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Encapsulation and controlled release of lysozyme from electrospun poly(ε-caprolactone)/poly(ethylene glycol) non-woven membranes by formation of lysozyme–oleate complexes

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Abstract In this study, the concept of hydrophobic ion pairing was adopted for incorporating lysozyme into electrospun poly(ε-caprolactone) (PCL)/poly(ethylene glycol) (PEG) non-woven membranes. The solubility of lysozyme in organic solvent was enhanced through the formation of lysozyme–oleate complexes, which could be directly loaded into PCL/PEG membranes using electrospinning technique. The resultant PCL/PEG nanofibers have a compact structure with an average diameter ranged from about 0.4 μm to 0.9 μm. The addition of PEG into the PCL nanofibers not only improved the hydrophilicity of the membrane, but also played an important role on in vitro lysozyme release rate. It was found that the release rate of lysozyme was enhanced with the increase of PEG content. In addition, the increase of salt concentration in the release medium accelerated lysozyme release. It has also been shown that the released lysozyme retained most of its enzymatic activity.

Introduction

The electrospinning technique can produce polymeric nanofibers with diameter in the range from tens of nanometers to several micrometers. These nanofibers are of considerable interest because of their unique properties such as high surface area, high porosity and interconnected fibrous network [1]. Recently, electrospun fibers based on biocompatible materials have been paid more and more attention for biomedical applications, such as wound dressings [2], tissue engineering [3–7] and gene delivery [8]. However, the applications of biodegradable electrospun nanofibers for controlled release are mainly limited to small molecular drugs up to date [9–13]. There were still few reports on the incorporation and controlled release of protein/peptides from electrospun fibers so far [14, 15]. A significant baffle is the poor solubility of protein/peptide in common organic solvents (e.g., dimethylformamide (DMF), dimethyl sulfoxide (DMSO) or chloroform), which are frequently used for dissolving the biodegradable polymers (e.g., poly(lactic acid) (PLA), poly(lactide-*co*-glycolide) (PLGA), poly(ε-caprolactone) (PCL) etc) during the electrospinning process. Wnek reported a "two-phase electrospinning" method for incorporation of BSA into poly(ethylene-*co*-vinyl acetate) fibers [15]. However, the method would result in uneven drug distribution through the polymeric fibers, which may lead to unpredictable release behavior of drugs from the membranes.

An effective method to solve above mentioned problem is to enhance the solubility of protein in organic solvents. Many literatures have indicated that proteins could have an altered solubility in organic solvents by means of hydrophobic ion pairing (HIP) [16, 17], where the electrostatic interactions between proteins and an appositely charged surfactant results in the formation of protein/surfactant complexes. In addition, it was reported that the stability of proteins could also be enhanced by HIP. The direct suspension or dissolution of neat proteins in organic solvents will always destroy the protein conformation, while the stability of proteins in organic solvents could be enhanced through complexation with ionic surfactant [17].

In the present study, lysozyme was selected as a model protein to be incorporated into the electrospun PCL fibers

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by formation of lysozyme–oleate complex. The lysozyme–fatty acid complexes have been extensively studied to enhance lysozyme solubility in common organic solvents. In addition, the effect of the electrospinning process on the protein stability could be conveniently investigated by determining the enzymatic activity of lysozyme. PCL was selected in this study due to its good biocompatibility, biodegradability and good fiber-forming properties [18]. In order to modulate release rate of lysozyme, a certain amount of PEG was added into the electrospun PCL fibers. The electrospun nanofibers were characterized by DSC and X-ray diffraction. The release and bioactivity of lysozyme from the non-woven membranes were also tested.

Experimental details

Materials

Lysozyme (from chicken egg white), its substrate, *Micrococcus lysodeikticus*, and oleic acid (*cis*-9-octadecanoic acid) sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). Poly(ε-caprolactone) (PCL) was synthesized in our own lab (M_n 35,000). Polyethylene glycol (M_n 6000) was obtained from Fluka (Buchs, Switzerland). Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dried with 4 Å molecular sieves prior to use. All other chemicals were of analysis grade and used as received.

