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RESEARCH PAPER

Development of antimicrobial chitosan based nanofiber dressings for wound healing applications

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ABSTRACT

Objective(s): Chitosan based composite fine fibers were successfully produced via a centrifugal spinning technology. This study evaluates the ability of the composites to function as scaffolds for cell growth while maintaining an antibacterial activity.

Materials and Methods: Two sets of chitosan fiber composites were prepared, one filled with anti-microbial silver nanoparticles and another one with cinnamaldeyhde. Chitosan powder was dissolved in trifluoroacetic acid and dichloromethane followed by addition of the fillers. The fiber output was optimized by configuring the polymer weight concentration (7, 8, and 9 w/w% chitosan) and applied angular velocity (6000-9000 RPM) within the spinning process.

Results: Scanning electron microscopy revealed fiber diameters ranging from 800-1500 nm. Cinnamaldehyde and silver nanoparticles helped to improve and control the anti-bacterial activity. Through a verified cell counting method and disk diffusion method, it was proven that the chitosan based composite fibers possess an enhanced anti-bacterial/microbial activity against gram-positive Staphylococcus aureus. Both composite systems showed anti-bacterial activity, inhibition zones fluctuating between 5 to 10 mm were observed depending on the size of the fiber mat and no bacteria was found within the mats. The developed fiber scaffolds were found to be noncytotoxic serving as effective three-dimensional substrates for cell adhesion and viability.

Conclusion: These results provide potential to use these scaffolds in wound healing and tissue regeneration applications.

Keywords: *Anti-microbial, Cell adhesion, Chitosan, Cinnamaldehyde, Forcespinning, Silver nanoparticles, Wound dressing*

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INTRODUCTION

It is estimated that over $25 million are spent annually on the treatment of chronic non-healing wounds [1], indicating there is a need to develop advance wound dressings that can last longer before replacement and can heal acute/chronic wounds rapidly. Bacterial infection is the primary culprit for delaying the wound healing process, this has resulted in a demand to incorporate antimicrobial agents within the wound dressings [2]. The estimated cost in the wound care dressing market, incorporating these antimicrobial agents, was estimated at over $7 billion in 2013 and is expected to grow to $10 billion by 2020 [3]. In addition, antimicrobial efficacy of a wound dressing alone is insufficient, and therefore other properties are needed to improve wound healing. For example, dressings composed of nanofibers and submicron fibers in particular have relevance in medical/biological applications since proteins and bacteria have dimensions on this order, and can encourage cell proliferation/wound healing [4, 5].

Chitosan is an abundant biocompatible amino based polysaccharide that has been added to wound

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dressings due its antimicrobial activity. Chitosan is the principal component of arthropods (crustacean shells, mollusks, and insects) [6], and consists of a deacetylated derivative of chitin, the second most abundant polysaccharide after cellulose [7]. The D-glucosamine structure is highly crystalline with many hydroxyl groups, which can form intermolecular hydrogen bonds, leading to its poor solubility in common organic solvents. The amino group on chitosan makes it slightly alkaline and insoluble at neutral or basic water; however, under acidic conditions (pH < 6.5) it is soluble due to the amino groups becoming protonated (cationic) and positively charged (Fig 1). These acidic systems include dilute aqueous acetic, lactic, malic, formic acids, and organic mixtures such as water-methanol, -ethanol, and acetone mixtures [8, 9]. The amino group on chitosan not only plays an important role in regards to processing but also in function. For instance, the amino groups can interact or chelate with several metals such as copper, cadmium, iron, mercury, and magnesium. This has resulted in the incorporation of chitosan/carbon fiber composites as effective filtration media [10, 11]. The cationic nature of chitosan, from the amino groups, also makes it a potent antimicrobial agent that inhibits growth and removes microorganisms such as bacteria and fungi [12].

In wound care management, chitosan acts as an analgesic drug and anti- inflammatory agent, which can exhibit a pleasant and soothing effect when applied to an open wound [13]. In vivo studies have demonstrated pain reduction when applying a nonwoven chitin fabric/membrane wound dressings to patients with burns, skin abrasions, skin grafts and ulcers [14]. The mechanism for pain relief has been studied by an in vitro chitosan suspension/ acetic-acid writhing test on mice, which indicated a reduction in inflammatory pain due to a pH increase from protonation of $–NH_2$ to $–NH_3^+$ (Fig 1) [15].

