

RESEARCH PAPER

New Chitosan/Poly(ethylene oxide)/Thyme Nanofiber Prepared by Electrospinning Method for Antimicrobial Wound Dressing

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ABSTRACT

A new natural and environmental friendly wound dressing was introduced for the first time that was prepared by electrospinning method. This new wound dressing has chitosan base, and poly (ethylene oxide) was added as co-spinning agent to improve spinnability of chitosan. Moreover, thyme extract as a natural antibacterial additive was introduced in the as electrospun nanofibers scaffold in order to increase those wound healing properties. Some parameters of electrospinning such as feed rate, nozzle-collector distance, voltage and content of thyme extract in nanofiber structure were studied and optimized. The average diameters of prepared nanofibers was determined by "Clemex vision professional edition" software. Morphology and structure of electrospun nanofibers was studied with use of scanning electron microscopy and Fourier transform infrared spectroscopy. The results showed that the antibacterial activity of nanofibers increased as the amount of thyme extract was increased, thus a chitosan/PEO containing 3% of thyme extract was selected as the best prepared nanofiber for wound dressing preparation. Chitosan/PEO/thyme nanofiber showed high stability in the buffer and good antibacterial activity against three understudy bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

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INTRODUCTION

Wound healing is a process in which the destroyed tissues of body are replaced by living tissue and usually comprises different phases which are not mutually exclusive and that can considerably overlap [1]. A good wound dressing system should be have different properties such as durability, gas permeability and the capacity to absorb fluids exuded from the wounded area as well as to, simultaneously, control water loss [2, 3]. One of the films and foams types or combinations of both can be used as wound dressing materials based on the wound's exudation level [4, 5]. Synthetic polymers such as

polyurethane, polyethylene and natural polymers like chitosan, gelatin and collagen were used mostly in order to prepare wound dressing [6]. As the natural polymers are usually biocompatible, biodegradable and inexpensive, thus their dermis application is easy and safe, therefore, they are attractive materials as wound dressing in healing process of dermal wounds [7, 8]. Chitosan is the deacetylated derivative of chitin [9]. Chitosan is a natural polysaccharide that is non-toxic, biocompatible, mucoadhesive, and cost effective, which makes it attractive for various biomedical and pharmaceutical applications [10-12]. Chitosan is a well-known biopolymer that showing a

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broad-spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria (This positive/negative reference is based on the bacterium's chemical and physical cell wall properties. To determine if a bacteria is gram-positive or gram-negative a microbiologist will perform a special type of staining technique, called a Gram Stain) and fungi [13, 14].

Nanofibers have good properties such as high surface area-to-volume ratio and high porosity that makes them good candidates for different biomedical applications such as tissue engineering and wound dressing production [15]. Electrospinning is one of the best methods for nanofiber fabrication. Electrospinning is a suitable technique to produce nanofibers due to its easy manufacturing process, which is governed by several parameters that can be controlled by the user [16-18]. Electrospun fiber mats are highly porous and permeable materials with high surface area that is ideal for wound dressings [19-21]. Since the chemical structure of chitosan is similar to glycosaminoglycans, it finds high attention for nanofiber production [22]. The main problem for the electrospinning of chitosan is its poor solubility. In order to overcome this limitation, chitosan can be blended with an easily electrospinnable polymer such as: PEO, poly (vinyl alcohol), polycaprolactone and silk fibroin [23-26].

Antimicrobial properties are important for wound dressings to provide a barrier against the growth of unwanted microorganisms. Critical colonization of the wound by bacteria or microbial substances can delay the healing process, can result in malodor and may increase pain. Therefore, additives have been used in wound healing dressings to render them antimicrobial [27]. The therapeutic efficacies of many indigenous plants, for various diseases have been described by traditional herbal medicine practitioners [28]. The presence of various life sustaining constituents in