Preparation of lysozyme–oleate complexes

The lysozyme–oleate complexes were prepared following the procedure previously described [16]. Briefly, certain amounts of lysozyme and sodium oleate (at the molar ratio of 1:6) were dissolved in 10 mM Tris/HCl buffer solution (pH 7.0), respectively, and mixed together under mild magnetic agitation. The resultant white precipitates were collected by centrifugation for 10 min at 2,500 rpm, washed with deionized water and freeze dried. The composition of the lysozyme/sodium oleate was analyzed by determining the amount of remaining lysozyme in the supernatant after centrifugation.

Electrospinning of PCL/PEG

Compared with free lysozyme, the resulting complexes could be dissolved in DMSO with enhanced solubility [16], but it is insoluble in other organic solvents (e.g., DMF, $CHCl_3$ or acetone). In this study, DMSO/$CHCl_3$ admixture was used for dissolving both polymer and the complex. The lysozyme–oleate complex (2.5% weight ratio to polymer) was first dissolved in DMSO, and then $CHCl_3$ was added with the volume ratio to DMSO at 4.5:5.5. After lysozyme solution was evenly mixed, PCL was added. The PCL concentration was 15 wt%. In some cases, PEG 6000 was also added (10, 20 and 50 wt% with respect to polymer) to the solution. The homogeneous solution was electrospun at 12–16 kV under a steady flow rate of ~1 mL/h using a spinneret with a diameter of 0.7 mm. The electrospun fibrous membrane was collected on an aluminum plate. The distance between the spinneret and the collector was 30 cm. The resulting membrane was vacuum dried at room temperature overnight.

Characterization of electrospun PCL/PEG nonwoven membranes

Morphology of the electrospun membrane was observed by SEM (Stereoscan 260, Cambridge). Before SEM, samples for SEM were dried under vacuum, mounted onto stubs using double-sided adhesive tape, and sputter-coated with gold-palladium.

To evaluate the hydrophilicity of the membranes, static contact angles were measured by sessile drop method at 25 °C using a digital optical contact angle meter CAM 200 (KSV Instruments Ltd., Helsinki, Finland). Each measurement was finished within the first 10–15 s after the deployment of the sessile drop. Angles were measured on five different regions of the membrane surface, and the measurements were the average of 15 contact angles at least operated on three membrane samples.

Thermal analyses were performed using a thermal analysis system (Pyris 1 DSC, Perkin Elmer Corp., Britain). Samples of the electrospun membranes (each weighed approximately 8 mg) were sealed in aluminum cassettes under dry conditions. DSC scans were taken first during heating from –100 °C to +100 °C at a rate of 10 °C per min. After 10 min of annealing at 100 °C, the samples were quenched again to –100 °C at a rate of 10 °C per min and the second scan was repeated at a rate of 10 °C per min. All DSC thermograms were taken under a nitrogen environment.

In vitro release study

Lysozyme release profiles from the electrospun PCL/PEG membranes were determined. The membranes with 120 μm thickness (approximately 60 mg per sample), with different PEG content, were immersed in 2 mL of PBS (pH 7.4) at 37 ± 0.1 °C. At predetermined time intervals, 1 mL of PBS was removed for analysis and the same amount of fresh PBS was added for continuing incubation. The lysozyme content in PBS was determined by a bicinchoninic acid assay (BCA) method. In addition, lysozyme

release behavior in PBS containing different ionic strength was also conducted with PCL/PEG (10:2) membranes.

Analysis of lysozyme activity

The biologic activity of lysozyme released from electrospun PCL/PEG membranes was determined by measuring the turbidity change in a *Micrococcus lysodeikticus* bacterial cell suspension [19]. Briefly, 150 μL of lysozyme sample was added to 2.5 mL *Micrococcus lysodeikticus* suspension in Tris/HCl (0.066 M) buffer solution (0.15 mg/mL, containing 15 mM NaCl) at room temperature, followed by immediate mixing. The decrease in turbidity at 450 nm (Cary 100 UV-VIS-NIR Spectrophotometer, USA) was measured for every 15 s for 3–4 min. The results were expressed as the initial kinetic rate. The lysozyme activity for the corresponding initial rate of a sample was calculated using a standard curve of log concentration versus initial rates.

