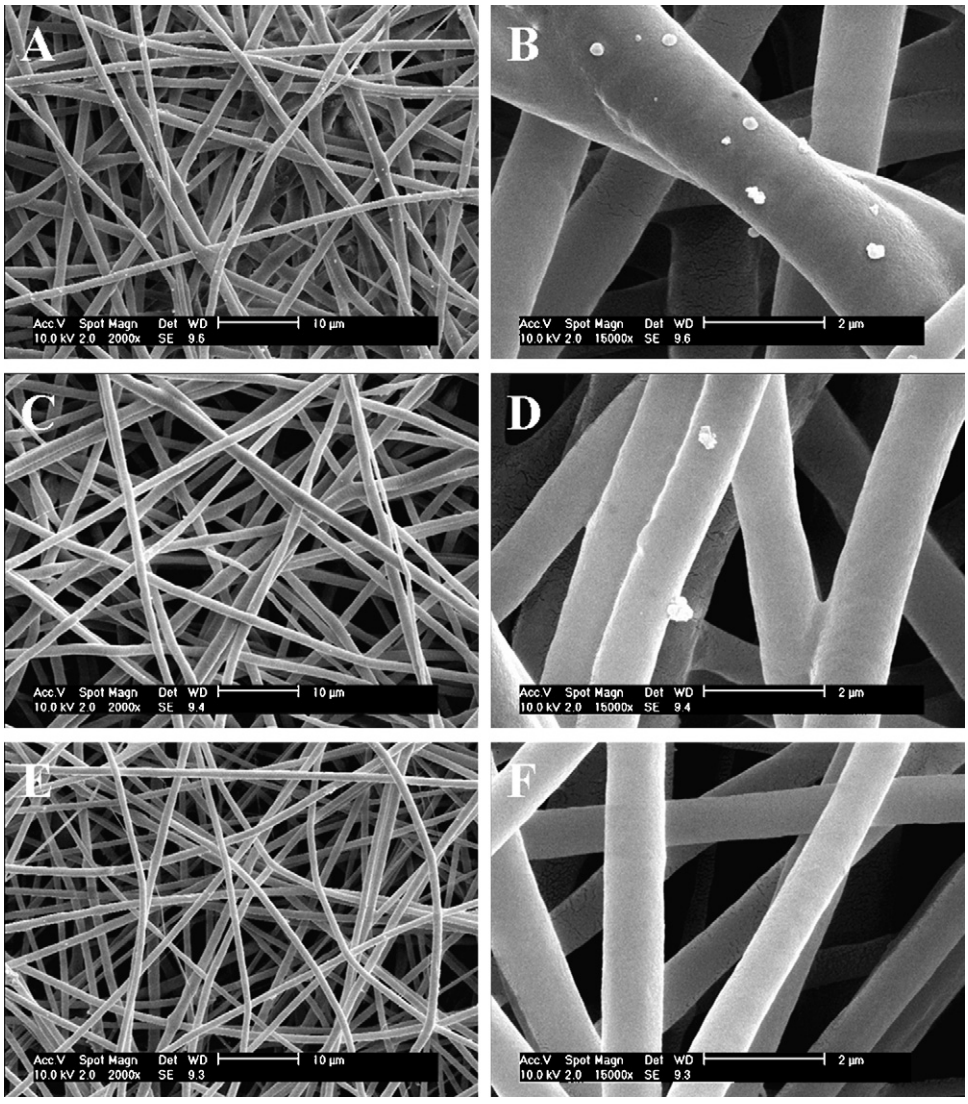

Fig. 1. SEM images of PEO/PLLA (A and B), PEO/PCL (C and D), and PEO/PLGA (E and F) electrospun fibers at a 70/30 blend weight composition.

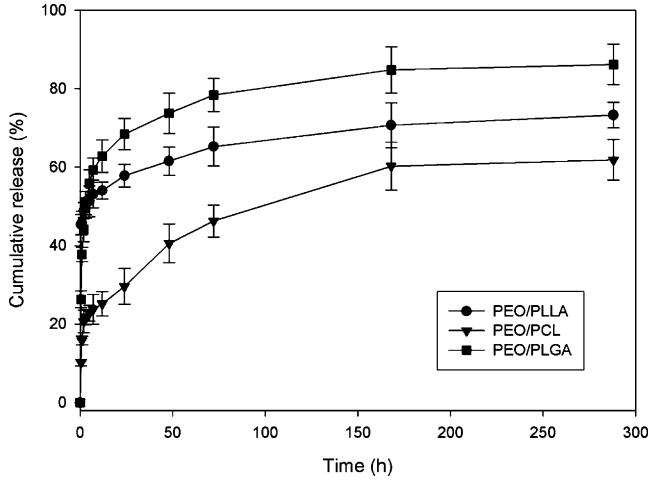

Fig. 2. Lysozyme release profiles from electrospun PEO/PLLA, PEO/PCL, and PEO/PLGA fibers with a 70/30 blend weight composition.

