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Encapsulation of proteins in poly(L-lactide-co-caprolactone) fibers by emulsion electrospinning

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ABSTRACT

This study was aimed at investigating emulsion electrospinning to prepare biodegradable fibrous mats with encapsulation of human-nerve growth factor (NGF). One of the best methods for fabricating a bio-functional tissue engineering scaffold is to load bioactive agent into the scaffold. In this work, the feasibility of incorporating NGF into poly(L-lactide-co-caprolactone) fibers by emulsion electrospinning has been studied. The release behavior of encapsulated bovine serum albumin (BSA) was investigated. The bioactivity of NGF released from fibrous mats was verified by testing the neurite outgrowth of rat pheochromocytoma cells (PC12). Furthermore, the process of fiber forming during emulsion electrospinning was discussed. The results demonstrate that emulsion electrospun fibers can successfully encapsulate proteins and release them in a sustained manner. The bioactivity of NGF released from emulsion electrospun fibers was confirmed by PC12 bioassays.

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1. Introduction

In contrast to conventional transplantation methods, tissue engineering provided a new medical therapy which used biodegradable polymeric materials with or without living precursor [1]. The ultimate purpose of tissue engineering is to re-establish the destroyed human tissues or organs by providing suitable scaffolds for functional cells regeneration. Nerve tissue repair is a precious treatment concept in health care as it directly impacts on the quality of human life [2,3]. The effective methods for nerve tissue engineering include fabricating a polymeric scaffold with the encapsulation of bioactive proteins (e.g., nerve growth factor) [4,5]. In human tissue, extracellular matrix (ECM) plays a pivotal role in supporting and controlling cells living, therefore, the tissue engineering scaffolds should be designed to mimic the natural ECM. To achieve this aim, the scaffolds made of electrospun fibrous mats which have the similar structure with natural ECM were getting increasing interest in recent years [6–11].

Electrospinning is a technique that utilizes electric force to drive the spinning process and to produce fibers from polymer solutions or melts [12–14]. Unlike conventional spinning techniques (solution- or melt-spinning), which are capable of producing fibers with diameters in the micrometer range, electrospinning produces fibers with diameters in the nanometer range. Electrospun fibers possess many extraordinary properties, such as small diameters and large specific surface areas. Additionally, the non-woven fibrous mats made of electrospun polymer fibers offer a unique capability of controlling the pore sizes. Unlike nanorods, nanotubes, and nanowires that are produced mostly by synthetic, bottom-up methods, electrospun fibers are produced through a top-down process, which results in continuous and low-cost fibers that are also relatively easy to align, assemble and process into applications. Till now, many synthetic and natural polymers including, but not limited to, polylactide (PLA) [15], poly(ε-caprolactone) (PCL) [16], poly(glycolic acid) (PGA) [17], poly(L-lactide-co-ε-caprolactone) (PLACL) [11], collagen [18], and chitosan [19] have been electrospun into fibrous mats as tissue engineering scaffolds.

Tissue engineering scaffolds with the incorporation of drugs or bioactive proteins have been investigated by many researchers, including the methods of blending [20], co-axial [21] and emulsion electrospinning [22,23]. The incorporated drugs or proteins in fibers fabricated by blending electrospinning have a burst release phenomenon in the initial stage [24], because drug particles tend to locate on the surface of the fibers under the impelling electric force during the process of electrospinning. One of the most feasible methods to resolve the problem of initial burst release is to encapsulate drugs or proteins into the electrospun fibers. Furthermore, the releasing of drugs or proteins from fibers would be more stable [24,25]. Co-axial electrospinning has been used for

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preparing fibers with core–shell structures, in which two components can be co-axially and simultaneously electrospun through different feeding capillary channels [21]. However, co-axial electrospinning is a dynamic process, and many factors such as flow rate of the inner and outer solutions, interfacial tension, and viscoelasticity of the two polymer solutions could affect entrainment and thus produce fibers without the required core–shell morphology [26]. In addition, a special apparatus and careful selection of operational parameters were also needed to ensure the desired results.

Recently, preparing core–shell type fibers by emulsion electrospinning has attracted growing interests [22,23,27–29]. The emulsions used for electrospinning (especially the water-in-oil type of emulsions) usually contain an oil phase which is obtained by dissolving polymers into organic solvent, and a water phase (or the emulsion spheres) with sizes of microns or sub-microns in which drug or protein dissolve. Under the electric force, emulsion containing the oil phase of polymer solution and the water phase of micron or sub-micron spheres could be elongated and converted into core–shell structure fibers. These fibers with drugs and proteins encapsulated would be fabricated into bioactive tissue engineering scaffolds. It is remarkable that unlike co-axial electrospinning which needs a special apparatus, the basic equipment for emulsion electrospinning is only a single needle.

