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Preparation and characterization of *Rana chensinensis* skin extract/poly(ε-caprolactone) electrospun membranes as antibacterial fibrous mats

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ABSTRACT: In this study, membranes composed of *Rana chensinensis* skin extracts (RCSEs) and poly(ε-caprolactone) (PCL) were fabricated by an electrospinning technique. The RCSEs were prepared by the extraction of *R. chensinensis* skin with acetic acid solution, and the electrospun membranes were prepared by the mixture of RCSEs and PCL in 1,1,1,3,3,3-hexafluoro-2-propanol before electrospinning. The membranes were characterized by scanning electron microscopy and Fourier transform infrared spectroscopy and were subjected to mechanical tests (tensile and nanoindentation) and antibacterial evaluation. The results indicate that the surface roughness of the fibers clearly decreased with the increase in the amount of PCL in the membranes. The mechanical test indicated that PCL played a dominant role in the mechanical strength of the RCSE/PCL electrospun membranes. As a potential bactericidal packaging material for practical applications, the antibacterial activity results indicate that the membranes had antibacterial effects against *Staphylococcus aureus* and *Escherichia coli*. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42030.

KEYWORDS: biomaterials; electrospinning; fibers; membranes; packaging

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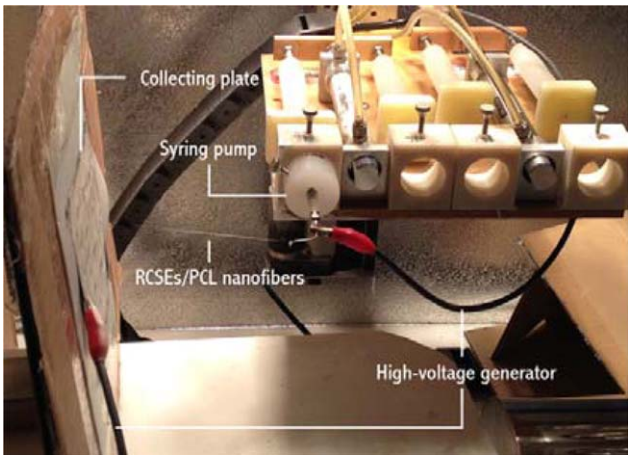
INTRODUCTION

Recently, effective and nontoxic antibacterial materials composed of biological components have attracted much attention because of their excellent properties, including a low toxicity, biodegradability, and antibacterial activity.[1] It was confirmed that antibacterial materials composed of bioactive peptides, chitosan, and hydrolysates of tissues can be used for food storage and medical treatments without any side effects. Moreover, polymeric nanomaterials with antibacterial properties are highly desired in biomedical applications, and many approaches have been reported in the literature for their production.[2–5]

Amphibian skin plays an important role as a defense against attack by pathogens, and it is an important component of the amphibian innate immune system. Extracts from amphibian skins have been structurally characterized for development as therapeutic agents.[6–8] Previous studies have shown that *Rana chensinensis* skin extracts (RCSEs) are mainly composed of peptides and proteins. In 1978, Cevikbas[6] reported that RCSEs exhibited a strong antibacterial activity on a variety of microorganisms. In 2009, six antimicrobial peptide precursors (preprobrevinin–1CEa, preprobrevinin–1CEb, preprotemporin–1CEa, preprotemporin–1CEb, preprotemporin–1CEc, and preprochensinin–1) were identified from *R. chensinensis*. All peptides inhibited the growth of Gram-positive bacteria.[9] In 2012, De-Jing Shang isolated and purified preprochensinin–1 from *R. chensinensis* skin (RCS). Preprochensinin–1 exhibited selective antimicrobial activity against Gram-positive bacteria but had no hemolytic activity against human erythrocytes.[10] In the same year, Wang *et al.*[11] purified, synthesized, and structurally characterized preprotemporin–1CEa. Preprotemporin–1CEa exhibited cytotoxicity to all of the tested cancer cell lines in a concentration-dependent manner. However, preprotemporin–1CEa showed no significant cytotoxicity toward normal cells. Compared to the other antimicrobial agents, the RCSEs had some excellent properties, including a low toxicity, degradability, immune-modulating activity, and good antibacterial properties.[10–13] Moreover, because RCSEs were prepared from the abandoned RCS, the environment was protected by the recycling of the waste of RCS.

An ideal antibacterial material must satisfy certain biological and mechanical requirements. Therefore, membranes have been made with polymer nanofibers by electrospinning for biological



Scheme 1. Preparation of the RCSE/PCL membranes via electrospinning. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

engineering purposes.[14–17] Electrospinning is an effective and versatile technique where electrostatic forces are used to produce microfibers or nanofibers, with diameters ranging from a few micrometers to tens of nanometers.[18] Synthetic polymers, such as poly(vinyl alcohol),[19,20] poly(ε-caprolactone) (PCL),[21] poly(lactic acid), and poly(lactic-*co*-glycolic acid), have been developed as electrospun biomaterials.[22] Apart from synthetic polymers, peptide copolymers and natural proteins have been electrospun into fiber membranes for biomedical applications.[23] Herein, a new type of antibacterial material was developed from RCSEs and PCL by an electrospinning technique.

In general, high-molecular-weight synthetic polymers are easily transformed into desired shapes with good mechanical properties.[24,25] Naturally derived polymers have specific biological interactions, but they possess poor mechanical strength.[16] RCSEs have good antibacterial activity but poor mechanical strength. PCL has good physicochemical properties, and it is currently used in polymeric membranes. Therefore, PCL was used as an additive to improve the mechanical properties of the RCSE/PCL membrane.

In this study, the main aim was to prepare and characterize RCSE/PCL membranes by the electrospinning technique. The effects of the weight ratio of RCSE/PCL on the morphology, mechanical properties, and antibacterial activity were also investigated. An additional aim of this study was to evaluate the potential of such electrospun membranes as an antibacterial packaging material through various tests.