than the other semi-crystalline polymers upon incubation. As a result, most of the entrapped lysozyme molecules were likely to be released out in an initial burst. Although the PEO/PLGA electrospun mesh showed good lysozyme loading and permeability, it exhibited a very large dimensional change in aqueous medium, resulting from hydration. The mesh shrunk more than 60% from its original area, which was very undesirable for use as drug delivery devices (Zong et al., 2003). The PEO/PCL mesh maintained its original dimension during the release period. Therefore, the PEO/PCL electrospun mesh was chosen for further studies. In order to visualize the distribution of lysozymes in the PEO/PCL electrospun fibers, fluorescent dye labeled lysozyme was incorporated, and the fluorescent image was observed under a confocal microscope. Fig. 3 shows that lysozyme molecules were homogeneously distributed along the fiber with the presence of a few lysozyme aggregates near the surface. This indicates that lysozyme could be molecularly incorporated into the bulk phase of PEO/PCL fibers.

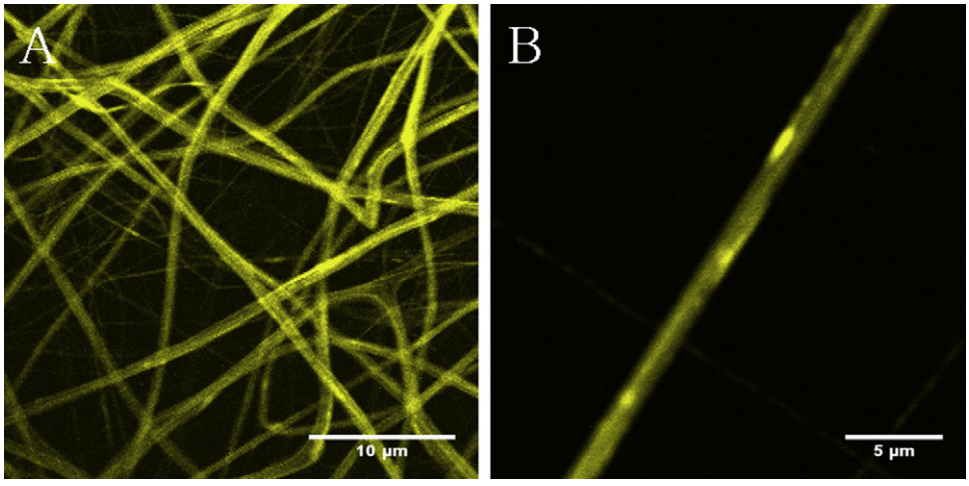

Fig. 3. LCSM image of (A) electrospun PEO/PCL (70/30) blend fibers encapsulated with fluorescently labeled lysozyme and (B) higher magnification image.