In this work, fibrous mats which electrospun from an emulsion made of PLACL solution, phosphate buffered saline (PBS, pH 7.4) solution contained proteins and sorbitan monooleate (Span80, an emulsifier/surfactant widely used in food products and oral pharmaceuticals) were investigated. PLACL was selected because of its good properties of attachment and proliferation of animal cells [11]. Fibrous mats with bioactive proteins encapsulated were successfully fabricated by electrospinning the emulsions made of PLACL, chloroform, Span80, NGF and PBS solution.

2. Materials and methods

2.1. Materials

Recombinant human β-NGF and DuoSet ELISA development system for human β-nerve growth factor were purchased from R&D Systems, Inc. A rat pheochromocytoma cell line, PC12, was obtained from Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Phosphate buffered saline (PBS), pH 7.4, containing no calcium chloride and magnesium chloride; and RPMI medium 1640 with L-glutamine were obtained from GIBCO, Invitrogen Corporation. Dichloromethane (99.8% anhydrous) and bovine serum albumin (BSA), fluorescein isothiocyanate conjugate bovine serum albumin (FITC-BSA) were obtained from Sigma–Aldrich Corporation. The serum-free RPMI cell culture medium consists of RPMI 1640 medium, 1% Hepes buffer, 1% sodium pyruvate, 0.275% of penicillin–streptomysin, and 0.556% glucose.

PLACL with molar ratio of 50% being L-lactide was purchased from the Sigma–Aldrich Co. (Milwaukee, Wisconsin). Span80 was purchased from the Zibo Haijie Chemical Industry Co., Ltd. (Zibo, China). Chloroform was purchased from the Shanghai Fine-Chemicals Co., Ltd. (Shanghai, China). All of the materials were used without further purification.

2.2. Emulsion electrospinning of PLACL fibrous mats

Span80 (0.04 mL) was first dissolved in 10 mL chloroform; subsequently, different amount of BSA/PBS solutions were added dropwise (there was no NGF adding in the PBS solution); this was followed by stirring the mixtures at 240 rpm for 20 min to obtain uniform emulsions. PLACL was then added into the emulsions, dissolved in the chloroform phase, and the final concentration of PLACL in emulsions was 6 wt%. In the case of the FITC-BSA encapsulated PLACL fibers, FITC-BSA and BSA solution were both used. For NGF-BSA encapsulated PLACL fibrous mats, NGF was added into PBS solution. BSA, which was used as a filler protein and stabilizer for NGF, was also dissolved in PBS. The resulting protein solution was added into chloroform/Span80 solution, giving a protein–polymer suspension, which was then stirred to distribute the protein suspension uniformly throughout the polymer solution to become an electrospun emulsion.

The experimental set-up used for conducting electrospinning included a voltage power supply (BGG DC high-voltage generator), purchased from the BMEI Co., Ltd. (Beijing, China), and a digitally controlled and extremely accurate syringe pump (789100C, Cole-Palmer, USA). During electrospinning, a positive voltage of 15 kV was applied at the tip of a syringe needle with the inner diameter of 0.9 mm. The electrospun fibers were collected on a piece of aluminum foil which was electrically grounded and placed at a distance of about 15 cm below the tip of the syringe needle. The flow rate of electrospun solution was maintained at 1.0 mL/h. The electrospinning was conducted under room temperature; and the collected fibrous mats had a thickness around 50 μm.

2.3. Characterization of emulsion electrospun fibrous mats

Morphology of fibrous mats with and without BSA encapsulation was observed by Digital Vacuum Scanning Electron Microscope (JSM-5600LV, Japan Electron Optical Laboratory) at the accelerating voltage of 15 kV. Samples for SEM observing were sputter coated with gold. Diameters of electrospun fibers were measured with image visualization software Image-J (National Institutes of Health, USA). Average fiber diameters and diameter distributions were determined by measuring about 100 random fibers from the SEM micrographs.

Protein distribution in the PLACL fibers was evaluated by observing the distribution of FITC-BSA encapsulated in the result fibers by an optical microscope (UL100HG Olympus Corporation, Japan).

