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전기방사를 사용한 항균성 컬큐민 함유 Zein 나노섬유의 제조

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Fabrication of Electrospun Antibacterial Curcumin-loaded Zein Nanofibers

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Abstract: Electrospinning was used to load curcumin (a natural compound that has antiinflammatory properties) into zein nanofibers. An emulsifier, Tween 80, was combined with curcumin in the zein nanofibers. The morphology of the curcumin-loaded zein nanofibers (CLZNFs) was observed using field emission scanning electron microscopy. Investigation of curcumin released from the zein nanofibers into phosphate buffer saline at pH 7 indicated that the Tween 80 had increased the amount of curcumin released from the CLZNFs. The antibacterial activity of the CLZNFs against *Staphylococcus aureus* (*S. aureus*) was determined by measuring the optical density of bacterial solutions containing CLZNFs. The zein nanofibers fabricated with 10 wt% surfactant and 1.6 wt% curcumin showed high (*i.e.*, 83%) efficiency in inhibiting the growth of *S. aureus* in the solution incubated for 21 h. These results suggest that the electrospun CLZNFs show potential application as antibacterial nonwoven mats.

Keywords: electrospinning, curcumin, Tween 80, zein, antibacterial property.

Introduction

Curcumin, a polyphenol, is an active principle in turmeric (*Curcuma longa* L.) that imparts the yellowish pigmentation to the plant. It has been widely used to protect skin by quenching free radicals thereby reducing inflammation and oxidative stress, and by increasing fibroblast and vascular density in wounds thereby improving wound healing and reducing wound-healing times.[1] Many interesting pharmacological activities such as antitumoral, antiinflammatory, antiviral, antioxidant,[2,3] wound healing activities[4] and low toxicity[2,3] have recently been reported for curcumin. Nevertheless, the use of curcumin is limited because of its low solubility in water under acidic or neutral conditions. In addition, it exhibits a high decomposition rate in alkaline media and photodegradation in organic solvents.[5] Many attempts have previously been made to improve the stability and bioavailability of curcumin, such as fabricating curcumin-eluting stents with biodegradable polymer coatings,[6] fabricating curcumin-eluting polylactic acid-*co*-glycolic acid films,[7] encapsulating curcumin in oil-in-water (o/w) nanoemulsions[8] or in biodegradable polymeric micelles,[9,10] and using Tween 20 as an emulsifier to incorporate curcumin into nonwoven fibrous chitin sheets.[5] According to the attempts, one way to improve the bioavailability of curcumin is to enhance the surface area.

Electrospinning (ES) is a simple and effective process for producing nanofibers with diameter range from a few nanometers to several micrometers which have high specific surface area. In this process, high voltage is applied to a capillary containing a polymer solution or a melt in order to induce the formation of a jet of charged liquid. The solvent rapidly evaporates while the jets of polymer solution move toward a collector, and a mat consisting of nanofibers is deposited onto the collector.[11-13] Hence, medicated nanofibers can be readily fabricated using a solution containing a mixture of a drug and a polymer.[14] Due to the high specific surface area, the ease of loading therapeutic compounds into the nanofibers and the

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simplicity of the process, the electrospun nanofibers make themself attractive for use in drug delivery systems such as tissue engineering and functional wound dressing.[15-17] In addition, polymer-nanofiber-based drug carriers have been shown to improve the therapeutic effect, reduce the toxicity,[18,19] and enhance the solubility of drugs.[20] Zein is a promising biopolymer for application as a drug-carrier because it is hydrophobic, nontoxic, biocompatible, renewable, and biodegradable. Zein is the major storage protein of corn, which comprises about 45-50% of its protein content.[21] It is one of the most hydrophobic proteins because its main constituents are proline and glutamine, which are apolar amino acids.[22] Besides, zein can be dissolved in aqueous polar organic solvents such as non-toxic aqueous ethanol. Because of its solubility and abundance, zein has been widely used as a coating/excipient for foods and drugs. Electrospun nonwoven zein mats have previously been investigated for application as novel scaffolds and drug carriers.[21-23] Recently, J.-M. Yang *et al.* produced zein nanofiber with poor water soluble drug, ferulic acid (FA), via core-shell electrospinning and found that the FA release 100% from composite zein nanofibers in 14 h.[24] Brahatheeswaran *et al.*[25] have incorporated curcumin into a zein nanofiber mats by using trifluoroethanol. They found that curcumin incorporated into zein matrix can maintain its biological activity and sustain its release.[25]