EXPERIMENTAL

Materials

RCS was purchased from a local market in ChangChun, China. RCS was purchased fresh and was frozen for storage. PCL (weight-average molecular weight = 52,000) was supplied by Shenzhen Esun Industrial Investment, Ltd., China. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from Tianjin Heowns Biochem LLC (China). All of the other chemicals were analytical grade and were used as received without further purification.

Preparation of RCSE from RCS

RCS (weight = 500 g) was immersed in an NaCl solution (15% w/v) to remove the impurities; this was followed by soaking in a n NaOH solution (10% w/v) to remove the pigments. The pigments in RCS were removed by soaking for 12 h, and then, the RCS was washed with distilled water and placed in an oven (DZF-1B, Shanghai Laboratory Instrument Work Co., Ltd., China) to remove moisture for 24 h. The dried RCS was then ground to grains (at 1800 rpm for 1 h) with a milling instrument (QM-3SP2, Nanjing University Instrument Factory, China). The grains were added to an acetic acid solution (5% v/v) with stirring for 3 days. The mixture was centrifuged (LD5-2B, Beijing Lab Centrifuge Co., Ltd., China) to separate soluble and insoluble substances (at 5000 rpm for 30 min). The supernatant was freeze-dried (ALPHA1–2, Marin Christ Co., Ltd., Germany) into RCSEs.

Preparation of the RCSE/PCL Membranes

Seven types of blend solution were prepared for the electrospun membranes by the addition of RCSEs and PCL in HFIP with RCSE/PCL ratios of 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, and 0/100 w/w with stirring for 12 h at room temperature. The concentration of each mixture was 12% w/v. The electrospinning machine (FM-1301, Beijing Material Sci-Tech Co., Ltd., China) was equipped with a high-voltage generator, a syringe pump, and a collecting plate, as shown in Scheme 1. The RCSE/PCL mixture was injected with a blunt needle (25 gauge) at a flow rate of 5 mL/h. A positive voltage supply of 20 kV was used in the system. The electrospun fibers were collected on the plate with a distance of 20 cm between the syringe tip and plate. Seven types of electrospun membranes with different RCSE/PCL ratios were obtained by this method.

Morphology

The fiber morphology of the RCSE/PCL electrospun membranes was observed with scanning electron microscopy (SEM; S-3400N, Japan Hitachi Co., Ltd., Japan) at an accelerated voltage of 10 kV after gold coating; five images were taken per sample. With the SEM micrographs, the average diameter and diameter distribution were determined with Nano Measure software (Nano Measurer 1.2, Fudan University, China); three measurements were taken per image.

Fourier Transform Infrared (FTIR) Analysis

An FTIR spectroscopic analysis of the RCSE/PCL electrospun membranes was performed with a Spectrum One instrument (IRPRESTIGE-21, Shimadzu Co., Ltd., Japan). The FTIR spectra of all of the samples were obtained with the KBr disk method in the range 4000–400 cm^{-1} at room temperature, and the resolution was 4 cm^{-1}.[26,27]

Tensile Test

The mechanical tensile properties of the RCSE/PCL electrospun membranes were determined with a materials testing machine (LLY-06ED, Laizhou Electronic Instrument Co., Ltd., China) at room temperature (20°C) and a humidity of 65%. All of the samples were the same size (50 × 5 mm^2) and were tested with a maximum load of 200 cN and a sensitivity of 0.01 cN at a crosshead speed of 5 mm/min. The thickness of each sample was measured with a caliper (103–256, Mitutoyo Co., Ltd.,