Results and discussions

The formation of lysozyme–oleate complex

Lysozyme has seven primary amine groups in its structure. In addition, other basic amino acid residues such as arginine, present at the molecular surface, make lysozyme a basic protein at physiological pH. Yoo et al. have discussed in detail about the complex formation of lysozyme with two anionic surfactants, sodium oleate and sodium dodecyl sulfate (SDS) [16]. It was found that lysozyme could be equally well ion paired with both of the two surface-active molecules and precipitated out of the solution. The resultant lysozyme/surfactant complexes showed increased solubility in DMSO. However, there was little effect of complex formation on the stability of lysozyme molecularly dissolved in DMSO.

In this work, lysozyme–oleate complexes were prepared by the method previously reported [16]. The solubility of lysozyme in DMSO was significantly improved through complexation with oleate. For example, the solubility of the free lysozyme was 12.6 ± 4.2 mg/mL for the free lysozyme, while increased to 21.1 ± 3.5 mg/mL when it was complexed with oleate. The results can be attributed to both the neutralization of lysozyme with oleate and the hydrophobicity of oleate.

Characterization of electrospun PCL/PEG non-woven membranes

Since lysozyme–oleate complexes can only be soluble in DMSO, a mixed solvent of DMSO and chloroform was used for electrospinning. Through our pretests, PCL concentration was fixed at 15 wt% and $CHCl_3$/DMSO volume ratio was set at 4.5:5.5 in order to realize the maximum lysozyme loading. The mixed solution was transparent with naked eye for all the studied blend composition. No apparent phase separation was observed. It was observed that stable jets could be formed when the electrospinning voltage exceeded 12 kV. Figure 1 shows surface morphology of PCL/PEG nanofibers when PEG content increased from 10% to 50%. The average diameters of the nanofibers decrease with the increase of PEG content (Table 1). A smooth surface can be observed in all the cases. When PEG content in the nanofibers exceeds 50%, fiber fusion can be occasionally observed. The contact angles of PCL/PEG non-woven membranes are listed in Table 1, which decrease with the increase of PEG fraction.

Figure 2 shows the results of DSC analysis. For pure PCL, two crystalline peaks could be apparently observed in curve a. The melting point of PCL is around 61.5 °C, which slightly decreased to 60.4 °C when PEG content increased to 20% (curve b). In curve c, the crystalline peak of PEG can also be observed, which is partially overlapped with that of PCL. Nevertheless, a crystalline peak of PCL can still be discriminated at about 59.8 °C. ΔH data of the blend fibers are listed in Table 1. The crystallinity of PCL in the blend fibers is difficult to be calculated since the crystalline peak of PEG overlaps with that of PCL. However, from the data of ΔH, it can be concluded that both PCL and PEG crystalline in all the fibers. Therefore, phase separation occurs in all the cases. No apparent glass transitions for both PCL and PEG in the blend fibers were detected in the traces of DSC. Lin et al. also reported similar results for PCL/PEG blend films prepared by casting method [20].

In vitro release study

The in vitro lysozyme release profiles from electrospun PCL/PEG membranes with four compositions are shown in Fig. 3. It can be seen that the lysozyme release rate from PCL non-woven membranes is extremely slow. For example, only 3.8% lysozyme was released within 48 days. The addition of PEG significantly improved the lysozyme release. All profiles are characterized by an initial burst release followed with a slowed release phase. In general, lysozyme release rate increases with the increase of PEG content, e.g., the initial burst release during the first day increases from 7.9% to 20% when PEG content increases from 10% to 50%. Since the degradation rate of PCL is very low [21], lysozyme is released from the nanofibers mainly through diffusion mechanism. The thick structure of PCL nanofibers, as well as the low solubility of lysozyme–oleate complexes in water, results in the slow