Surfactants have been exploited in a wide range of potential application because their driving force to adsorb at an interface is to reduce surface tension of the solution in which they are dissolved. One of the most potential applications is that surfactants can be added to solutions to improve the solubility of hydrophobic active compounds such as curcumin since surfactants tend to form aggregates, so-called “micelles”.[26] The concentration at which micelles start to form is called critical micellar concentration or CMC.[26] These micellar solutions can serve as vesicles to solubilize lipophilic functional ingredients thereby further enhancing the functionality of nanofibers.[27] For example, Ratanajiajaroen *et al*. reported that Tween 20 had increased the water solubility and release ability of curcumin from β-chitin sheets.[5] Besides, J. Zeng *et al*. showed that addition of small amounts of surfactants to polymer solutions can reduce the diameter and narrow the diameter distribution of electrospun fibers.[18] T. Lin *et al.* also prepared polystyrene nanofibers containing surfactants and observed that surfactants can stop bead formation and lead to thinner fibers.[28]

In this study, electrospun CLZNFs were fabricated for application in antibacterial nonwoven zein mats. Surfactant Tween 80 was used to encapsulate the curcumin into micelles in order to increase the bioavailability of the curcumin when it was released into phosphate-buffered saline (PBS). The morphology of the zein nanofibers fabricated with different percent of Tween 80 and loaded with various amounts of curcumin was observed using field emission scanning electron microscopy (FE-SEM). The amount of curcumin released from the electrospun CLZNFs was measured using ultraviolet (UV) spectroscopy. The antibacterial activity of the CLZNFs against *Staphylococcus aureus* (*S. aureus*) was evaluated *in vitro* by measuring the optical density of bacterial solutions containing unloaded and CLZNFs for potential application as antibacterial nonwoven zein mats.

Experimental

Materials. Zein from corn (grade Z3625, 22-24 kDa) was purchased from Sigma-Aldrich (U.S.A). Curcumin powder was supplied by the Vietnam Institute of Industrial Chemistry (Hanoi, Vietnam). Tween 80 was purchased from Samchun Chemical Co. (Korea). Distilled water was used to prepare all the solutions. All the chemicals were used without further purification.

Fabrication of Curcumin-loaded Zein Nanofibers. A 25-wt% solution of zein was dissolved in a mixture of 75 wt% ethanol and 25 wt% acetic acid, which was then used for electrospinning. A solution of curcumin/Tween 80/zein was prepared by dissolving curcumin (0.4, 0.8, 1.2 and 1.6 wt%, based on the weight of the zein) in 7.5 g ethanol/acetic acid, followed by adding Tween 80 (2, 5, and 10 wt%, based on the weight of the zein) with a vigorous stirring until a transparent solution was obtained. And then 2.5 g of zein powder was added to the mixed solution of Tween 80 and curcumin. The lowest concentration of Tween 80 in the solutions containing zein and curcumin was 5×10^{-3} M, which is above the CMC of Tween 80, 0.012×10^{-3} M. The solution mixtures were homogenized using a magnetic stirrer. A control solution containing only 25 wt% pure zein was prepared using the same method.

The solutions were introduced into a standard 5 mL plastic syringe attached to a blunt 22-gauge stainless steel hypodermic needle, which was connected to a high-voltage supply (Chungpa EMI, Korea). A syringe pump (KD scientific, Model KDS200, U.S.A) was used to control the flow rate of the solution. Electrospun nanofibers were collected on a rotating collector covered with an aluminum sheet. All the electrospinning processes were performed at +15 kV, an 11 cm needle-tip-to-collector distance, and an 8 μL/min solution flow rate.

Characterizations

Morphology Characterization. The viscosity of the polymer solution was obtained with a Brookfield DV-II+ viscosmeter (USA). The surface tension of the polymer solution was measured using a surface tension meter (Quantachrome Sigma 70, U.S.A).

The morphology of the electrospun CLZNFs was characterized using field emission scanning electron microscopy (FE-SEM) (HITACHI S-4700, Japan). Visualization software (TOMORO ScopeEye 3.6, U.S.A) was used to determine the average diameter and distribution of the nanofibers from the FE-SEM photographs.