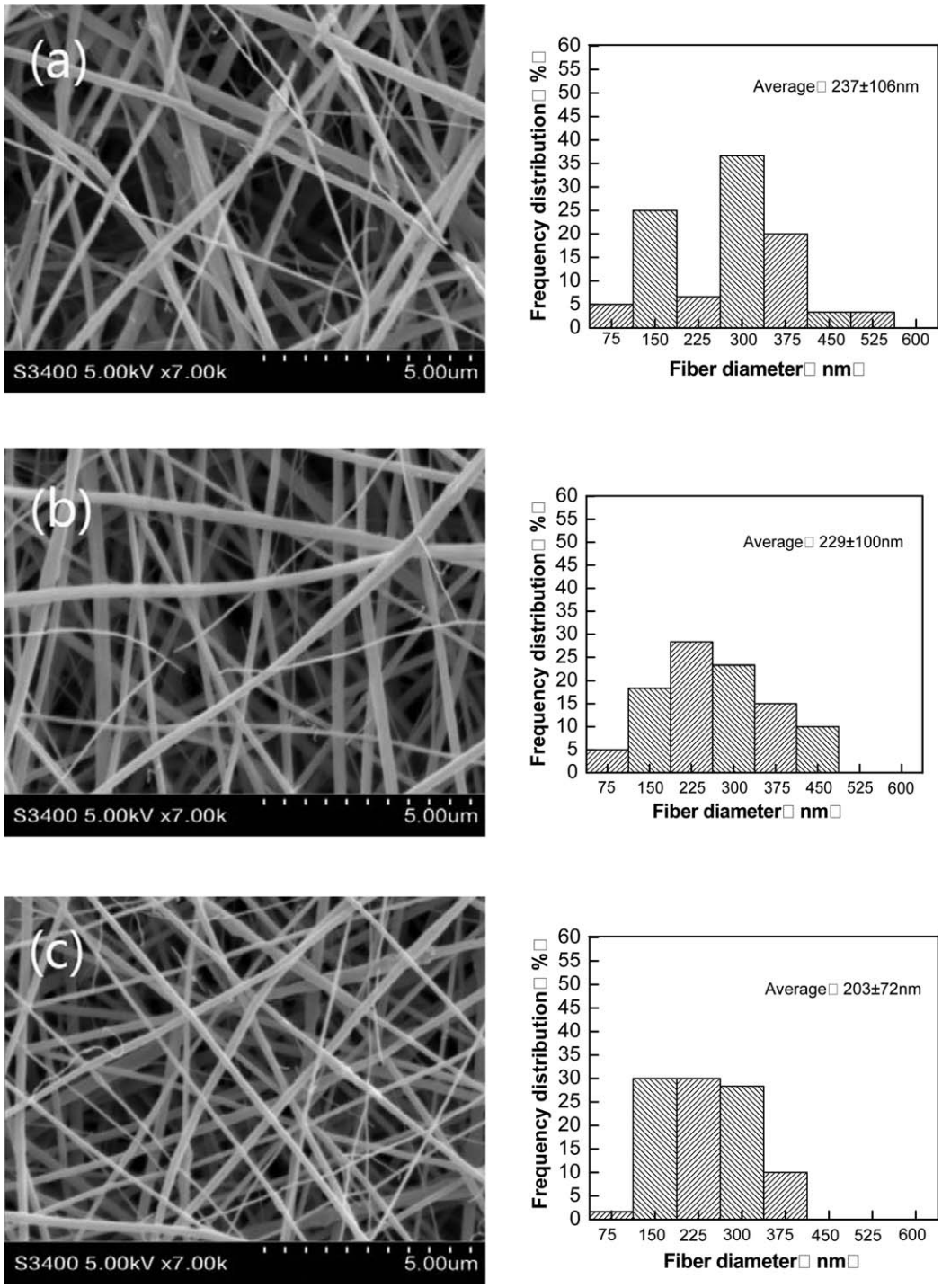


Figure 1. SEM images and fiber diameters of the RCSE/PCL membranes fabricated via electrospinning (electric voltage = 20 kV, flow rate = 5 mL/h, tip–target distance = 20 cm, concentration = 12% w/v): (a) RCSE/PCL = 100 : 0, (b) RCSE/PCL = 90 : 10, (c) RCSE/PCL = 80 : 20, (d) RCSE/PCL = 70 : 30, (e) RCSE/PCL = 60 : 40, (f) RCSE/PCL = 50 : 50, and (g) RCSE/PCL = 0 : 100.

Japan) with a precision of 0.02 mm. Five measurements were taken per sample.

Nanoindentation

Nanoindentation was performed to evaluate the elastic modulus and hardness of the scaffolds[28,29] with an Agilent Nano Indenter G200 (U9820A, Agilent Technologies., Inc.) at room temperature. A conical diamond flat-punch indenter with a diameter of 50 nm was used. Five measurements were conducted per sample as follows: a strain rate of 0.4/s was maintained constantly during the addition of the load until the indenter reached a depth of 2000 nm on the surface of the scaffold. The load was maintained at a maximum value for 10 s. The indenter was then withdrawn from the surface of the scaffold, unloaded to 10% of