For determination of hydrophilicity of emulsion electrospun fibrous mats, the contact angles of non-woven scaffold were measured by a contact angle analyzer (Data Physics Corp., San Jose, CA, USA). During the measurements, fibrous mats were cut into square specimens with the size of 1 cm × 1 cm, followed by placing them on the testing plate. Subsequently, 0.03 mL distilled water was carefully dropped onto the prepared specimens. The contact angles between water droplets and fibrous mats were measured using photos taken at various time points (1, 2, and 3 s). Five samples were used each test. The average value was reported with standard deviation (±SD).

2.4. Release behavior study

For the protein release behavior study, fibrous mats were generated from the emulsion had BSA contained in the spheres of water phase. Three samples that were electrospun by the same electrospinning parameters were used in this study. Electrospun fibrous mats, each weighing about 100 mg, were soaked in glass vials with 5.0 mL of PBS. The fibrous mats were incubated at 37 °C in the presence of 5% carbon dioxide. At various time points, 1 mL of supernatant was retrieved from the vial and an equal volume of fresh medium was replaced. The concentration of BSA in the supernatant was then determined by an UV-vis spectrophotometer (WFZ UV-2102 Unique Technology Shanghai) at an optical wavelength of 280 nm. As a control, BSA release behavior from blending electrospinning was also tested.

2.5. Bioactivity of released NGF

PC12 cells, which differentiate to a neuronal phenotype in the presence of bioactive NGF, were used to test for the bioactivity of the NGF released from the emulsion containing BSA dispersed phase. PC12 cells were cultured in collagen type IV-coated 24-well plates at a density of 1×10^4 cells/cm^2. A volume of 400 μL of the NGF supernatant from the PLACL fibers was added to each well of PC12 cells and serum-free RPMI was added to top up the medium volume to 1.0 mL/well. As a positive control, 8 μL of 50 μg/mL of NGF solution was added to the PC12 cell culture medium, and the total volume of medium was then topped up to 1.0 mL with serum-free RPMI. A negative control in which no NGF was added to the serum-free RPMI medium was also used. Each set of samples was repeated twice. Images of the PC12 cells were taken after the supernatant was added into the culture medium for 48 h.

3. Results

3.1. Morphology of electrospun PLACL fibrous mats

Fig. 1(a) shows the SEM micrographs of PLACL fibrous mat without encapsulating of any protein, which was fabricated under the conditions described in Section 2.2, and the inset displays the corresponding fiber size distribution. The average diameter of PLACL fibers in Fig. 1(a) was 910 ± 20 nm.

Fig. 1(b) shows the SEM micrographs of PLACL with BSA encapsulated, and the weight ratio of BSA to PLACL was 1:12. For this emulsion, 0.5 mL 10 wt% BSA/PBS solution was added into 10 mL 6 wt% PLACL/chloroform solution with 0.04 mL Span80 in it. There was no significant difference in fibers morphology between pure PLACL and PLACL/BSA composite fibers. However, the average diameter of PLACL/BSA was 610 ± 20 nm, and the diameter distribution was narrower than that of pure PLACL fibers. When the weight ratio of BSA/PLACL increased to 1:6, the average diameter was 630 ± 20 nm, and the diameter distribution was similar to that of the fibers in Fig. 1(b), as shown in Fig. 1(c).

Moreover, it is clear to find from the SEM images that the electrospun fibers were adhered at their junction zones. This seemed to show that fibers were not adequately dried during the electrospinning process, because of the short collector/spinneret distance and/or reduced evaporation rate of solvent when the surfactant was added.

3.2. Proteins distribution in fibers

As the SEM images cannot provide evidences that proteins were successfully incorporated into polymer fibers, FITC-BSA was conducted for the study of proteins distribution in fibers. FITC-BSA was used as part of carried materials, and the composite fibers of FITC-BSA/BSA/PLACL were observed by Fluorescence Microscopy (FM, IX71-A12FL, OLYMPUS, Japan). The low amount of FITC-BSA in the electrospun solution had no significant impact on the electrospinning process, allowing the same electrospinning parameters for BSA-encapsulated fiber formation to be used. Fig. 2 indicates the distribution of proteins in PLACL fibers. FITC-BSA/PLACL fibers were collected on a glass slide, and then observed by optical microscopy (OM) and FM at the same collected point. As shown in Fig. 2(b), the