Measurement of Amount of Curcumin Released from Zein Nanofiber Mats. The curcumin was released from the CLZNFs into PBS pH 7. A certain amount of each sample (25 mg) was immersed in 5 mL of the buffer solutions and incubated at a physiological temperature of 37 ℃ with shaking rate of 120 rpm. At a specific immersion period, 1.0 mL of the sample solution was dipped out and then an equal amount of fresh buffer was added back into the vial. The absorbance of curcumin release was determined using UV spectrophotometer (UV-1601, Shimadzu, Japan) at a wavelength of 425 nm. The amount of curcumin released from the CLZNFs was calculated based on a calibration curve for curcumin in PBS containing 2% v/v Tween 80, and 4% v/v ethanol (*i.e.*, B/E/T). The B/E/T solution was used as the medium to determine the calibration curve because the solubility of curcumin in PBS is limited. The experiments were carried out in triplicate and the results were reported as average values.

The actual amount of curcumin released from the CLZNFs was determined as follows: Each 25 mg test sample was dissolved in 2.5 mL of 2:1 v/v ethanol/Tween 80, and 0.6 mL of the solution was added to 9.4 mL of PBS. The actual amount of curcumin released was measured using a UV spectrophotometer (UV-1601, Shimadzu, Japan) at a 425 nm wavelength. The actual amount of curcumin in the CLZNFs was back-calculated using the same method. The amount of curcumin released was calculated using the following eq. (1):[5]

$$\text{Release of curcumin (\%)} = \frac{\text{Released curcumin}_{t=t}}{\text{Actual amount of curcumin}_{t=0}} \times 100 \tag{1}$$

where t represents a specific immersion time.

Antibacterial Test. The optical densities of bacterial solutions were used to evaluate the antibacterial activity of the CLZNFs against the common bacterium *Staphylococus aureus* (Gram positive; ATCC 6538; *S. aureus*).[28] The bacterial suspensions were prepared as follows: 100 μL of bacterial solution containing 10^8 colony-forming units per milliliter (CFU/mL) was diluted to 10^5 (CFU/mL) in Difco™ nutrient broth solution. A 50 mg fragment of the CLZNF mats was introduced into 5 mL of the diluted bacterial solution. The mixtures were cultured at 37 ℃ in a shaking incubator for 21 h. The turbidity of the medium, which represents the growth of the bacterial cells, was measured using a spectrophotometer (SpectraMax Plus 384, Molecular Devices, U.S.A) at a 600 nm wavelength four times during incubation. The antibacterial efficiency of the CLZNFs was then calculated from eq. (2):[29]

$$\text{Antibacterial efficiency(\%)} = \left(1 - \frac{OD_2}{OD_1}\right) \times 100 \tag{2}$$

where OD_1 and OD_2 represent the optical densities of the bacterial solutions containing unloaded and CLZNFs, respectively, for certain incubation times.

Results and Discussion

Morphology of Electrospun Nanofibers. Prior to electrospinning, both the pure and the curcumin-loaded solutions were measured for their viscosity and the surface tension, and the results are summarized in Table 1. The presence of curcumin in the zein solution led to change on solution viscosity from 161 to 184 cp. Meanwhile, the addition of the surfactant had only a little effect on solution viscosity. Upon addition of the surfactant, the change in surface tension was observed. The surface tension of all solutions with the surfactant was slightly lower than that of solutions without it. The presence of curcumin had little influence on the surface tension. Electrospinning of these solutions was performed at a fixed electric field of 15 kV/ 11 cm. The morphologies of the electrospun unloaded and CLZNFs are shown in Figures 1 and 2, respectively. The electrospun zein nanofibers are shaped like a ribbon and are several hundreds of nanometers in diameter, which is consistent with the results previously published in the Torres-Giner report.[21] Figure 1(a) and 1(b) show that the average diameters of the electrospun unloaded and CLZNFs fabricated without Tween 80 are ~543±248 nm and ~668±216 nm, respectively, indicating that incorporating curcumin into the zein nanofibers could increase their average diameter. This is

Table 1. Viscosity and Surface Tension Data of Pure and Curcumin-loaded Zein Solutions as well as Diameters of Electrospun Unloaded and Curcumin-loaded Zein Nanofibers

Type of zein solution	Viscosity (cP)	Surface tension (mN/m)	Fiber diameters (nm)
Pure zein	161	23.79	543±248
With 0.8 wt% curcumin	184	23.87	668±216
With 0.8 wt% curcumin and 2 wt% Tween 80	177	22.04	475±174
With 0.8 wt% curcumin and 5 wt% Tween 80	175	22.48	449±141
With 0.8 wt% curcumin and 10 wt% Tween 80	176	22.43	480±133
With 0.4 wt% curcumin and 10 wt% Tween 80	178	22.53	488±123
With 1.2 wt% curcumin and 10 wt% Tween 80	178	22.57	438±119
With 1.6 wt% curcumin and 10 wt% Tween 80	180	22.99	462±130