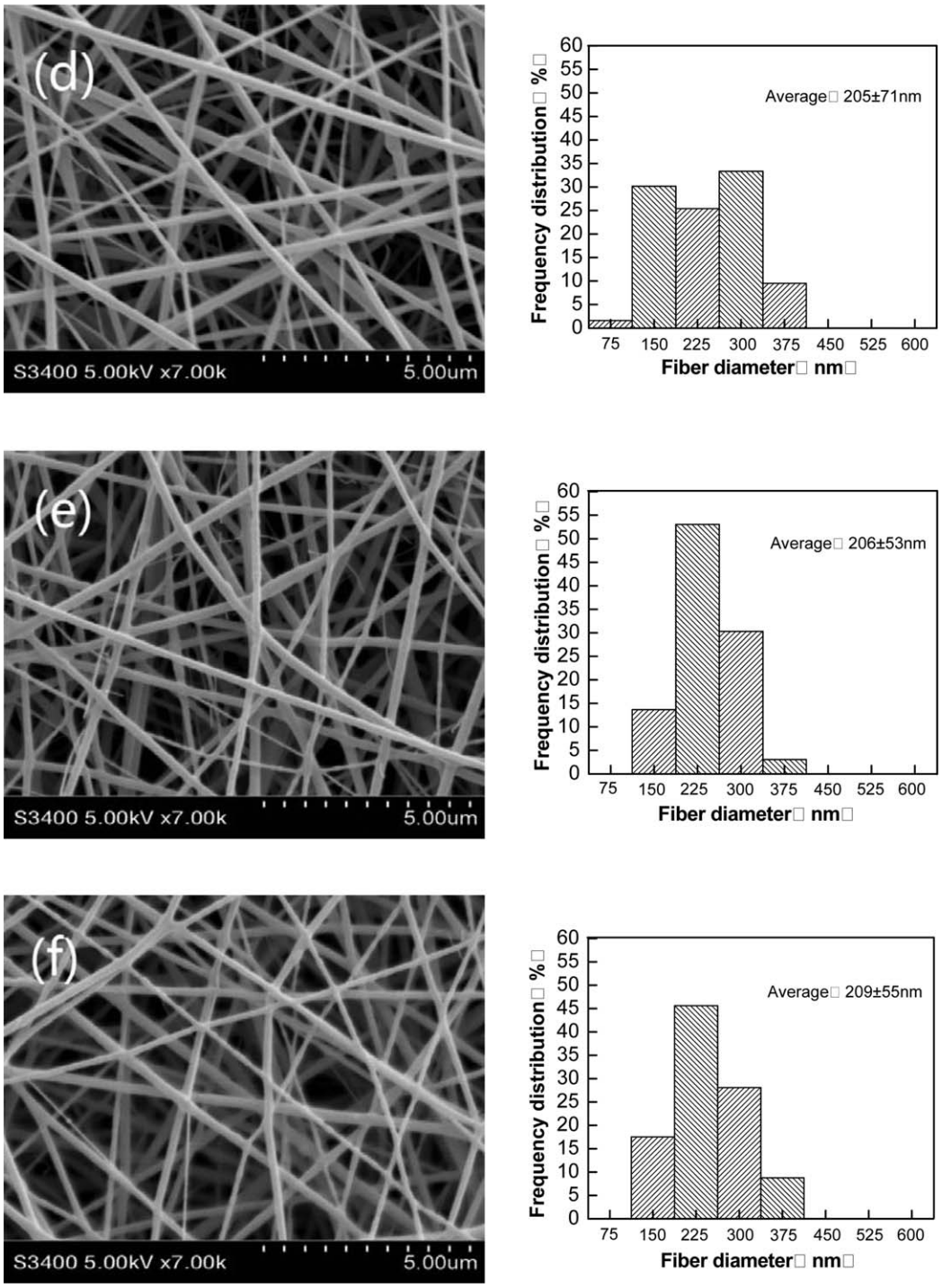


Figure 1. *Continued*

the maximum load. To ensure the reliability of these nanoscale deformation measurements, at least five indents were performed on each sample to obtain effective data.

Antibacterial Activity

To test the antibacterial activity, the antibacterial efficiencies of the RCSE/PCL electrospun membranes were quantitatively evaluated with the viable cell-counting method.[30–32] The Gram-positive bacteria *Staphylococcus aureus* (a suspension of 5×10^7 cfu/mL) and Gram-negative bacteria *Escherichia coli* (a suspension of 5×10^7 cfu/mL) were used as the model organisms. All of the samples (six types of RCSE/PCL membranes and one blank sample) with the same diameter (as disks of 1.5 cm) were placed in a Petri dish. Each sample was coated evenly with 20 μL of the bacteria suspension to make a full contact. After 24 h, each sample was added to 20 mL of distilled water with stirring for 30 min; therefore, the remaining bacteria on the samples were dispersed in the distilled water. We calculated the number

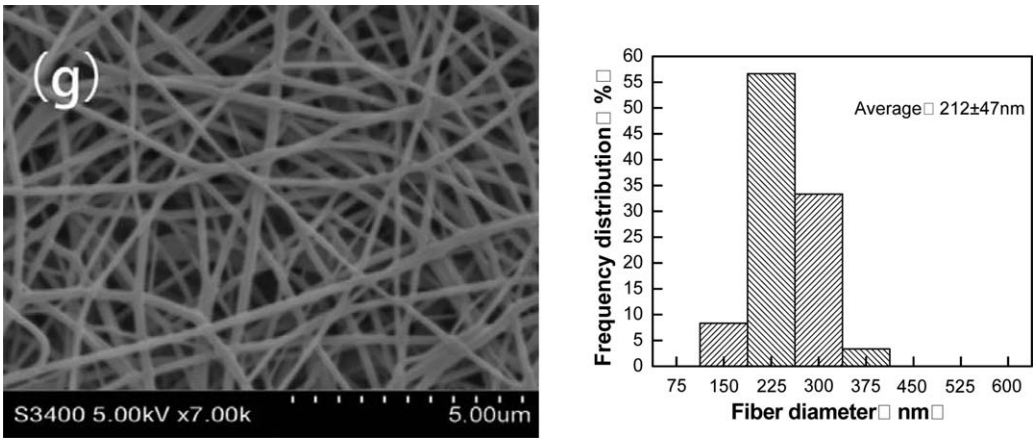


Figure 1. continued

of remaining bacteria by plating 20 μL of distilled water onto lysogeny broth (LB) agar plates and culturing them in an incubator for 24 h. Six measurements were conducted per sample, and all of the operations were conducted in a sterile environment.

Data Analysis and Statistical Methods

All of the average values were reported as the average plus or minus standard deviation. To determine statistical difference when comparing two mean values, we used the Student t test to verify which pair of mean values was statistically different ($p < 0.05$).

RESULTS AND DISCUSSION

Morphological Analysis

Figure 1 shows the SEM micrograph of the RCSE/PCL electrospun membranes under a magnification of 3500×. Figure 1(a–g) shows the SEM images and fiber diameters of the RCSE/PCL membranes fabricated by electrospinning. Figure 2 shows the average diameters of the fibers and fiber diameter distribution.

The figures show that the RCSEs and PCL were distributed uniformly in HFIP. Fractured and irregular fibers were clearly observed when the PCL content in the RCSE/PCL blend was less than 30% w/w, as shown in Figure 1(a–c), but the nanofibers of pure PCL were continuous and uniform, as shown in Figure 1(g). With increasing PCL content in the electrospun scaffold fibers, the amount of surface roughness and irregular fibers clearly decreased, and the fibers became smooth and straight. This showed that the RCSEs and PCL were dissolved in HFIP uniformly without agglomeration, and this phenomenon showed that PCL helped to improve the continuity of the blend and probably affected the mechanical properties of the membranes.

With increasing content of PCL, the fiber diameter distribution of RCSE/PCL remained in a narrow range from 75 to 375 nm, as shown in Figure 1, and the average diameters and standard deviations decreased, as shown in Figure 2. This indicated that the fibers became more uniform; thus, the mechanical properties of the membranes were directly improved. However, on the

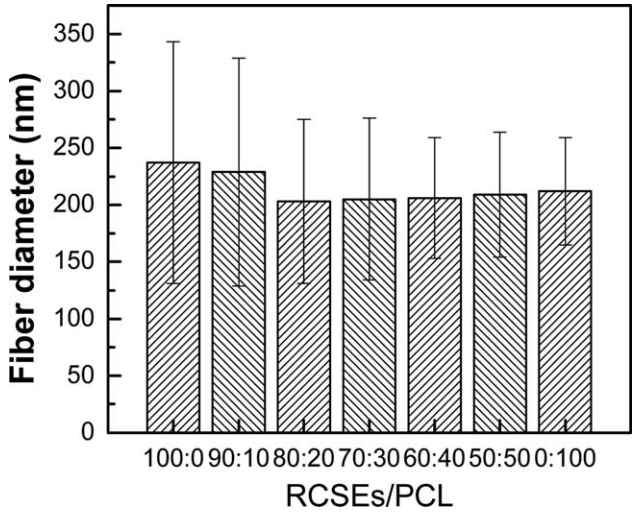


Figure 2. Average diameter of the RCSE/PCL electrospun fibers and fiber diameter distribution.