Fibers can be produced via electrospinning, a versatile method to process polymer solutions into fibers, with diameters ranging from tens of nanometers to a few micrometers [4, 16]. Electrospun fibers have made promising wound dressings in the biomedical field. For example, electrospun fibrous membranes composed of hybrid chitosan/collagen/PEO fibers, showed an increase in the Young's modulus under aqueous conditions when cross-linked [17, 18]. It was further shown, compared to traditional gauze and commercial collagen dressings, that these membranes can mimic the native extracellular matrix resulting in improved wound healing and in vitro tissue regeneration (e.g. bone, cartilage). Hybrid chitosan fiber dressings, compared to topical agents, are also expected to speed up the healing process and recovery time by inhibiting bacterial growth and spread of infection. The bio-activity of these fibers is further improved when combined with agents or enzymes, which can promote wound healing and pain alleviation. For instance, Jiang et al. developed PLGA/ PEG-g-chitosan fibers containing ibuprofen, which showed a controlled and sustained release rate of ibuprofen [19], and exhibited mechanical properties similar to skin. Production of fine fibers through electrospinning can be challenging due to the low fiber yield, which averages ~0.01-1.0 gram per hour at the lab scale [4, 20, 21]. The Forcespinning® (FS) method has shown the capability to produce fine fibers from melt and solution through centrifugal spinning [22-24]. The FS method does not require electric fields, and broadens the choice of materials to be spun into fibers [22, 23, 25]. The process is highly controllable at the industrial scale and has shown production rates of up to hundreds of meters per minute depending on desired basis weight. Previous FS studies have successfully produced wound dressings composed of cellulose acetate fibers embedded with silver nanoparticles (AgNPs), and ternary composites fibers dressings such as pullulan/tannic acid/chitosan fiber and polyvinyl alcohol/chitosan/tannic, all of which showed anti-microbial activity [26-28].

In this study, chitosan binary nonwoven fine fiber composite scaffolds composed of chitosan/ cinnamaldehyde (CA) and chitosan/ AgNPs, were produced using FS technology. Cinnamaldehyde and silver are known to possess strong antimicrobial properties [29] and therefore its effect in these binary composites is evaluated. The added agents, CA and AgNPs, were shown to exhibit improved antimicrobial activity against *Staphylococcus aureus,* in addition to cell biocompatibility, which was evaluated with mouse embryonic fibroblasts (NIH 3T3). To increase the stability of the developed membranes in conditions resembled in wounds (pH < 6), a ternary composite containing a cross-linking reagent, tannic acid (TA), was developed by adding TA to the chitosan/CA and the chitosan/AgNPs solution, followed by FS and alkaline hydrolysis to initiate cross-linking. The ternary composite dressing structural stability under various pH conditions. The structural morphology and anti-microbial activity against the gram-positive *Staphylococcus aureus* were also investigated.

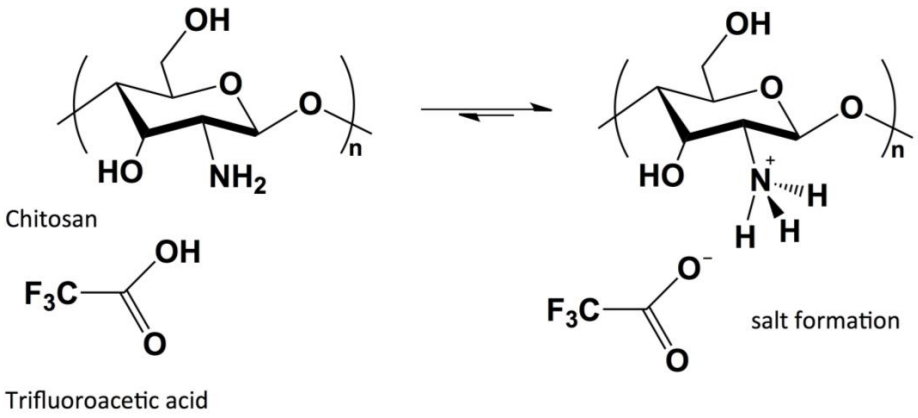

Fig 1. Chitosan is a de-acetylated derivative (1,4-linked 2-amino-2-deoxy-beta-D-glucopyranose) of chitin (-NH-COCH3). Chitosan also contains acetylated groups, however, most of these contain a free amino group (-NH2), which can act as a proton acceptor (Bronsted-Lowry base) in water. Chitosan is soluble in trifluroacetic acid, which creates an acidic aqueous environment (pH < 6), and allows for the amino group to undergo protonation and form a salt with the acid