plants has urged scientists to work on these plants with a view to discovering its wound healing properties [29]. Thyme (*Thymus vulgaris* L) is an herb of the Labiate family and has long been used as flavoring agent in various food products [30]. Due to the presence of carvacrol and thymol in the thyme structure, it shows considerable antibacterial properties [31]. Chemical structures of carvacrol and thymol are shown in Fig. 1. The antimicrobial activity of several essential oils has been attributed to the presence of phenolic compounds, i.e. the efficiency of thyme was assigned to thymol and carvacrol. The essential oil of Thyme subspecies contains between 5% and 75% of carvacrol. The inhibitory effect of phenols could be explained by interactions with the cell membrane of micro-organisms and is often correlated with the hydrophobicity of the compounds. Among phenolic compounds, carvacrol, or cymophenol, $C_6H_3CH_3(OH)(C_3H_7)$, a monoterpenoid phenol, was reported to have one of the strongest antimicrobial activity. Also, thymol (2-isopropyl-5-methylphenol) is a monoterpene phenol, $C_6H_3CH_3(OH)(C_3H_7)$, isomeric with carvacrol, found in oil of thyme. Numerous studies have demonstrated that naturally occurring biocides such as thymol and carvacrol reduce bacterial resistance to antibiotics through a synergistic effect. However, to the best of our knowledge, the use of thyme as natural antimicrobial agent, has not been studied to date, in wound dressing production. Thus, the objective of the present study was to determine the effect of thyme extract on the healing efficacy of chitosan/PEO electrospun nanofiber.

MATERIALS AND METHODS

Acetic acid, tween 80 and nutrient agar cultivation environment were prepared from Merck. Medium molecular weight chitosan and PEO with 900000 Mw were purchased from Sigma-Aldrich, Three different bacteria including *Escherichia coli* (*E.coli*) and *Pseudomonas aeruginosa* (*P.aeruginosa*) as gram-negative bacteria, and *Staphylococcus aureus* (*S.aureus*) as a gram-positive bacteria were used.

Electrospinning process was performed with an electrospinning apparatus model ES1000. A scanning electron microscope (model LEO1455VP) was employed to study the surface morphology of prepared nanofibers. FT-IR model Nicolet 800 was used to record IR spectrum of prepared nanofibers. Heater and magnetic stirrer, model IKA-RCT-B. UV-

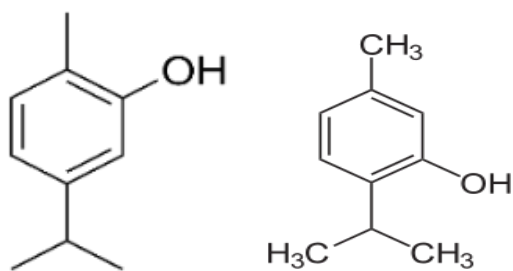


Fig. 1. Chemical structures of carvacrol (a) and thymol (b)

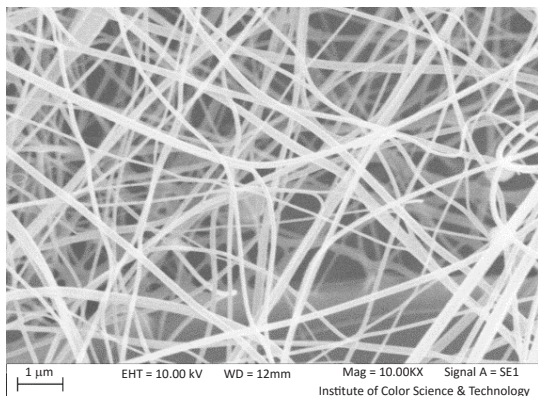


Fig. 2. SEM image of chitosan/PEO nanofiber

Vis spectrometer model Spekol 2000 was used to study the drug delivery template of thyme extract from nanofiber scaffold.

Preparation of chitosan/ PEO /thyme extract solution

Solution of chitosan/ PEO was prepared by slowly adding 0.27 g of chitosan powder with medium molecular weight and 0.04 g PEO on the appropriate volume of 50% acetic acid. Then, 0.25 mL tween 80 was added to this solution as an emulsifier. This solution was stirred on a magnetic stirrer at 300 rpm for 12 h at 37 °C to yield a homogeneous solution. Finally, Shirazian thyme extract was added to the chitosan/PEO solution to obtain solutions containing 0.5, 1, 2 and 3% of thyme extract.