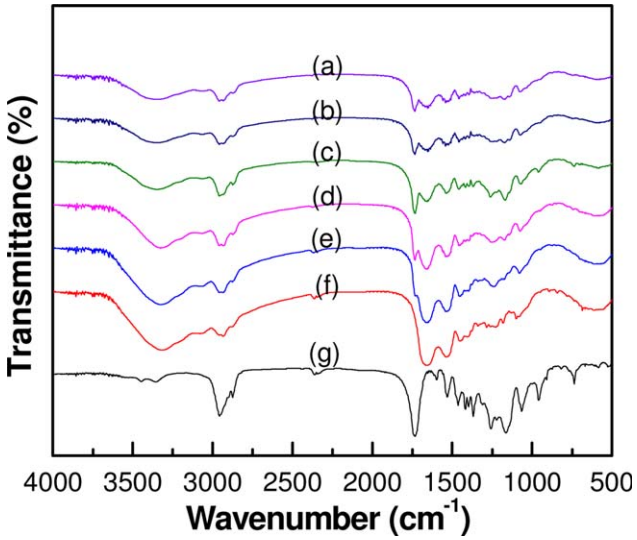


Figure 3. FTIR images of the different formulations: (a) RCSE/PCL = 100 : 0, (b) RCSE/PCL = 90 : 10, (c) RCSE/PCL = 80 : 20, (d) RCSE/PCL = 70 : 30, (e) RCSE/PCL = 60 : 40, (f) RCSE/PCL = 50 : 50, and (g) RCSE/PCL = 0 : 100. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

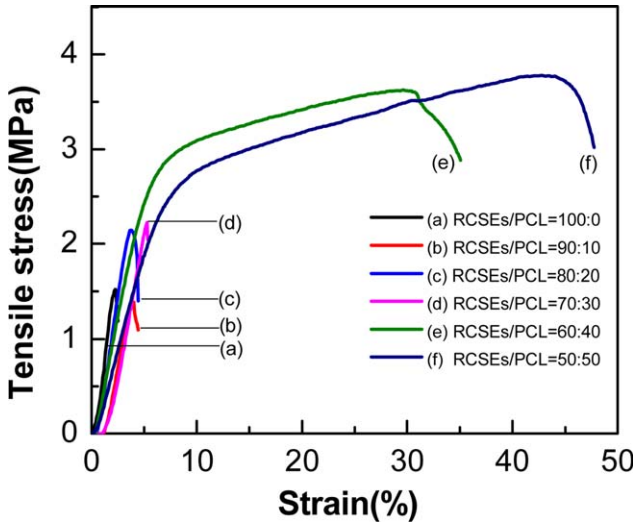


Figure 4. Stress–strain curves of the RCSE/PCL electrospun membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

other hand, a higher amount of RCSEs did, to some extent, improve the bioactivity of the membranes.

FTIR Analysis

The presence of RCSEs and PCL in the electrospun membranes was qualitatively evaluated by the KBr disk method. Figure 3 shows the spectra of the electrospun membranes with RCSE/PCL blend ratios of 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, and 0/100. Stretching vibration bands were observed in the RCSE/PCL membranes. With respect to RCSEs only, the NH stretching vibrations, CH_2 vibrations, C═O vibrations of amide linkages, NH bending vibrations, CH_2 bending vibrations, and C—N vibrations were observed at 3326, 2928, 1735, 1553, 1375, and 1239 cm^{-1}, respectively. The C═O stretching at the 1735-cm^{-1} band was the characteristic of polypeptide secondary structure.[17] Therefore, the C═O vibrations of the blend gradually disappears from Figure 3(a) to 3(f). These spectral band regions were directly related to the polypeptide conformation present in the RCSEs, as it was mainly composed of active peptides and protein.[17] The peaks observed for only PCL at 2930, 2870, and 2821 cm^{-1} were attributed to CH_2 vibrations. The intense sharp peak at 1728 cm^{-1} were attributed to C═O vibrations, and the CH_2 bending vibrations appeared at 1465, 1407, and 1362 cm^{-1}. The COO vibrations appeared at 1248 and 1181 cm^{-1}. The vibrations of the two typical bands shifted with increasing PCL content in the membranes. Thus, the increase in the intensity of the characteristic bands from Figure 3(a–f) was directly related to the addition of PCL.