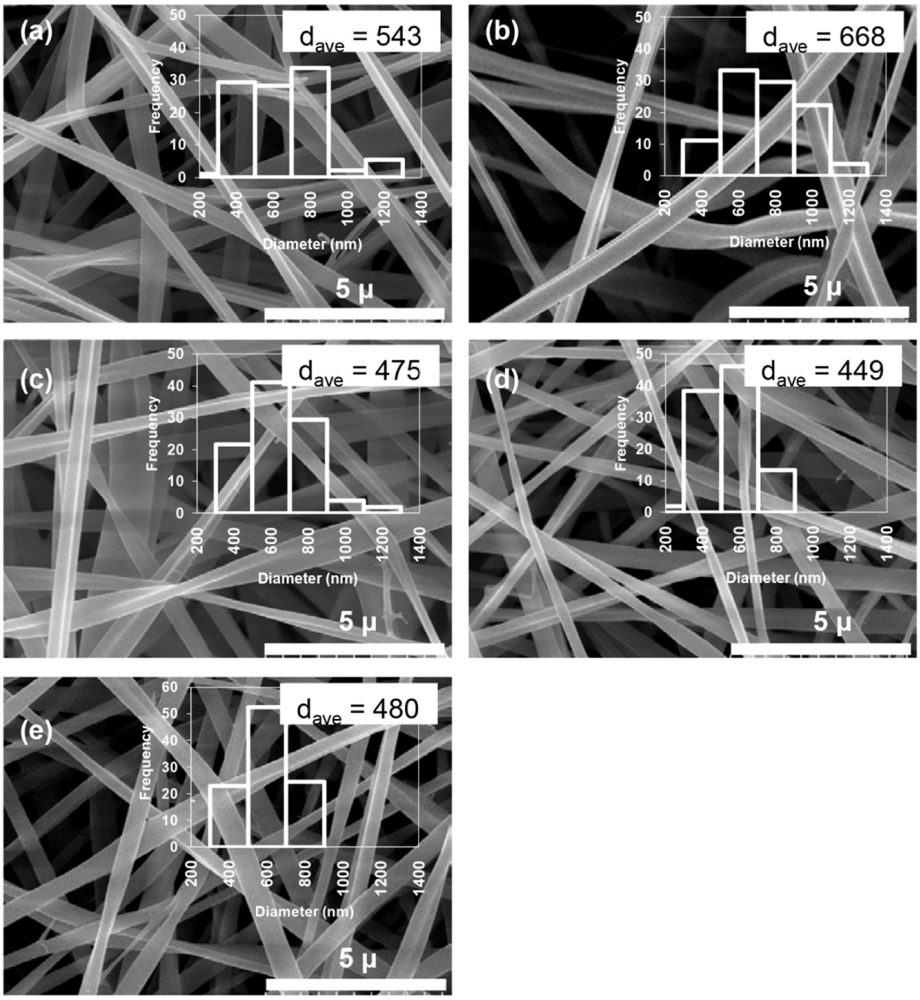

Figure 1. FE-SEM images, average diameter (d_{ave}) and diameter distribution of (a) electrospun unloaded zein nanofibers and (b-e) electrospun curcumin-loaded zein nanofibers fabricated with 0.8 wt% curcumin and various contents of Tween 80 of (b) 0 wt%; (c) 2 wt%; (d) 5 wt%; (e) 10 wt% (respect to the weight of the zein). The polymer concentration was 25 wt% in a mixture of 75 wt% ethanol and 25 wt% acetic acid.

probably due to the change in viscosity of the solution caused by the addition of curcumin. The results obtained in this work are similar to the phenomenon that Y. Li *et al*. previously reported.[30] They showed that zein nanofibers electrospun with and without (-)-epigallocatechin gallate (EGCG), a green tea polyphenol, showed similar ribbon-like morphologies and that the fibers containing EGCG tended to be wider than those that did not. Brahatheeswaran *et al*. showed that the present of curcumin slightly increase in the diameter of the zein nanofibers.[25] However, the average diameter of CLZNFs in Brahatheeswaran's report[25] was affected by concentration of curcumin in the polymer solutions rather than by the addition of the surfactant. The average diameter of CLZNFs fabricated with Tween 80 ranged between ~449±141 nm and ~480±133 nm regardless of the actual content of Tween 80 in the nanofibers, while the average diameter of the CLZNFs fabricated without Tween 80 was ~668±216 nm, indicating that the Tween 80 had reduced the diameter of the electrospun CLZNFs and had narrowed the distribution of the nanofiber diameters. Adding a nonionic surfactant such as Tween 80 to a polymer solution slightly lowers the surface tension of the polymer solution, which may explain this finding and is consistent with the findings that J. Zeng *et al*. reported.[18] C. Kriegel *et al.* supposed that the addition of nonionic surfactant led hydrophobic interaction between surfactant and polymer, thus quite lowered the surface tension.[27]