RESULTS AND DISCUSSION

Electrospinning process

For investigate the effect of thyme extract and chitosan nanofibers, chitosan/PEO (90:10 ratio) without thyme extract and containing 0.5, 1, 2 and 3% of thyme extract, were used as stock solutions for nanofiber preparation. First the prepared solutions were supplied into a 5 mL syringe with a stainless steel capillary needle. All fibers were spun at 15-20 kV, keeping a constant tip-to-collector distance (TCD) of 10 cm and the flow rate was kept at 0.3-0.6 mL/h and collector rotated at 400 rpm. In order to obtain nanofibers with appropriate thickness, electrospinning was performed for 10 h. For the completely removal of solvent and water from the electrospun samples, they were drying at room temperature for 24 h.

As mentioned above, addition of PEO to chitosan solution, increase its spinnability. Hence we used a mixture of chitosan/PEO with the ratio

of 90:10(w/w) for the chitosan based nanofiber production by electrospinning method. This mixture results to a uniform nanofibers with the average diameters 63.2 nm containing a few beads (Fig. 2).

SEM images of the chitosan/PEO containing different concentration of thyme extract are shown in Fig. 3. As it can be seen, the nanofibers diameter increase by increasing the amount of thyme. The average diameters of the chitosan/PEO/thyme nanofibers were varied from 68.2, 76.8, 81.3 and 84.9 nm for 0.5, 1, 2 and 3% of thyme extract respectively. Determination of nanofibers diameters were performed with "clemex vision professional edition" software. This results showed that the best uniform nanofibers including no droplet were obtained at 19 kV voltage with the 0.5 mL/h feeding rate.

Infrared (IR) spectra of the composite fibers were recorded with a Nicolet 800 FTIR spectrometer in transmittance mode at room temperature. All samples were scanned from 4000 to 400 cm^{-1} . We used this technique for investigation of chemical changes during the nanofiber preparation and confirming the chemically attachment of thyme extract to the chitosan. FT-IR spectrum of chitosan/PEO/thyme (3%) nanofiber is shown in the Fig. 4. Also, FT-IR spectrum of chitosan/PEO was used for comparison [32]. As shown in Fig. 4(b), the broad band at around 3400 cm^{-1} is assigned to the stretching mode of the O-H and N-H bonds in the chitosan, O-H bond in the PEO backbone and O-H bond in the carvacrol and thymol. Moreover, the bands located in 1734 cm^{-1} ascribed to the stretching mode of the C=O bond and band in the 1612 cm^{-1} assigned to the bending vibration of the N-H group in the chitosan. Moreover, by addition of thyme extract to chitosan/PEO, the intensity of band between 2900-2800 cm^{-1} increased that can be attributed to addition of C-H bonds. Furthermore, the peaks located between 950-600 cm^{-1} refer to the C-H bond in the aromatic carvacrol and thymol compounds in the thyme structure.

Nanofibers stability study

For simulation of human skin in order to examine the stability, degradation and swelling of prepared nanofibers, we used a buffer with pH 5.5 and 37 °C temperature.

The stability of the nanofibers were studied by immersing fragments of chitosan/PEO/thyme (3%) nanofiber mats in this solution for 24 h. After this