Tensile Property Assessment

Figure 4 shows the stress–strain curves of the RCSE/PCL electrospun membranes. The tensile strength, elongation at break, and Young's modulus are shown in Figure 5. In this test, the data of pure PCL electrospun scaffold (RCSE/PCL = 0 : 100) was beyond the scope of testing; this showed that PCL had good mechanical performance. Figure 4 shows that the scaffold

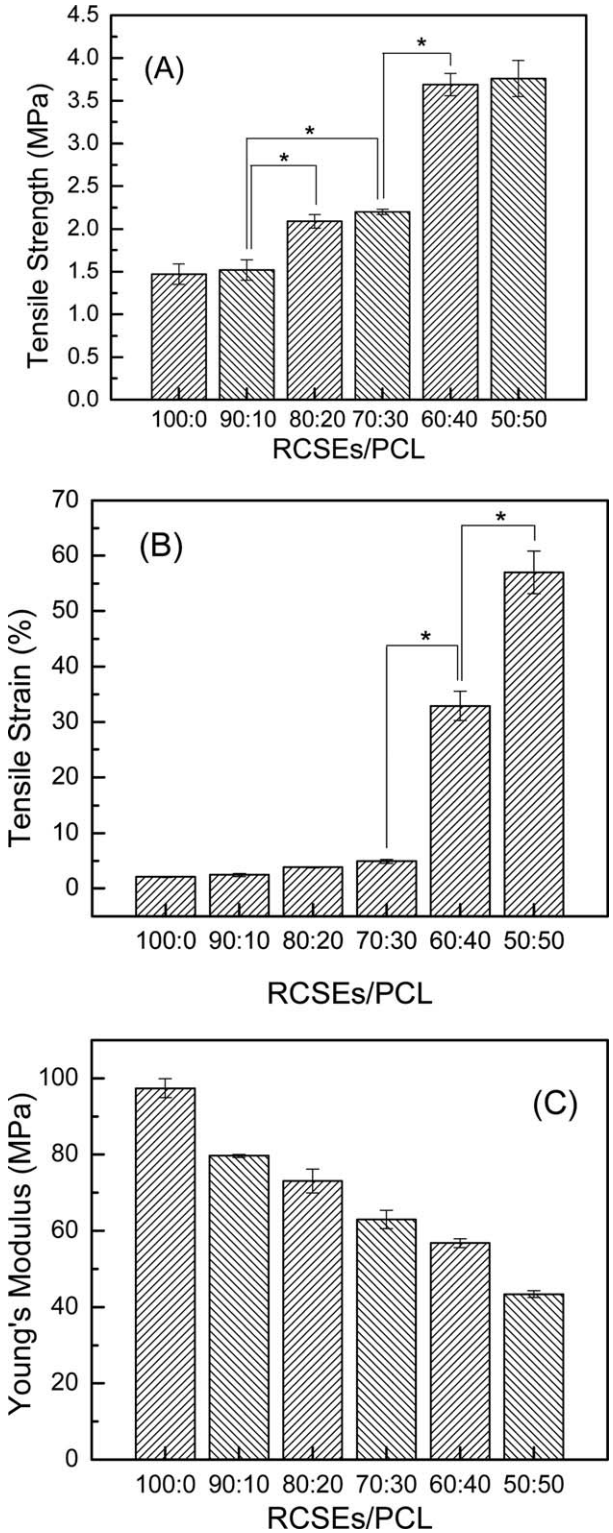


Figure 5. Mechanical tensile properties of the RCSE/PCL electrospun membranes ($*p < 0.05$): (A) ultimate tensile strength, (B) tensile strain, and (C) Young's modulus.

prepared from pure RCSEs (RCSE/PCL = 100 : 0) had lower mechanical tensile properties, as it was mainly composed of protein substances, and a large variation in fiber diameter. The

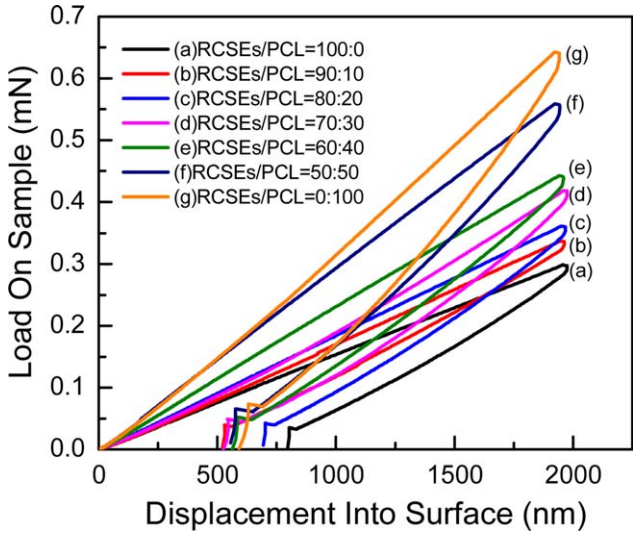


Figure 6. Nanoindentation load–displacement curve for the RCSE/PCL electrospun membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

strength, toughness, and elastic deformation ability of the membranes increased with increasing PCL content in the electrospun fiber membranes, as shown in Figure 5.

This was probably because the addition of PCL made the fibers uniform and continuous, and the mechanical performance of the nanofibers increased. A homogeneous distribution of RCSEs and PCL increased the tensile properties. When the ratios of RCSEs and PCL were 100 : 0, 90 : 10, 80 : 20, and 70 : 30, the elongation at break values increased by 3, 38, and 5%. However, the elongation at break increased significantly ($p < 0.05$) when the ratios reached 60 : 40 and 50 : 50. The membrane with an RCSE/PCL ratio of 60/40 showed a 68% increase in the elongation at break compared to the membrane with an RCSE/PCL ratio of 70/30. The Young's modulus was calculated from the initial slope of the curve; it decreased gradually with increasing PCL content. This indicated that the fiber membranes had good elastic deformation ability.

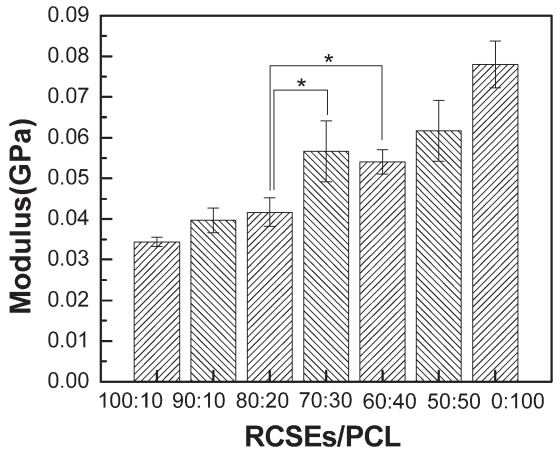


Figure 7. Average modulus values and standard deviations of the RCSE/PCL electrospun membranes (*$p < 0.05$).