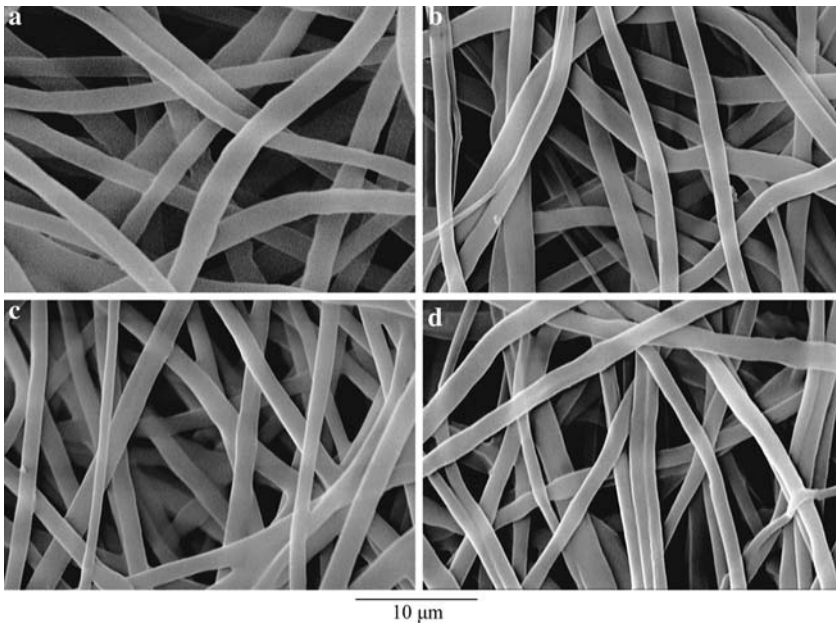

Fig. 1 SEM micrographs of the electrospun PCL/PEG membranes. PCL concentration was 15 wt%, PEG content to PCL was (**a**) 0%, (**b**) 10%, (**c**) 20% and (**d**) 50% respectively. DMSO/chloroform (5.5/4.4, v/v) was used as solvents, the voltage for electrospinning was 14 kV, the feed rate of the solution was 1 mL/h, and the distance between the electrode and the collecting plate was 30 cm

Table 1 Properties of electrospun PCL/PEG membranes at different PEG contents

PEG content (to PCL, wt%)	Average diameters (μm)	Contact angle	ΔH (J/g)
0	0.84	78	49.5
10	0.78	65	/
20	0.73	46	51.1
50	0.69	32	120.3
100	0.58	18	/

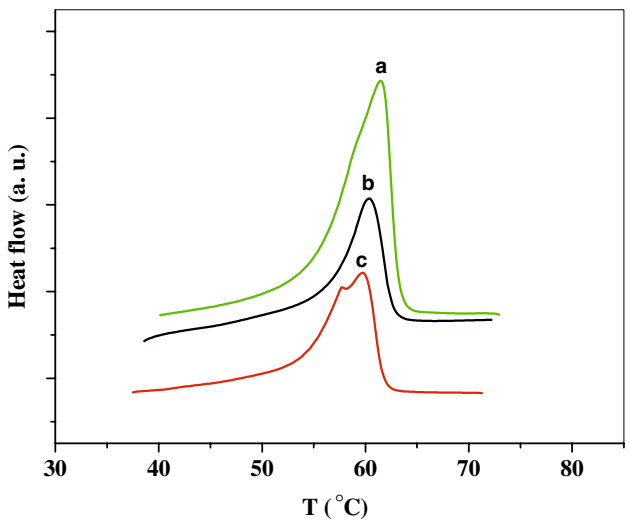

Fig. 2 DSC thermograms of lysozyme–oleate complex-loaded electrospun PCL/PEG membranes with (a) no PEG; (b) 20% PEG and (c) 50% PEG content

diffusion of lysozyme through PCL matrix. The addition of PEG into PCL nanofibers improves their hydrophility, thereby enhances the water uptake of the electrospun membranes. In addition, PEG in the nanofibers can act as porogen due to its water-soluble nature. The morphology of the PCL/PEG nanofibers incubated in release media is shown in Fig. 4. It can be observed that the nanofibers become porous after 7 days incubation in PBS and the porosity increases with release period of time.