Figure 2 shows the effect of the content of curcumin on the morphology of the electrospun zein nanofibers fabricated from zein solutions containing 10 wt% Tween 80. The average diameters of the CLZNFs varied between ~438±119 nm and ~488±123 nm with no particular dependency on the initial content of curcumin. It may be assumed that the Tween 80 had affected the morphology of the zein nanofibers much more than the quantity of curcumin in the nanofibers had.

Measurement of amount of curcumin released from zein nanofibers.

Effect of Tween 80 on Amount of Curcumin Released from Curcumin-loaded Zein Nanofibers. Figure 3 shows the effect of the content of Tween 80 on the amount of curcumin released into the PBS from the CLZNFs containing 0.8 wt% curcumin. A burst of curcumin was released from the zein nanofibers immersed in PBS for 2 h. After 8 h, the release amounts of all the samples reached maximum values. About 2.5% of the curcumin were released from the zein nanofibers fabricated without Tween 80 at 72 h of immersion, while 7.2, 9.0, and 11.5% of the curcumin was released from the zein nanofibers fabricated with 2, 5, and 10 wt% Tween 80, respectively for the same immersion time. More curcumin was released from the zein nanofibers fabricated with Tween 80 than those fabricated without it, suggesting that the Tween 80 had encapsulated the curcumin into micelles, leading to higher release of curcumin into buffer solution. Thus, it could be explained that although the increase of surfactant content does not decrease the diameter of CLZNFs, the highest released amounts of curcumin were increased. Releasing of curcumin

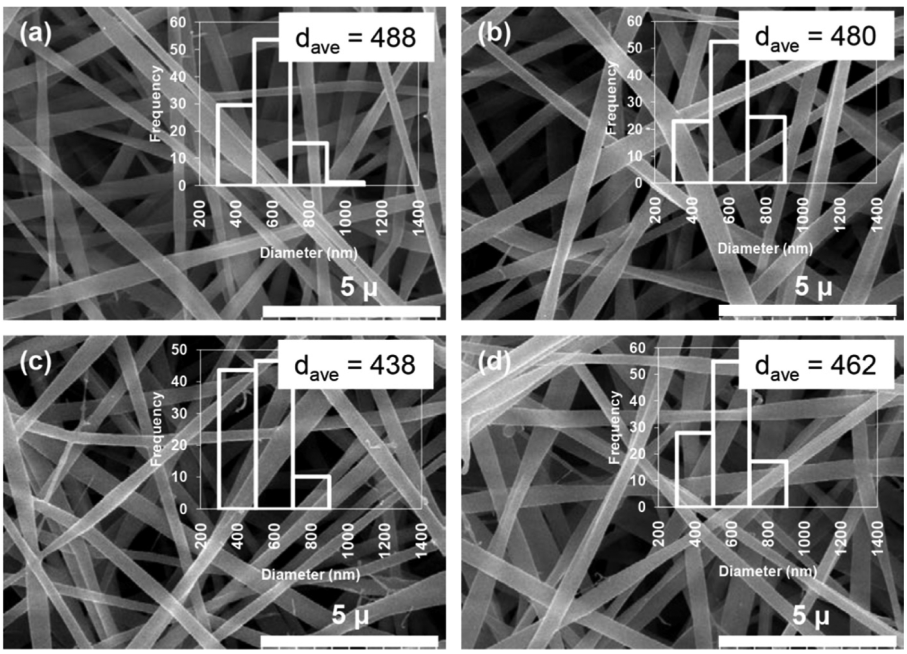

Figure 2. FE-SEM images, average diameter (d_{ave}) and diameter distribution of electrospun curcumin-loaded zein nanofibers fabricated with 10 wt% surfactant and different curcumin content of (a) 0.4 wt%; (b) 0.8 wt%; (c) 1.2 wt%; (d) 1.6 wt% (respect to the weight of the zein). The polymer concentration was 25 wt% in a mixture of 75 wt% ethanol and 25 wt% acetic acid.