As observed, PCL played a dominant role in the mechanical strength of the RCSE/PCL electrospun membranes. From the application perspective, considering the three aspects of strength, elongation at break, and Young's modulus, when the ratio of RCSEs to PCL became lower than 60 : 40, the electrospun membranes showed obvious tensile properties.

Nanoindentation Assessment

The probable improvement of the mechanical properties of the RCSE/PCL electrospun membranes under compressive forces was investigated by a nanoindentation test. Figure 6 shows the representative load–hold–unload of nanoindentation on the electrospun scaffold with RCSE/PCL blend ratios of 100/00, 90/10, 80/20, 70/30, 60/40, 50/50, and 0/100. In this test, the indenter was loaded onto the electrospun membranes with a constant strain rate (0.4 s^{-1}); then, the load was held for 10 s after the indenter penetrated the membranes at a depth of 2000 nm. The unloading rate was set equal to the loading rate. In this process, the load was measured by the increase (or decrease) in the indenter with increasing (or decreasing) contact area between the indenter tip and the fiber sample. The curves shifted upward with increasing PCL concentration. This indicated that the load on the indenter tip increased gradually with the increase in the PCL content.

Figure 7 shows the average modulus and standard deviation of the RCSE/PCL electrospun membranes. In the case of the membrane with 10% w/w PCL (RCSE/PCL = 90 : 10), a 16% increase in the average modulus was observed compared to the pure RCSEs electrospun scaffold (RCSE/PCL = 100 : 0). The average modulus of the membranes increased with increasing PCL concentration. The membrane with an RCSE/PCL ratio of 70/30 showed a 65% increase in the average modulus compared to the pure membrane, and the membranes showed a significant increase ($p < 0.05$) in the average modulus when the PCL content in the RCSE/PCL blend became higher than 30% w/w.

Antibacterial Activity Assessment

The antibacterial activity of the RCSE/PCL electrospun membranes against *S. aureus* and *E. coli* is shown in Figure 8. Figure 8(A–F) shows the remaining number of *S. aureus,* and Figure 8(a–f) shows the remaining number of *E. coli* after full contact with the RCSE/PCL membranes with blend ratios of 100/0, 90/10, 80/20, 70/30, 60/40, and 50/50. Figure 8(G,g) shows the remaining number of bacteria after contact with the blank samples.

The result shows that the number of cells surviving on the electrospun membranes with a higher proportion of RCSEs was less than the others. We observed that the survival numbers of *S. aureus* and *E. coli* cells decreased with increasing amount of RCSEs in the composite membranes. Thus, the antibacterial activity of RCSEs in the composite RCSE/PCL membranes was verified. Moreover, this phenomenon showed that the electrospinning process itself did not change the antibacterial properties of the RCSE/PCL membranes.

More importantly, Figure 9 shows the antibacterial rate of the RCSE/PCL membranes. Clearly, the number of *S. aureus* cells