3.2. Erosion study of PEO/PCL blend meshes

Based on the observation that the PEO/PCL blend mesh was good to use for delivering lysozyme, subsequent experiments were performed to optimize lysozyme release profiles with varying blend ratios between PEO and PCL (90/10, 70/30, and 50/50, w/w). Fig. 4 shows mass erosion behaviors of PEO/PCL blend meshes with different blend ratios. As expected, increasing the amount of PEO in the blend fiber resulted in a more rapid mass erosion. In the case of 90/10 PEO/PCL blend mesh, very sharp mass erosion occurred within a day, indicting that the hydrophilic PEO part was dissolved out immediately. At the end of the incubation period, the remaining weight percentages for 90/10, 70/30, and 50/50 blend meshes were 14, 35, and 55%, respectively, which were close to the initial weight percents of PCL in the blend mixture. This reveals that the hydrophilic PEO part was mostly dissolved out into the incubation medium, while the hydrophobic PCL part remained in the mesh. The PEO/PCL blend fibrous meshes produced by electrospinning maintained their structural integrities in the form of a fiber, although massive dissolution of PEO occurred. This is in contrast to the polymer casting film having an identical blend composition, but exhibited severe physical disintegration within a short period of incubation time (less than 24 h) (data not shown). It is not clear why the PEO/PCL blend fiber was more resistant to physical disintegration than the PEO/PCL blend film having the same composition. This might be due to the effect of electrospinning on the degree of phase separation between the PEO and PCL domains. It was likely that the sizes of phase separated PEO and PCL domains in the fibrous structure were much smaller than those in the film prepared by solvent evaporation. Electrified rapid ejection of a blend polymer solution through a narrow nozzle with quick solvent evaporation might kinetically arrest phase separation between PEO and PCL, leading to smaller domain sizes than those in the film that were formed by much slower solvent evaporation. Hence, the blend fiber mesh could maintain its dimension and shape because the remaining PCL domains constituted a fibrous skeletal structure even after the PEO domains were leached out massively. Fig. 5 shows SEM pictures for 90/10 and 70/30 PEO/PCL blend fibrous meshes as a function of incubation time. For the 90/10 blend mesh, it can be seen that the fibrous morphology was still maintained with a swollen and rugged surface structure even after 12 day incubation. The average fiber diameter changed from 1.16 to 1.55 μm. This was clearly due to the high water absorbing capacity and rapid dissolution of PEO domains embedded in the fiber, making the individual fiber to be in a swollen state (Miller-Chou and Koenig, 2003). No significant change, however, occurred in the 70/30 blend mesh, because a higher PCL percent prevented the blend fibers from swelling to some extent. In order to examine morphological changes occurring in the 70/30 PEO/PCL blend mesh during incubation, differential scanning calorimetric study was performed. As shown in Fig. 6, melting temperatures for PEO and PCL as-spun fibers were observed at 68.6 and 61.6 °C, respectively. When blended, melting temperature of PEO appears at 66.1 °C, while that of PCL was imbedded in the broad melting endotherm of PEO, suggesting that PEO and PCL are slightly phase-mixed. In semi-crystalline polymer blends, it is known that miscible polymer blends show a shift in each melting temperature to be

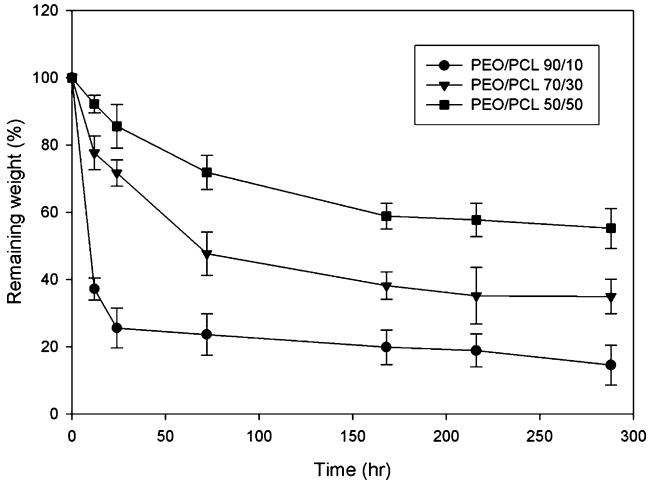

Fig. 4. Remaining weight of various electrospun PEO/PCL blend fibers (90/10, 70/30, and 50/50).