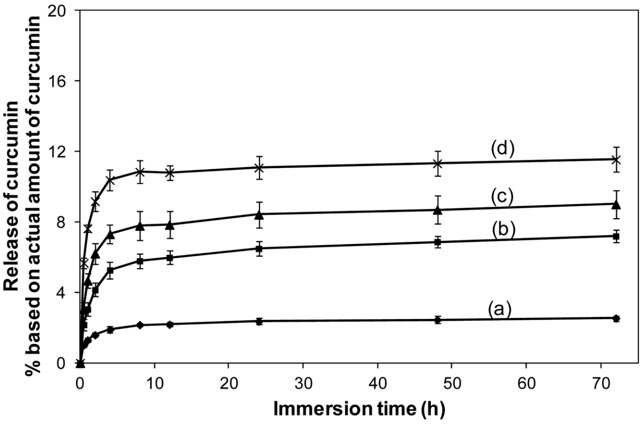

Figure 3. Effect of Tween 80 content on amount of curcumin released from curcumin-loaded zein nanofibers fabricated with 0.8 wt% curcumin and various contents of Tween 80 of (a) 0 wt%; (b) 2 wt%; (c) 5 wt%; (d) 10 wt% (respect to the weight of the zein).

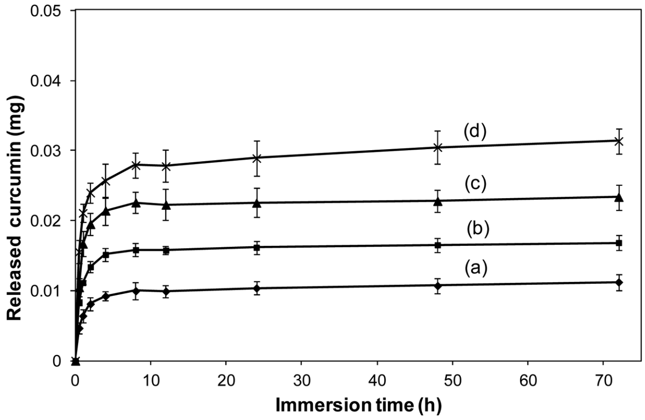

Figure 5. Effect of curcumin content on amount of curcumin released from curcumin loaded zein nanofibers fabricated with 10 wt% Tween 80 and different curcumin contents of (a) 0.4 wt%; (b) 0.8 wt%; (c) 1.2 wt%; (d) 1.6 wt% (respect to the weight of the zein).

from the nanofibers was quite low since zein was resistant to degradation on the PBS release medium, which was reported by Hurtado-López and Murdan.[31] Morphology of the electrospun fibers after immersion observed in Figure 4 agreed well with the previous report. Figure 4 shows that incubating the electrospun zein nanofibers in PBS for 2 days increased the fiber diameter regardless of with or without Tween 80, but maintain the morphology of the electrospun zein nanofibers.

Effect of Content of Curcumin on Amount of Curcumin Released from Curcumin-loaded Zein Nanofibers. As shown in Figure 5, significant amounts of curcumin were very rapidly released from the zein nanofibers containing 10 wt% Tween 80 into the PBS during the first 2 h that the nanofibers were immersed in the PBS. Almost the maximum amounts of curcumin were released from the nanofibers immersed in the