MATERIALS AND METHODS

Materials

Chitosan (medium molecular weight, CAS 9012-76-4 (448877-250G), Brookfield viscosity 200 cps), trifluroacetic acid (CF_3CO_2H), dichloromethane (DCM), tannic acid, potassium hydroxide (KOH), sodium hydroxide (NaOH), and cinnamaldehyde were purchased from Sigma Aldrich. The silver nanopowder was acquired from US Research Nanomaterials, Inc. For fiber production, 30 gauge (half-inch) bevel needles (PrecisionGlideTM) were purchased from Fisher scientific.

Solution preparation

Chitosan fiber preparation

A 7, 8, and 9 w/w% chitosan solution was made by dissolving 0.75, 0.87, and 0.99 grams of chitosan to a 7:3 by weight trifluroacetic (TFA)/ dichloromethane (DCM), which amounted to 7 grams or 4.7 ml of TFA, and 3 grams or 4 ml of DCM, respectively. The chitosan solution was sonicated in a water bath for one hour or until the solution was homogenous, and left to rest in a rolling machine for two to three days.

Chitosan / silver nanoparticle (AgNPs) fiber preparation

Samples containing different concentration (0.05 to 0.6 w/w%) of AgNPs were prepared. It was observed that the sample containing 0.2 w/w% (22 mg) AgNP within the 8 w/w% CA solution was shown to be the optimum concentration when considering fiber output and homogeneity.

Chitosan / Cinnamaldehyde

Two cinnamaldehyde solutions were prepared at different concentrations. The first solution, ~2 w/w% CA was prepared by mixing 0.22 g CA to an 8w/w% chitosan solution as prepared above, this concentration was too high and fiber homogeneity was affected. Concentration was dropped to 0.8 w/w% CA, which was prepared by adding 90 mg of CA to an 8 w/w% chitosan solution. Fibers output was consistent with the AgNP loaded fibers though with a rougher fiber texture.

Chitosan / tannic acid (TA)

Tannic acid was incorporated (10 w/w%) in the 8 w/w% chitosan solutions.

Fiber production

To produce the chitosan-based fibers from the prepared solutions, a lab scale Cylone™ L-1000M (FiberRio Technology, Corp.) was used. Approximately 1 mL of the prepared solution was injected into a cylindrical spinneret that contained two 30 gauge (half inch) bevel needles at each end. To determine the optimal fiber output and change in fiber diameter, the chitosan solution was tested at different angular velocities (6000-9000 RPM), which allowed the polymer solution to be extruded through the orifices of the needles. The fibers were allowed to collect on a set of column collectors (12 x 1 inch). The nonwoven mat was prepared by taking a square frame (7x7inch), which was used to collect the fibers that deposited between the columns.

Measurements and characterization

The fiber morphology, diameter, and homogeneity of the produced fiber mats were examined using a scanning electron microscope (SEM-Sigma VP Carl Zeiss, Germany). Average fiber diameter was determined by measuring 100 unique nanofibers from the SEM images, for each prepared mat. Matplotlib was utilized to plot the normal fiber distribution using the calculated mean (μ) and standard deviation (σ) for the given sample size (100 fibers).

Alkaline induced crosslinking

Although chitosan is insoluble under basic conditions, the fibers can dissolve under acidic environments (pH <7). Crosslinking was carried out to enhance the structural integrity of the fibers for potential applications as wound dressing in different environments. To initiate crosslinking, the fiber mats containing TA were soaked for one hour in a potassium hydroxide (KOH) solution for 1 hour at ambient conditions. The soaked fibers were then rinsed in deionized water several times until the fibers had no traces of chemical residue or when the pH was neutral, which was confirmed by carrying out a pH test during washing. The stability of the chitosan composite based fiber mat was then tested under acidic, neutral, and basic conditions. Three separate and dried mats, were submerged in three different solutions of 1M acetic acid, distilled water (pH=7), and 1 M NaOH (pH=13). The composite fiber mats were removed after being immersed for 30 minutes at ambient conditions.