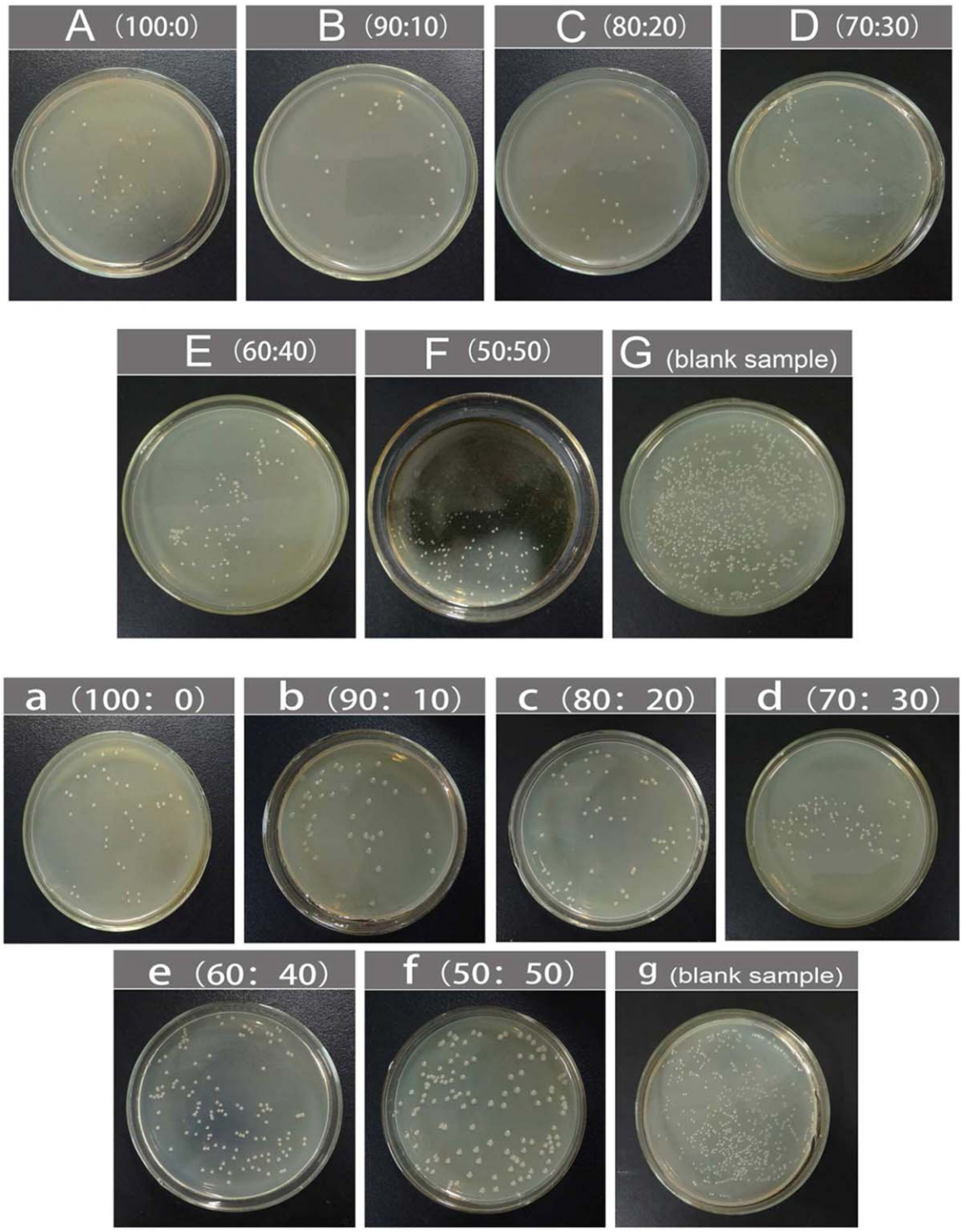


Figure 8. Antibacterial activity of the RCSE/PCL electrospun membranes against (A–G) *S. aureus* and (a–g) *E. coli*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with an initial concentration of 5×10^7 cfu/mL decreased when the cells were exposed to the electrospun membranes. The antibacterial rate was higher than 52% when the PCL content in the RCSE/PCL blend became higher than 50/50 w/w. This showed that the RCSE/PCL electrospun membranes had good antimicrobial activity against Gram-positive bacteria. The number of *E. coli* cells decreased when the cells were exposed to the electrospun membranes.

The antibacterial rate was higher than 41% when the PCL content in the RCSE/PCL blend was higher than 30% w/w. The antibacterial rate increased gradually with increasing PCL

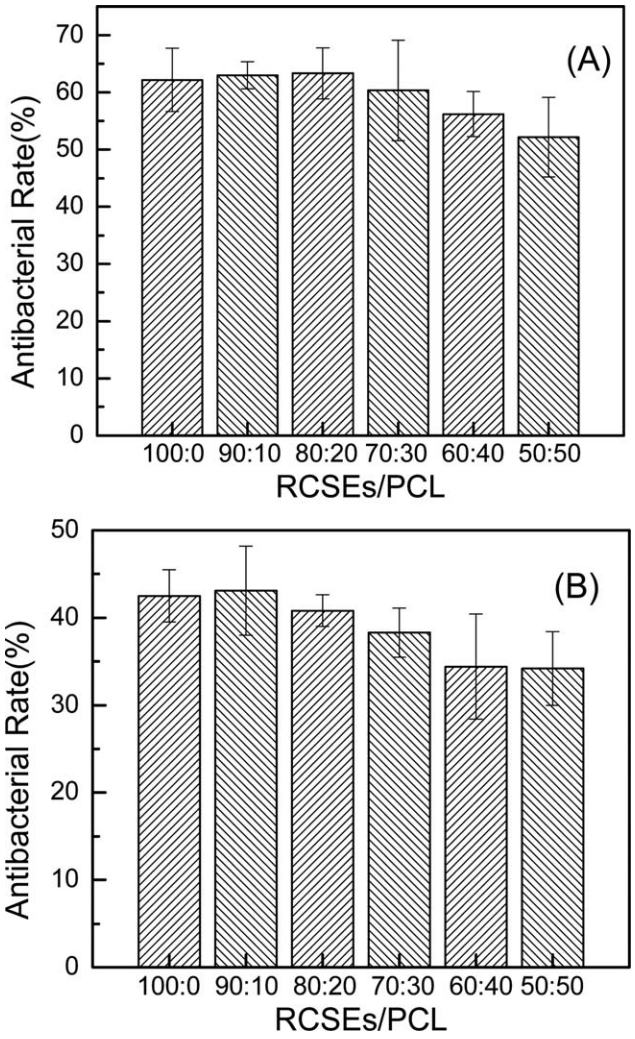


Figure 9. Antibacterial rate of the RCSE/PCL membranes with blend ratios of 100/0, 90/10, 80/20, 70/30, 60/40, and 50/50: (A) *S. aureus* and (B) *E. coli.*

content in the electrospun scaffold fibers. The result indicates that the antibacterial activity of the RCSE/PCL electrospun membranes was associated with the content of RCSEs. The RCSE/PCL electrospun membranes with RCSE contents in the RCSE/PCL blend showed antimicrobial activity against *E. coli.*

CONCLUSIONS

In this study, we successfully fabricated composite membranes composed of RCSEs and PCL by electrospinning. The SEM images showed that the membrane of the pure RCSEs had a rough surface and contained irregular fibers. These drawbacks were minimized with increasing PCL content in the membranes. The mechanical tests, including mechanical tensile evaluation and nanoindentation tests, showed that the tensile strength increased significantly when the RCSE/PCL ratio reached 60 : 40 and 50 : 50, and the hardness and elastic modulus of the membranes increased gradually when the PCL content was increased. Furthermore, the antibacterial experiment indicated that the RCSE/PCL electrospun membranes had good antibacterial activity against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli.* The antibacterial activity of the membranes was dependent on the content of RCSEs. The antibacterial rate increased gradually with increasing PCL content in the electrospun scaffold fibers. The result indicates that the antibacterial activity of the RCSE/PCL electrospun membranes was associated with the content of RCSEs. However, the presence of PCL in the RCSE/PCL membranes improved the mechanical properties, and the presence of RCSEs improved the antibacterial activity of the membranes. Thus, these membranes could serve as a good food storage and packaging material.

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