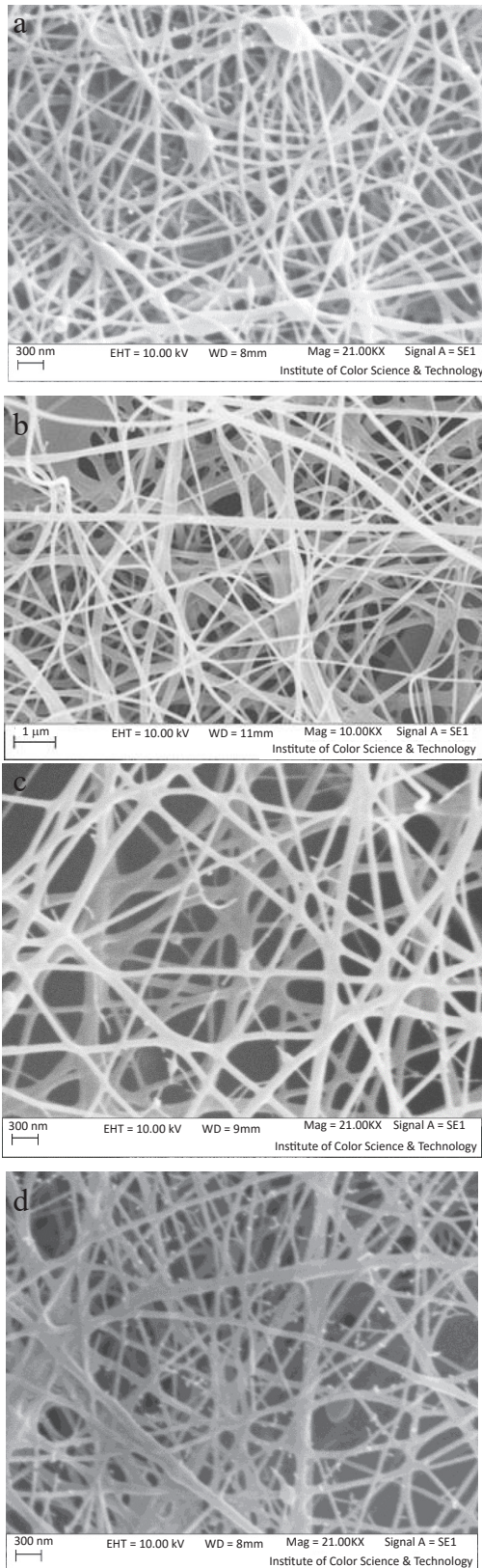


Fig. 3. SEM images of electrospun chitosan/PEO nanofiber containing; a): 0.5%, b): 1%, c): 2% and d) 3% of thyme extract.

time, nanofibers were dried at room temperature for another 24 h. Then, the morphology of the nanofibers were studied by scanning electron microscope. Fig. 5 shows the SEM image of chitosan/PEO/thyme (3%) nanofibers after placing those in buffer. As it can be seen, prepared nanofiber has stable structure and its morphology didn't differ even after 24 h. This high stability showed the bonding of chitosan chains together that prevent from water entering to the nanofiber scaffold.

Releasing template of thyme extract from nanofiber scaffold

Releasing template of thyme extract from composite nanofiber scaffold was studied by UV-Vis spectrometry. First, standard solutions of thyme extract with different concentrations were prepared in buffer and calibration curve was drawn at 274 nm. Afterward, 30 mg of chitosan/PEO/thyme (3%) extract nanofiber were placed in 100 mL of buffer at 37 °C, that was stirred at 200 rpm on a magnetic stirrer, in order to thyme extract be released into the buffer solution. Sampling from this solution was performed after each 40 h and the UV-Vis spectrum of released active substance