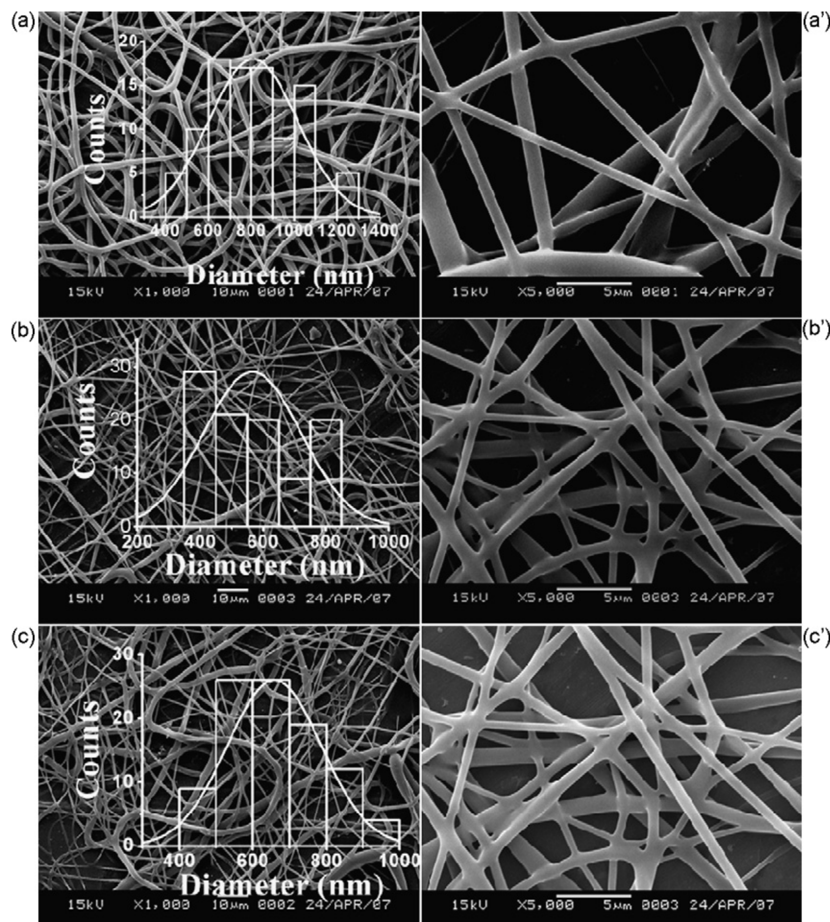

Fig. 1. SEM micrographs of PLACL fibers with and without BSA. (a–c) Micrographs of PLACL fibers, BSA/PLACL fibers with weight ratio of 1:12, and BSA/PLACL fibers with weight ratio of 1:6, respectively. (a′–c′) High-magnification images of (a)–(c). The insets show the fiber size distributions.

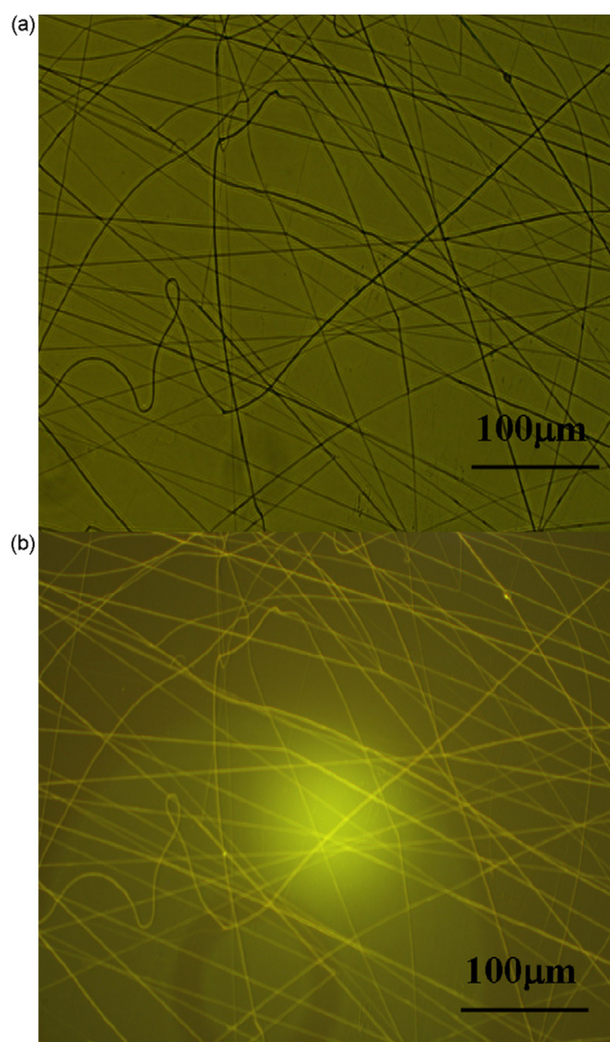

Fig. 2. Distribution of proteins and core–shell structure of BSA/PLACL fibers: (a) OM image of BSA/PLACL fibers on glass slide and (b) FM image of FITC-BSA/PLACL fibers.