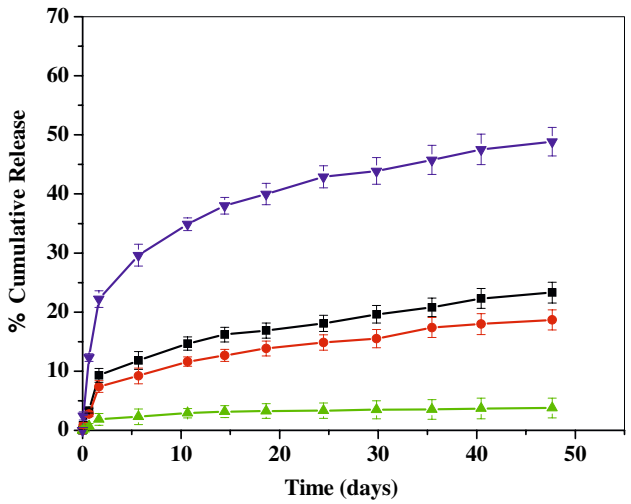

Fig. 3 In vitro release of lysozyme from the electrospun PCL/PEG membranes containing 2.5% lysozyme–oleate complexes with different PEG ratio to PCL in 0.1 M PBS (pH 7.4, 37 °C) (n = 3). ▲—Not containing PEG; •—with 10% PEG; ■—with 20% PEG; ▼—with 50% PEG. DMSO/chloroform (5.5/4.4, v/v) was used as solvents, the voltage for electrospinning was 14 kV, the feed rate of the solution was 1 mL/h, and the distance between the electrode and the collecting plate was 30 cm

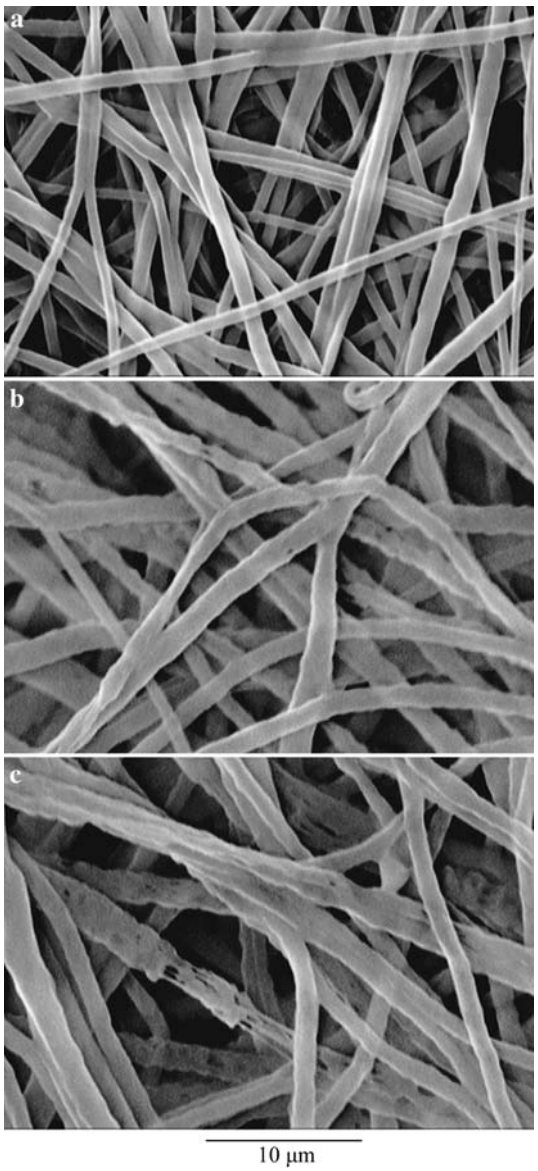

Fig. 4 Scanning electron micrographs of the electrospun PCL/PEG (10:2) membranes after degradation for (**a**) 0, (**b**) 7 and (**c**) 21 days in 0.1 M PBS (pH 7.4, 37 °C)

From Fig. 5, it can be seen that the increase of salt concentration in release medium accelerates the lysozyme release. This may be resulted from the rapid dissociation of lysozyme–oleate complexes in the presence of small molecular salts. Similar results were also reported previously [16].