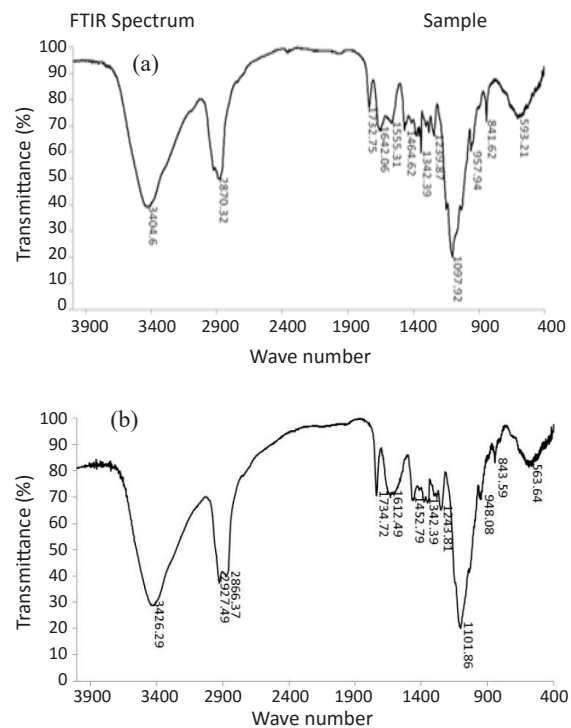


Fig. 4. FT-IR spectra of (a) chitosan/PEO and (b) chitosan/PEO/thyme (3%) nanofibers

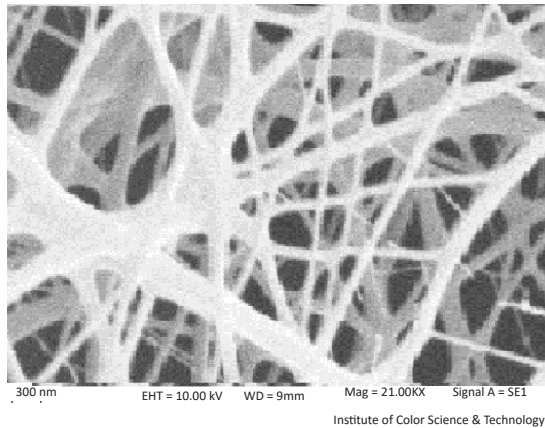


Fig. 5. SEM image of electrospun chitosan/PEO/thyme (3%) nanofibers after placing in the buffer

was recorded. As it could be seen in Fig. 6, thyme extract wasn't released suddenly and its delivery was happened moderately during the 10 days. This releasing template can be attributed to the porous structure of prepared nanofiber that cause to penetration of active substance to the nanofiber pores thus the releasing process occurs slowly. Moderate delivery of active substance during some days result in better healing and curing of wound.

Antibacterial activity of nanofibers

The effect of the incorporation of thyme in nanofibers and anti-bacterial properties in coatings was investigated by agar plate method by culturing of the bacteria E.coli, P. aeruginosa and S. aureus. Nanofibers were first sterilized using ethanol 75% treatment. Each bacteria separately suspensions of 0.5 McFarland number of bacteria is prepared and containing $1-2 \times 10^8$ CFU/mL of each series were removed and placed on the

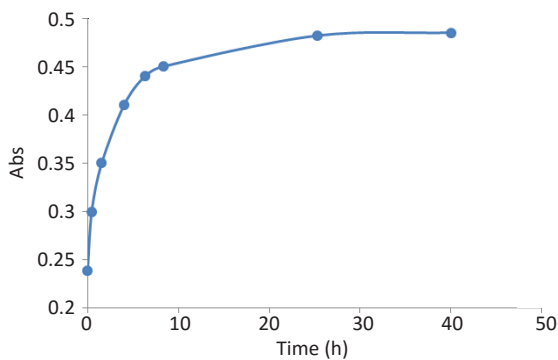


Fig. 6. Releasing template of thyme extract from chitosan/PEO/thyme (3%) nanofiber scaffold

surface of mueller- hinton agar plates seeded with S. aureus and E.coli and were inoculated at 37°C. After 24 h of incubation, the zones of inhibition (diameter of the inhibition circle around paper disks) were measured and considered as antibacterial deal of prepared nanofibers. Antibacterial activity was studied for thyme, polymeric solutions and nanofibers that prepared from these polymer solutions. The antibacterial activity results obtained for chitosan/PEO solution containing different amount of thyme extract are listed in table 1.

As it can be seen, polymer solutions have bactericide antibacterial effect and inhibition zone was created in the presence of both Gram-negative and Gram-positive bacteria. Table 2 summarized the antibacterial results of chitosan/PEO/thyme nanofibers.