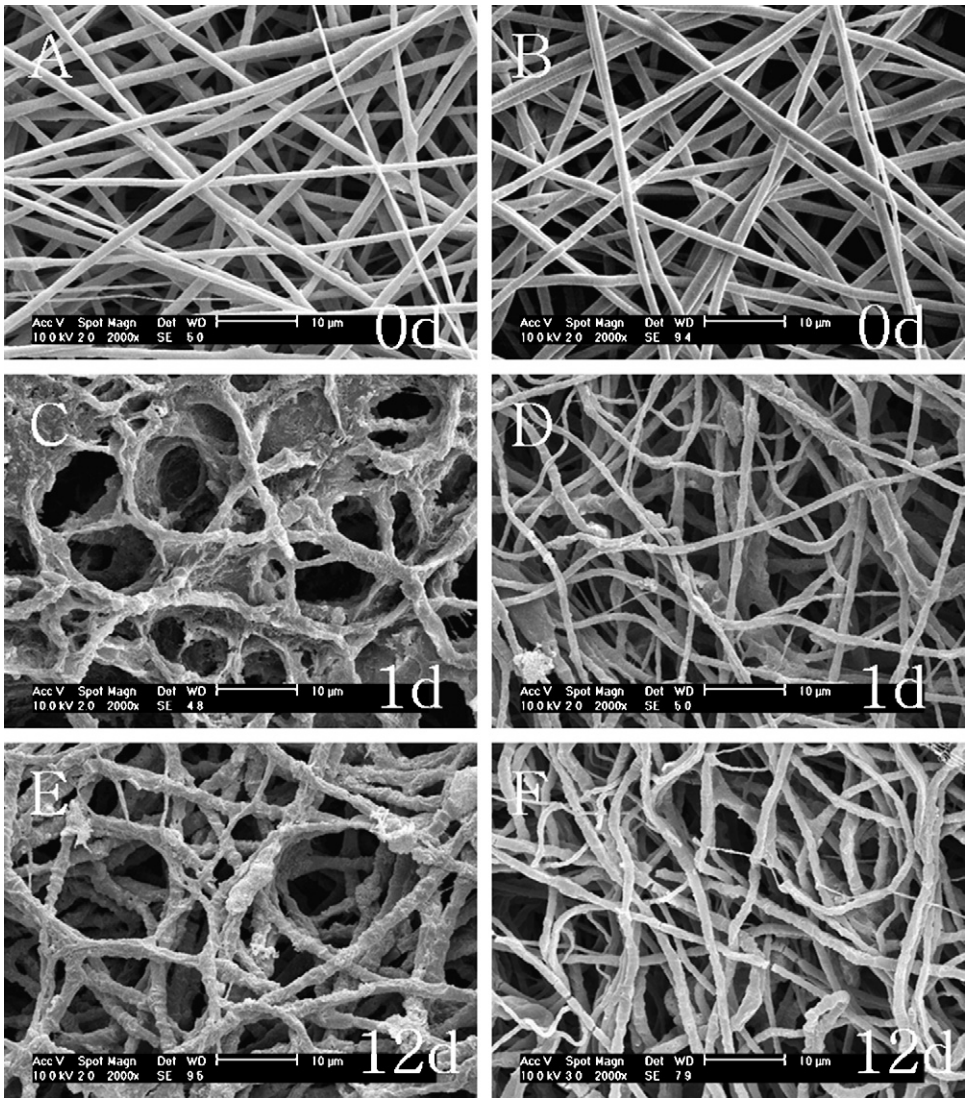

Fig. 5. SEM images for morphological changes of 90/10 (A, C, and E) and 70/30 (B, D, and E) PEO/PCL electrospun blend fibrous mesh as a function of incubation time.

close together (Na et al., 2002). The DSC result was indicative of partial miscibility between PEO and PCL, especially in the amorphous region. The melting endotherm of crystalline PEO domains gradually disappeared, while that of crystalline PCL domains gradually appeared with incubation time. At day 12, the melting endotherm of PCL was only observed, demonstrating that PEO domains were almost dissolved out, leaving behind the PCL skeletal fiber structure. The DSC results are in good agreement with the mass erosion results presented in Fig. 4.

3.3. Lysozyme release from PEO/PCL blend meshes

Fig. 7 shows lysozyme release profiles from PEO/PCL blend meshes with different blend ratios. It can be seen that lysozyme is released out more rapidly when the amount of PEO increases in the blend. For the 90/10 blend mesh, the cumulative lysozyme release percent reached to about 87% after 12 day incubation. For the 50/50 blend mesh, however, about 32% was released out during the same period. The extent of initial burst release was much higher for the fiber mesh containing higher PEO. The results suggest that the dissolution rate of PEO domains in the fiber structure controlled the release rate of lysozyme. Since lysozyme molecules were likely partitioned into the amorphous phase of PEO domains in the blend mixture, entrapped lysozyme species were released out through the aqueous fluid filled porous and interconnected channels that were created from gradual dissolution of phase separated PEO domains. Thus the incomplete release observed in the 70/30 blend mesh was also caused by poor inter-connectivity between the PEO domains, restricting the diffusion of lysozyme entrapped deep inside the fiber. It should be noted that protein stability problems such as aggregation and non-specific adsorption additionally contributed to protein release behaviors from biodegradable devices to varying extents (Perez et al., 2002; Kim and Park, 2004; Dai et al., 2005). In this sense, the observed lysozyme

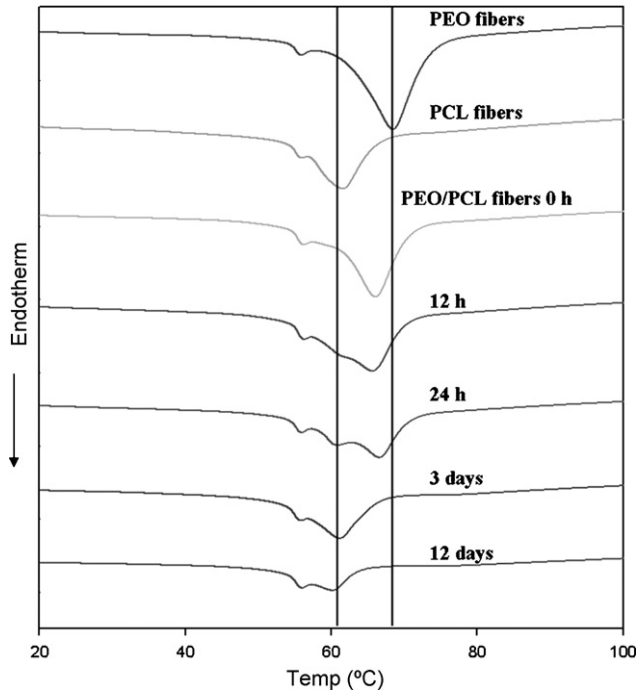

Fig. 6. Differential scanning calorimetric thermograms of electrospun PEO/PCL (70/30) blend fibrous mesh as a function of incubation time.