fibers emitted fluorescence light, suggesting the presence of FITC-BSA, and the fibers formed changed little compared with OM image in Fig. 2(a). These results confirmed that proteins were distributed in all parts of the PLACL fibers. Since the electrospinning parameters of fabricating FITC-BSA/PLACL were the same as the fabricating of NGF-BSA/PLACL fibers, the distribution of FITC-BSA could reflect the NGF-BSA distribution in the PLACL fibers.

3.3. Hydrophilicity test

As a tissue engineering scaffold, the hydrophilicity of electrospun fibrous mats is an important criterion when evaluating their overall performances. In the present work, the hydrophilicity test was conducted for the aim of investigating the condition of surfactant Span80 distribution on electrospun fibrous mats. Table 1 shows the results of contact angle measurement. The results show that the water contact angles immediately (close to 3 s) reached 0° after the

Table 1
Contact angles measurement of PLACL with and without Span80 incorporation.

Substrate	Contact angle at 1 s (°)	Contact angle at 2 s (°)	Contact angle at 3 s (°)
PLACL fibrous mats	119 ± 5	110 ± 8	104 ± 8
PLACL/Span80 fibrous mats	38 ± 3	9 ± 2	0

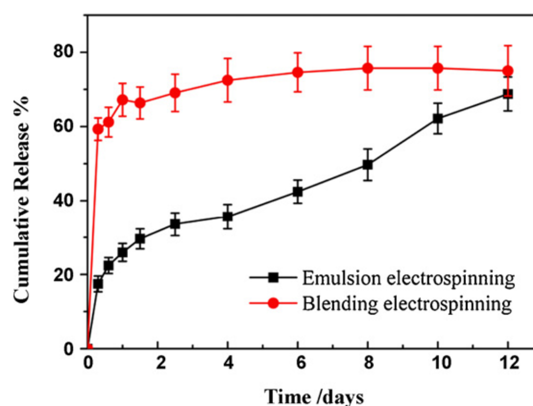

Fig. 3. *In vitro* release study of BSA encapsulated in emulsion electrospun fibrous mats.

water droplets arrived at the PLACL/Span80 fibrous mats. On the other hand, the water droplets on pure PLACL fibrous mats could maintain much larger contact angles for a long time. It is clear that the pure PLACL mats were hydrophobic and PLACL/Span80 fibrous mats were much more hydrophilic. Hence some of the surfactant Span80 molecules distributed on the surface of PLACL/Span80 fibrous mats.

3.4. In vitro protein release study

As show in Fig. 3, BSA released from blending or mix electrospun fibrous mats presents a burst release (about 60–70%) at the initial stage. Thereafter, the release rate was slowed down, and the ultimate proportion of released BSA in 12 days was around 70–80%. Xu et al. [22] have investigated a similar emulsion electrospinning system which was used to generate core–shell structure fibers generated from water in oil type emulsion. In their research, the emulsion electrospinning system was composed of water-soluble drugs and distilled water as the aqueous phase and poly(ethylene glycol-co-lactic acid)/chloroform solution as the oil phase. The report demonstrated that the release behavior of drugs was controlled by the distribution of the drugs. It is easy to understand that drugs located on fibers surfaces were easily dissolved in PBS solution at the initial stage. BSA is one of the well-known proteins which have more charged groups (such as $-NH_2$ and $-COOH$) than PLACL. Therefore, BSA was compelled to move onto the fibers surface by forces of electric.