As the results show, these nanofibers have bacteriostatic antibacterial effect and affected only on growing up of the Gram-positive bacteria but shows no effect on the Gram-negative bacteria. Additionally, in order to study the antibacterial effect of different thyme species, two type of thyme plant were investigated including broad-leaved and narrow-leaves thyme. Fig. 7 shows the antibacterial activity of these two thyme species.

Moreover, obtained results for these two species are shown in Table 3. According to these results broad-leaved thyme shows higher antibacterial activity than narrow-leaf type in the presence of all three bacteria.

Table 1. Antibacterial activity of chitosan/PEO/thyme Solutions.

Polymer solution	Inhibition zone diameter (mm)		
	E.coli	P.aeruginosa	S.aureus
Chitosan/PEO/Thyme (0.5%)	0	12	30
Chitosan/PEO/Thyme (1%)	0	15	35
Chitosan/PEO/Thyme (2%)	0	17	37
Chitosan/PEO/Thyme (3%)	0	20	40

Table 2. Antibacterial activity of chitosan/PEO/thyme nanofibers

Thyme species	Inhibition zone diameter (mm)		
	E.coli	P.aeruginosa	S.aureus
Broad-leaf thyme	13	10	19
Narrow-leaf thyme	11	8	15

Table 3. Antibacterial activity of different thyme species.

Nanofiber type	Inhibition zone diameter (mm)		
	E.coli	P.aeruginosa	S.aureus
Chitosan/PEO/ Thyme (0.5%)	0	8	24
Chitosan/PEO/ Thyme (1%)	0	11	30
Chitosan/PEO/ Thyme (2%)	0	15	35
Chitosan/PEO/ Thyme (3%)	0	19	39

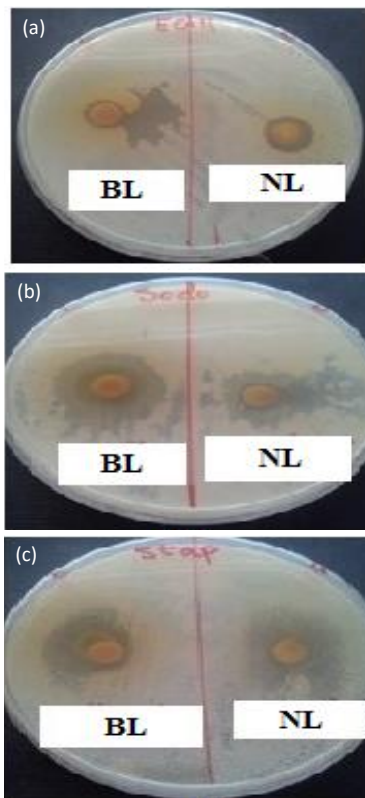


Fig. 7. The antibacterial activity of tow thyme species extract including B.L (broad-leaf) and N.L (narrow-leaf) species in the presence of (a): E.coli, (b) P.aeruginosa and (c): S.aureus bacteria.

CONCLUSION

Electrospinning is a simple and relatively cheap method that is able to produce nanofibers with nano size diameters. The prepared nanofibers are high porous and can be used as wound dressing. Chitosan-based nanofibers blending with PEO were fabricated by use of electrospinning method and were successfully applied as wound dressing. Since thyme leaf extract has different biological and pharmacological properties such as high antibacterial activity, hence, it was introduced

in the prepared nanofibers scaffold as a natural antibacterial agent to improve the healing effects of chitosan based nanofibers. Scanning electron microscopy (SEM) and FT-IR were used in order to study the morphology and structure of prepared nanofibers. New prepared nanofiber composite showed high stability and good antibacterial activities versus three type under study bacteria. Moreover, UV-Vis study revealed that thyme extract releases moderately from the nanofiber scaffold during the 10 days that can be result in a better and more effective wound healing. In sum, it can be said that the new introduced chitosan/PEO/thyme nanofiber shows good healing activity and can be applied as a new ideal wound dressing preparation that cause faster and better wound healing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publicaton of this manuscript.

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