release patterns can not be solely explained from the combined mechanism of polymer erosion and lysozyme diffusion. Fig. 8 shows biological activities of lysozyme released from the 90/10 PEO/PCL blend mesh at early incubation stage. It can be seen that the released lysozyme fraction after 12 h incubation still retained about 90% of its catalytic activity compared to that of native lysozyme. This reveals that lysozyme survived during the electrospinning process involving direct dissolution in a mixed organic solvent of DMSO and chloroform and rapid jet-streamline ejection through a nozzle under high electric voltage conditions.

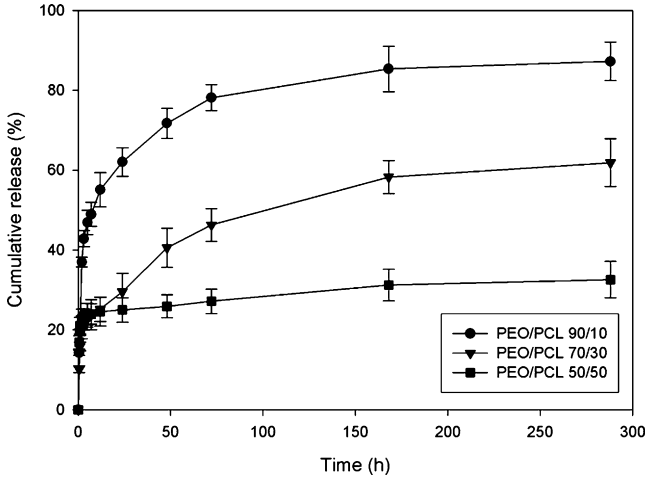

Fig. 7. Lysozyme release profiles from electrospun PEO/PCL fibrous mesh with varying blend weight compositions (90/10, 70/30, and 50/50).

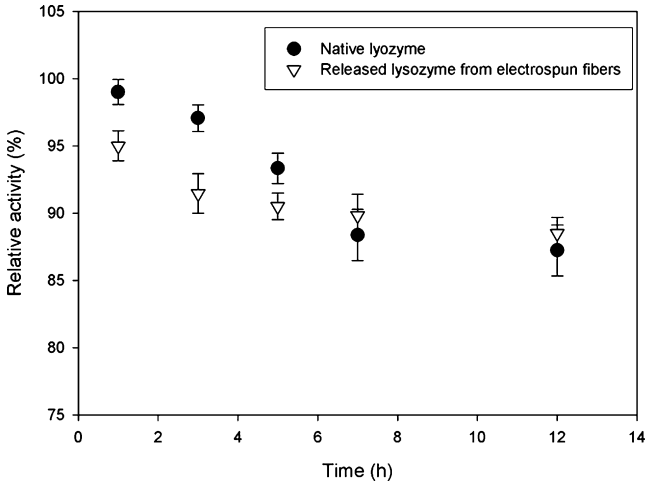

Fig. 8. Relative activities of lysozyme released from electrospun PEO/PCL fibrous mesh as a function of time. Lysozyme activity for each released fraction was normalized to the activity of free lysozyme (100%).

4. Conclusions

In this study, we demonstrated that PEO/PCL blend electrospun meshes released bioactive lysozyme in a sustained manner over a 1 week period. Among the biodegradable polymers (PLLA, PCL, and PLGA), PCL was the best candidate polymer for blending with PEO. The PEO/PCL electrospun meshes maintained structural integrity over the release period, although they were massively eroded due to dissolution and extraction of the hydrophilic PEO part from the blend mixture upon incubation. The lysozyme release rate was primarily controlled by the dissolution rate of PEO domains that were phase-separated in the fiber bulk phase. Self-generation of inter-connected pores produced by slowly diminishing PEO domains seems to be an important route for controlled release of lysozyme from the fibrous PEO/PCL blend mesh. The current protein delivery strategy based on electrospun nano- and micro-fiber meshes can be potentially applied for various wound dressing and tissue engineering devices that require sustained release of angiogenic growth factors to the tissue defect site.

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