Antibacterial resistance measurements

The antibacterial activity was determined using the viable plate counting method against gram-positive bacteria *Staphylococcus aureus* (ATCC6538), which is the common cause of skin and other human infections. The anti-microbial test was based on the Kirby Bauer disk diffusion method [27, 51]. Bacteria were grown in nutrient agar broth at 37°C for 24 hours in a rotary shaker. The concentration of the suspension was adjusted to 10^4 cell per ml^{-1}. Aliquots of 100 μL of a bacterial suspension were spread onto the surface of agar plates and uniformly dispersed with a sterile L-shape glass rod. Then by means of sterile forceps, the fiber mats were carefully deposited on the surfaced of Mueller Hilton agar plates. Inoculated plates with fibers were incubated at 37°C for 48 hours. Measurements of the zones of inhibition were taken on each of the four replicated plates and treatments compared.

Cell culture and spreading assays

NIH 3T3 mouse embryonic fibroblasts were seeded onto the nanofiber composite mats in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), and cell viability was investigated. The control was prepared by allowing the cells to grow on glass cover slips. All tests were conducted in a humidified incubator at 37 °C and 5% CO_2. The composite fibers were placed in a 6-well cell culture plate and treated via UV light for 10 minutes and washed with a phosphate buffered saline (PBS) solution. UV irradiation was used to aid in promoting crosslinking within the chitosan fibers therefore enhancing stability in aqueous medium needed for cell studies [52]. 250,000 cells were then seeded onto the composite fiber mats and cultured overnight.–Following incubation, cells were stained with 40 nM MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA) in fresh media for 20 min. at 37°C. The cells were rinsed twice with the PBS solution, and fixed with 4% paraformaldehyde in PBS for 30 min. Cells were subsequently stained with 4',6-diamino-2-pheylindole (DAPI) for 10 min. to visualize cell nuclei. The cell/fiber matrix was subsequently washed 3 times with PBS and mounted with 50% glycerol in PBS. The cells were viewed and analyzed using an Olympus FV10i confocal laser-scanning microscope.

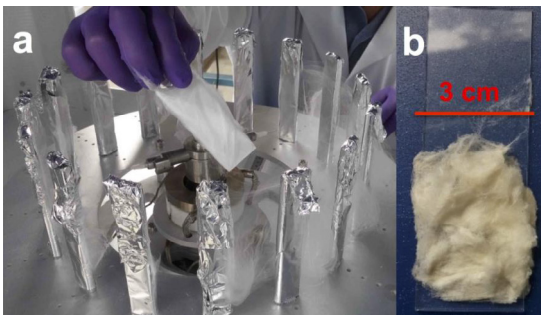

Fig 2. Production of fine fibers using Forcespinning® (FS) technology. (a) The FS apparatus consists of a spinneret, where the solution is injected, and a set of column collectors that allow for formation of the fiber webs (left). The collected chitosan/AgNPs fibers have a slight grey coloration in contrast to (b) pure chitosan fiber mats

RESULTS AND DISCUSSION

Fiber morphology

Previous reports have shown chitosan fiber production via electrospinning, either from a chitosan solution containing concentrated aqueous acetic acid [30, 31] or in trifluoroacetic acid (TFA) [32]. TFA can bind to chitosan in the form of a salt allowing for improved solubility (Fig 1).