By contrast, BSA released from emulsion electrospun fibrous mats showed stable and sustained release behavior in Fig. 5. Many researchers [27–29] have reported that emulsion electrospinning could generate the core–shell structure fibers and incorporate drugs and/or proteins in the core part. We believed that the stable and sustained release behavior of BSA in this study was caused by the location of proteins in fibers. To consider the electrospinning as an unstable process, some of the BSA would locate on the fibers surface. Therefore, there was a 10–20% initial burst release stage on the curve of BSA releasing from emulsion electrospun fibrous mats. After the initial stage, the curve presented a sustained and stabled behavior, and the ultimate proportion of released BSA from emulsion electrospun fibrous mats was closed to 70% at the end of 12 days test.

3.5. In vitro bioactivity study

The bioactivity of NGF released from emulsion electrospun fibers was analyzed by observing the differentiation of PC12 cells into neurons, in the presence of the supernatant obtained from the electrospun NGF encapsulated fibers. The differentiation of the

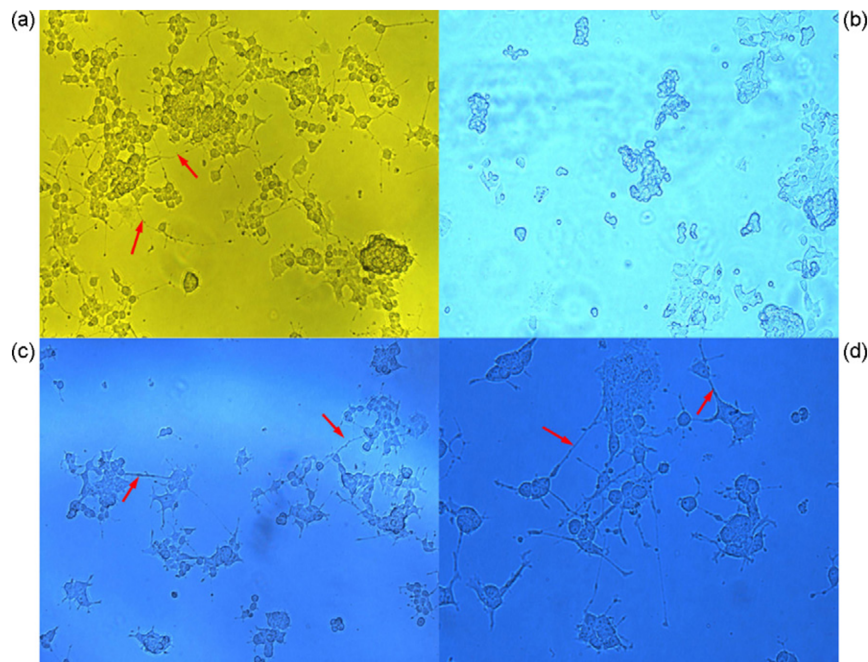

Fig. 4. (a) Positive control of PC12 cells in soluble NGF, (b) negative control of PC12 cells in pure serum-free RPMI medium, (c) PC12 cells after adding the supernatant at the 1st day, 200×, and (d) PC12 cells after adding the supernatant at the 10th day, 400×. The red arrows in (a), (c) and (d) indicate the PC12 cells neurite. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

PC12 cells into neurite in the supernatant and in the controls is shown in Fig. 4. It is likely that there existed denatured NGF, so attempts to quantify the percent bioactivity retained by the NGF released from the fibrous mesh was difficult. Culture-to-culture variability is typical in such cellular assays. Positive controls which had lead PC12 cells to differentiate into neurite were using fresh NGF at a concentration of 200 ng/mL. As shown in Fig. 4(d), the study of adding supernatant obtained from NGF released solution at the 10th day into PC12 cells culture medium showed a positive result. This experiment did indicate that the NGF released from the PLACL fibers retained at least some degree of bioactivity for up to 10 days.

3.6. Forming process of emulsion electrospun fibers

During the emulsion electrospinning in present study, the emulsion droplets moved perpendicularly to achieve their enrichment in the axial region. These emulsion droplets were stretched into elliptical shape in the direction of the fiber trajectory. It is difficult to monitor the whole forming process of electrospinning. Therefore, three points were selected to represent the changes of fibers morphological details at the distances of 0.2–0.5 cm, 2.0–2.5 cm, and 3.0–3.5 cm from the electrospun Taylor cone. Fibers were collected on a glass slide which fleets at certain distances from the electrospinning needle. In Fig. 5(a), it is clear to see that the emulsion was elongated, but the resulting product was a band-like material. Fig. 5(b) and (c) showed some elliptical-shape drops in electrospun fibers. Therefore, we believed that some of the fibers were formed by the elongation of these elliptical drops with the evaporation of solvents and solidification of electrospinning emulsion.