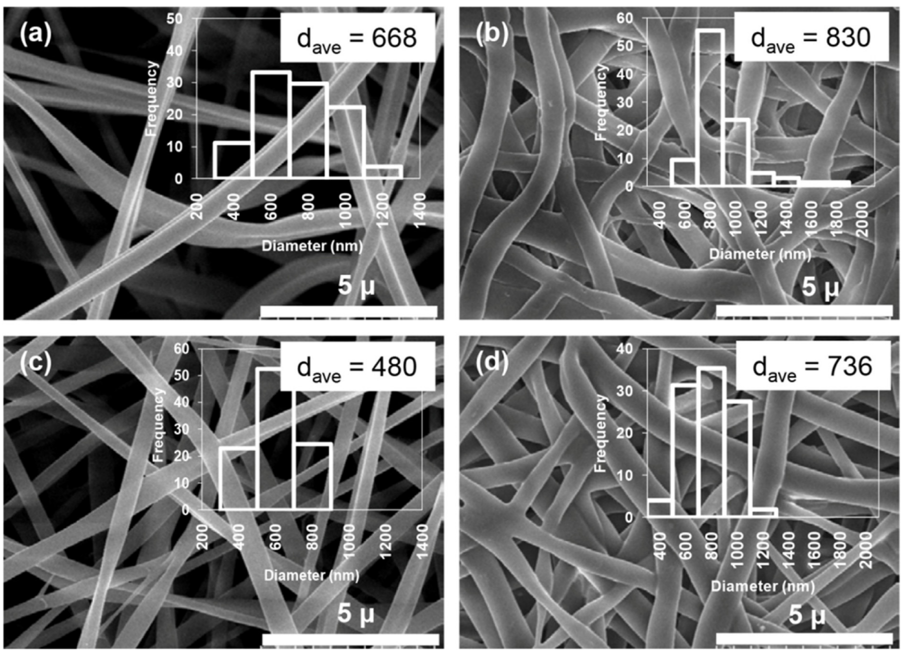

Figure 4. FE-SEM images of curcumin-loaded zein electrospun nanofibers from ethanol/acetic acid 75:25 w/w before and after incubation in PBS for 2 days. Curcumin-loaded zein nanofibers fabricated without surfactant (a) before incubation; (b) after incubation. Curcumin-loaded zein nanofibers fabricated with surfactant (c) before incubation; (d) after incubation.

PBS for 8 h. The maximum amounts of curcumin were released from the CLZNFs containing 10 wt% Tween 80 at 72 h of immersion. These results show that the amount of curcumin released from the zein nanofibers increased with increasing content of emulsified curcumin in the nanofibers.

Antibacterial Activity of Curcumin-loaded Zein Nanofibers. One of the aims of this study was to develop antibacterial CLZNFs for application as functional antibacterial nonwoven zein mats. Thus, the antibacterial activity of the CLZNFs was investigated using the bacterium *S. aureus*. Although only a small amount of curcumin was released from the CLZNFs, they exhibited good antibacterial activity. Figures 6 and 7 show the optical density (OD) curves plotted as functions of incubation time for the medium containing 10^5 CFU/mL of *S. aureus* and the CLZNFs. Bacterial propagation can be evaluated from the turbidity of bacterial solutions since bacterial cells are opaque; that is, the lower the OD of a bacterial solution, the fewer bacteria that propagated in the solution. The growth rate of the bacteria in the solutions was determined by measuring the OD of the solutions incubated for various amounts of time.

As shown in Figures 6 and 7, the unloaded zein nanofibers did not inhibit the growth of *S. aureus*. The OD of the bacterial solution containing the unloaded zein nanofibers increased with increasing incubation time, and the corresponding graph shows a slope of 0.023 OD/h. However, the CLZNFs did show antibacterial activity. Figure 6 shows the effect of the content of Tween 80 on the antibacterial activity of the CLZNFs fabricated with various contents of Tween 80 and loaded with 0.8 wt% curcumin. Although the ODs of the bacterial solutions containing the CLZNFs fabricated without Tween 80 were lower than that of the bacterial solution containing the unloaded zein nanofibers, the CLZNFs fabricated without Tween 80 showed insignificant antibacterial activity. By contrast, the ODs of the bacterial solutions containing the CLZNFs fabricated with Tween 80 were lower than those of the bacterial solutions containing the CLZNFs fabricated without it, suggesting that the CLZNFs fabricated with Tween 80 exhibited effective antibacterial activity against *S. aureus* by inhibiting bacterial growth for at least the first 9 h of incubation. The higher the content of Tween 80 in the zein nanofibers, the longer the nanofibers inhibited bacterial growth. The zein nanofibers fabricated with 2, 5, and 10 wt% Tween 80 inhibited bacterial growth for 9, 12, and 15 h, respectively, after which the bacteria in all the solutions gradually grew at the same rate. The zein nanofibers fabricated