The activity of lysozyme released from the electrospun fibers

It was frequently reported that substantial loss in bioactivity occurred when lysozyme was directly encapsulated into poly(lactic-*co*-glycolic) acid (PLGA) microspheres in

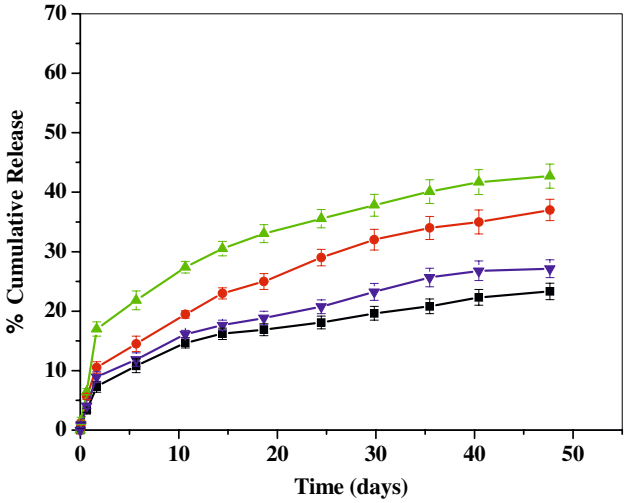

Fig. 5 In vitro release of lysozyme from the electrospun PCL/PEG membranes containing 2.5% lysozyme–oleate complexes in 0.1 M PBS (pH 7.4, 37 °C) containing different concentration of sodium chloride. ■—Not containing NaCl; ▼—with 0.1 M NaCl; •—with 0.2 M NaCl; ▲—with 0.5 M NaCl. PEG/PCL weight ratio was 0.2, DMSO/chloroform (5.5/4.4, v/v) was used as solvents, the voltage for electrospinning was 14 kV, the feed rate of the solution was 1 mL/h, and the distance between the electrode and the collecting plate was 30 cm

the absence of stabilizing additives [22]. Therefore, it is essential to investigate the bioactivity of the released lysozyme from electrospun PCL/PEG membranes. The results are shown in Fig. 6. Generally, no significant decrease in bioactivity of the released lysozyme was observed within 50 days. The released lysozyme retains over 90% of its bioactivity. This phenomenon can be mainly ascribed to the improved stability of lysozyme through the formation of lysozyme–oleate complexes [16]. In addition, the pres-

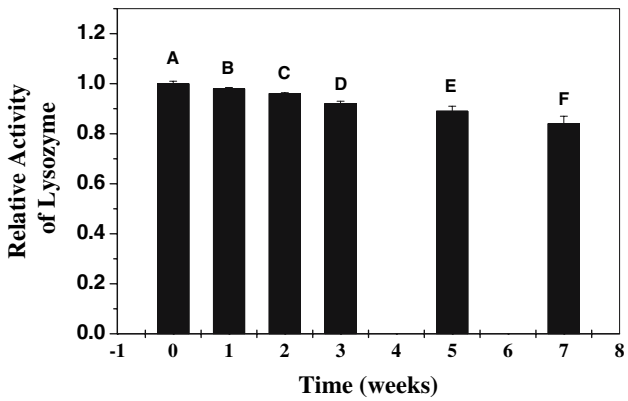

Fig. 6 Bioactivity of lysozyme (100 ng/mL) remaining upon release from electrospun PCL/PEG non-woven membranes with 20% PEG content in 0.1 M PBS (pH 7.4, 37 °C) (n = 2). (A) Native lysozyme; Lysozyme released after (B) 1 week; (C) 2 weeks; (D) 3 weeks; (E) 5 weeks and (F) 7 weeks

ence of PEG in the nanofibers can also enhance the stability of incorporated lysozyme [23].

Conclusions

Lysozyme could be directly loaded into electrospun PCL membranes by electrospinning the mixed solution of PCL (or PCL and PEG) and lysozyme/oleate complexes in the admixture of DMSO/chloroform. Its release rate can be modulated by PEG fraction in the nanofibers, which increases with the increase of PEG content. The released lysozyme displays comparable bioactivity with the native one.

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