The distribution process of Span80 in the present emulsion electrospun fibers was schematically shown in Fig. 6. During the process of emulsion electrospinning, the polymer jet can be elongated up to 10,000 times in tens of milliseconds, and the emulsion spheres/droplets would be elongated as well along with the polymer jet, which are accompanied by the rapid jet-solidification. The jet-solidification is caused by the evaporation of solvents (such as chloroform and water in this study), which is removed within 0.1 s after bending instability. Since Span80 is a viscous liquid at the room temperature (it is noted that all the experiments were conducted under room temperature), it is expected to have relatively high mobility in the electrospun polymer jet [30]. It is reasonable to believe that Span80 could move from the emulsion spheres to the outer region and even the surfaces of fibers due to the charge repulsion as shown in Fig. 6(b).

The ultimate distribution of Span80 in PLACL/Span80 fibrous mats was shown in Fig. 6(c) and (d). In the present emulsion electrospinning, Span80 was divided into two parts together with the

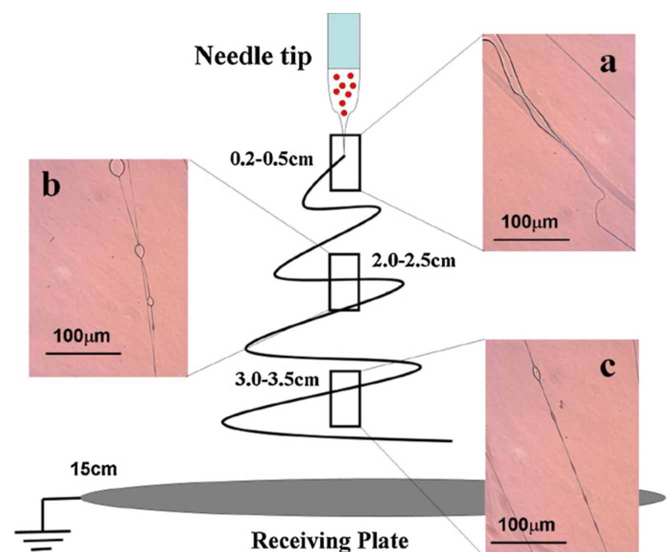

Fig. 5. Optical images of BSA/PLACL fibers collected on glass slide between the nozzle and collecting plate by emulsion electrospinning. These fibers were selected to show the morphological details of forming process during electrospinning. (a–c) The emulsion stretched into fibers at the distance of 1 cm, 2 cm, and 4 cm from the electrospun Taylor cone, respectively.