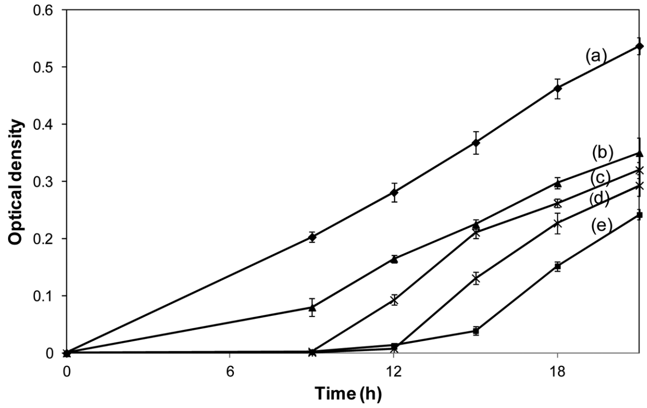

Figure 6. OD as function of time for (a) unloaded electrospun zein nanofibers and curcumin-loaded zein nanofibers fabricated with 0.8 wt% curcumin and various content of Tween 80 of (b) 0 wt%; (c) 2 wt%; (d) 5 wt%; (e) 10 wt% (respect to the weight of the zein). All solutions initially contained approximately 10^5 (CFU/mL) of *S.aureus* before incubation.

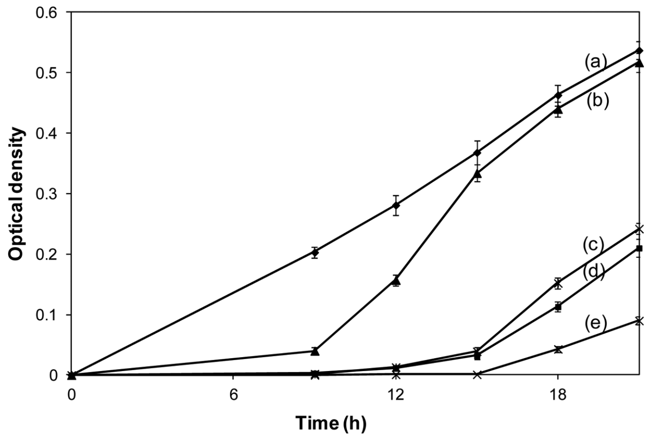

Figure 7. OD as function of time for (a) unloaded electrospun zein nanofibers and curcumin-loaded zein nanofibers fabricated with 10 wt% surfactant and different curcumin content of (b) 0.4 wt%; (c) 0.8 wt%, (d) 1.2 wt%; (e) 1.6 wt% (respect to the weight of zein). All solutions initially contained approximately 10^5 (CFU/mL) of *S.aureus* before incubation.

with 10 wt% Tween 80 showed 90 and 61% efficiency for inhibiting the growth of *S. aureus* after 15 and 21 h, respectively.

Figure 7 shows the effect of the content of curcumin loaded into the zein nanofibers fabricated with 10 wt% Tween 80 on the growth of *S. aureus*. The high OD of the bacterial solution containing the zein nanofibers loaded with 0.4 wt% curcumin indicated that these nanofibers did not show any significant antibacterial activity: only 44 and 4% antibacterial efficiency after 12 and 21 h, respectively. However, the ODs sharply decreased for the bacterial solutions containing the zein nanofibers loaded with 0.8, 1.2, and 1.6 wt% curcumin. This result indicates that the zein nanofibers loaded with 0.8 wt% or more curcumin showed good antibacterial efficiency by inhibiting the growth of *S. aureus*. The antibacterial efficiencies of the zein nanofibers loaded with 0.8, 1.2, and 1.6 wt% curcumin were 90, 92, and 99%, respectively, for the bacterial solutions incubated for 15 h and were 55, 61, and 83%, respectively, for the solutions incubated for 21 h.

Although the antibacterial activity of the electrospun CLZNFs fabricated without Tween 80 showed a trend similar to that of the antibacterial activity of those fabricated with it, the CLZNFs fabricated with Tween 80 showed more effective antibacterial activity than those fabricated without it.

Conclusions

Curcumin-loaded zein nanofibers (CLZNFs) were successfully fabricated by electrospinning process. Tween 80 was employed as an emulsifier to improve the bioavailability of the curcumin, enhancing the release ability of the loaded curcumin from the zein nanofibers. Adding the Tween 80 to the zein solution reduced the average diameter of electrospun CLZNFs, which could be attributed to the decrease in solution surface tension. More curcumin was released from the electrospun CLZNFs fabricated with Tween 80 than from those fabricated without it, suggesting that the Tween 80 had solubilized the hydrophobic curcumin by encapsulating it into micelles. In addition, the CLZNFs fabricated with Tween 80 exhibited effective antibacterial activity against *S. aureus*. The CLZNFs fabricated with 10 wt% Tween 80 and loaded with 1.6 wt% curcumin showed a maximum antibacterial efficiency of 83% for the bacterial solution incubated for 21 h. The antibacterial CLZNFs show potential for applications such as antibacterial nonwoven zein mats.

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