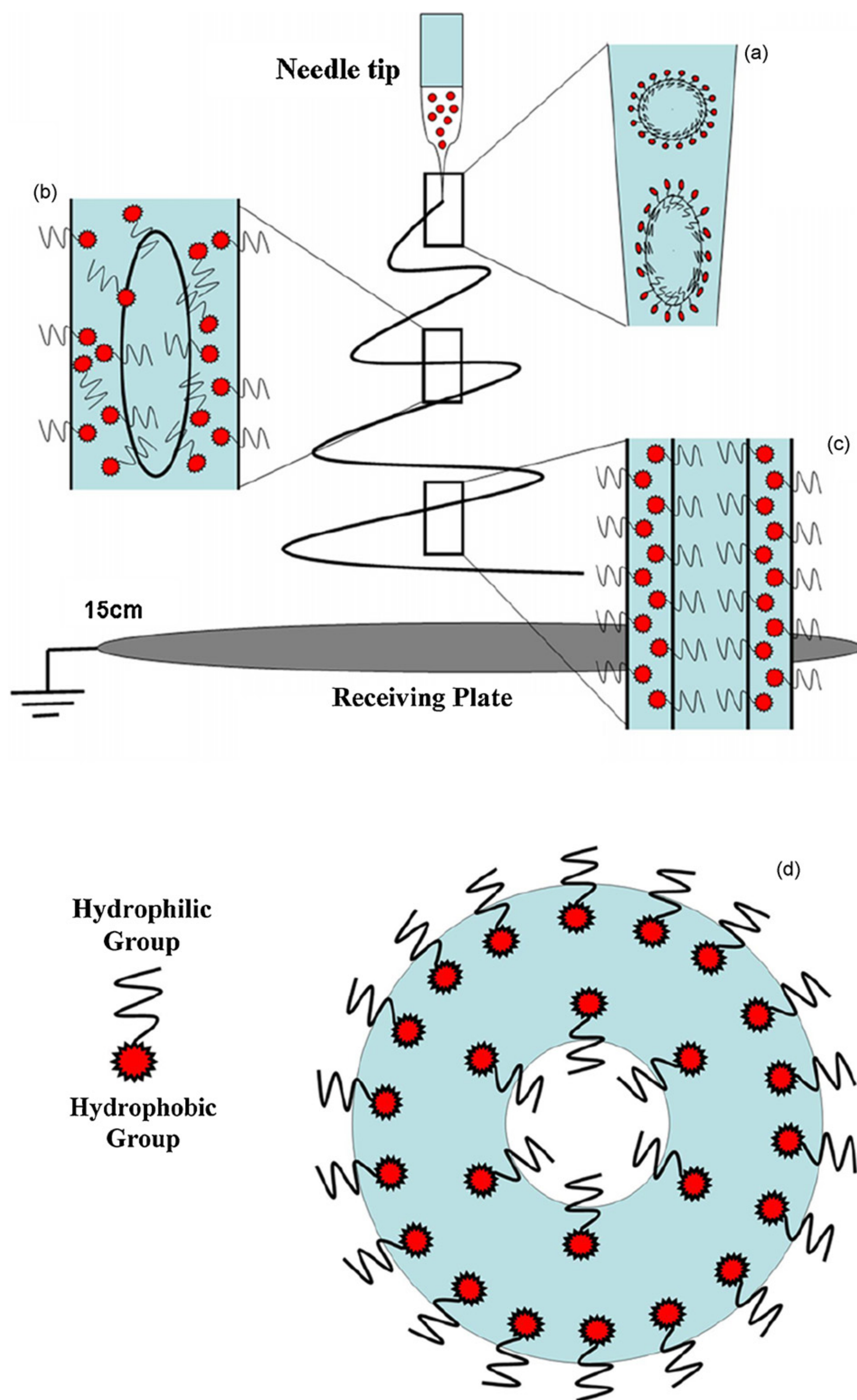

Fig. 6. Scheme of core–shell structure forming process and Span80 distribution in nanofiber cross section: (a) water spheres which stabilized by Span80 go through needle tip under the electric force; (b) some of the Span80 molecules immigrate from the interface of oil and water solution to the surface of nanofiber; (c) Span80 distribution on the longitudinal section of nanofiber; (d) Span80 distribution on the cross section of nanofiber.

polymer PLACL to fabricate the core–shell type fibers. One part was located with protein in the fibers, and the Span80 arrayed with their hydrophobic group in the shell of polymer fibers. The other pare of Span80 was distributed on the surfaces of fibers, with their hydrophilic group in the air.

Moreover, to improve the overall biocompatibility of emulsion electrospun fibrous mats, the incorporation of natural polymer and bioactive proteins to form the core–shell type fibers could be considered. Nonetheless, it is noted that most of the natural polymers could only dissolve into aqueous solutions. For the purpose of using emulsion electrospinning to fabricate core–shell type fibers with natural polymer as the shell, other type of emulsion must be chosen. That is, the oil phase contained bioactive proteins being emulsion spheres, which distribute in aqueous phase with or without surfactant. However, there is a major drawback of natural polymer such as gelatin and chitosan that their mechanical properties are usually

poor. The production of composite fibrous scaffolds generated by heterogeneous natural and synthetic polymers with suitable ratio would provide a desired option for nerve tissue engineering.

4. Conclusion

The objectives of this study were to investigate electrospinning of emulsions for preparation of bio-functional fibrous mats. PLACL fibrous mats electrospun from emulsions could possess unique combined characteristics of cell-growth scaffolds and controllable drug releasing agents. In this study, PLACL/Span80 fibrous mats were successfully prepared by electrospinning of emulsion made of PLACL, chloroform, Span80, PBS, and distilled water. The diameter of the resultant fibers ranged from 600–900 nm. The hydrophilicity test suggested that some Span80 molecules located on the surface of fibers. The release behaviors of NGF from fibrous mats were tested, and the results demonstrated that the method of emulsion electrospinning could generate fibrous mats with the capability of sustained and stable release of proteins. Furthermore, PC12 bioassay study confirmed that NGF maintains its bioactivity after being encapsulated in and released from emulsion electrospun fibers. The study of fiber forming process in emulsion electrospinning proved that the emulsion spheres were elongated along with the polymer solution under the applied electric force.

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