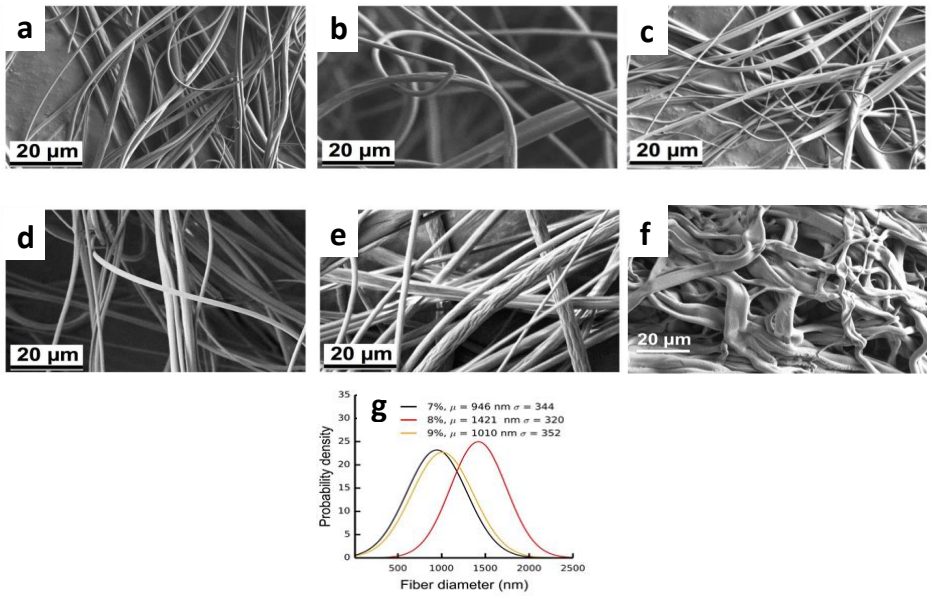

Fig 3. SEM images of (a) 7 %, (b) 8 %, and (c) 9 % chitosan spun at 8K RPM. Fig (d) shows fibers containing 0.2 % AgNPs and (e) 2 % CA within the solution. Fig 3f shows chitosan fibers containing tannic acid and dipped in a solution of KOH. Fig 3g shows the fiber diameter distribution for 7, 8, and 9 wt.% chitosan with mean (μ) and standard deviation (σ) with a sample size of 100 fiber diameters for each distribution

The addition of DCM to the chitosan/TFA mixture has been shown to improve the homogeneity/uniformity of the fiber with little or no bead formation, while also decreasing the inter-connectivity of the electrospun fibers [32]. To produce the nonwoven chitosan fiber mats using FS, the optimum parameters to maximize output while still maintaining a fine fiber structure were considered to be 7-9 w/w% chitosan solution in TFA and DCM, spun at 8000 RPM. The produced fibers are shown in Fig. 2.

Fig 3 shows the fiber distribution and average fiber diameter obtained for pure chitosan fibers produced from the 7-9 w/w % chitosan solutions. The mean fiber (μ) diameter for the chitosan solution at 7, 8, and 9 w/w% was found to be 946, 1421, and 1010 nm, with standard deviations (σ) of 344, 320, and 352 nm, respectively.

The chitosan fine fibers prepared from a 7 w/w% chitosan solution were found to be homogenous with no beads, as shown in the SEM micrographs (Fig. 3a). An 8 w/w% chitosan solution, spun at 8000 RPM, was found to produce an optimal output of chitosan fibers, however, had the greatest mean fiber diameter. Fibers produced at 9 w/w% chitosan were also spun at 8000 RPM, however, a lower fiber yield was obtained with decreased fiber diameter. An 8 w/w% solution was used for preparation of the AgNP and CA based solutions. The 0.2 w/w% AgNP composite fibers showed smooth homogenous fibers (Fig. 3d), versus a rough texture found for the 2w/w% CA solution.

Crosslinking of chitosan fiber with TA

Tannic acid is a hydrolysable phenolic compound derived from oak wood, and is found in many foods such as tea and cocoa beans. Tannic acid also has anti-microbial activity given that it contains hydroxyl groups making it a preeminent ligand that can chelate and reduce silver[*56*]. Tannic acid is a nontoxic agent that can cross-link with a polymer through alkaline hydrolysis, e.g. the hydroxyls group on tannic acid can react with the amino group in chitosan, which can change its solubility and mechanical properties[*58, 59*]. The possible binding mechanisms of TA to the amine containing group in chitosan can be due to hydrogen bonding and hydrophobic interactions[*60*], and complexes that depend on temperature and pH[*61*].

By treating the chitosan composite/TA fibers with KOH, through a soaking and rinsing process, a crosslinking effect results and fibers became stable at pH values commonly found in wounds [58, 62]. As shown in Fig 3f, the resultant fiber morphology is non-uniform, with a ribbon-liked fused fiber network.

Evaluation of anti-microbial activity of FS chitosan composite fibers from gram-positive bacteria Staphylococcus aureus

Chitosan-hybrid fibers have been known to demonstrate good antibacterial activity against gram-negative bacteria *E-coli* and gram-positive bacteria *Staphylococcus aureus* [33, 34]. The mechanism by which chitosan inhibits growth of gram-negative bacteria, is believed to be attributed to the formation of a polycationic complex between the amino group on chitosan and the outer cell membrane of the bacteria. The binding results in disruption of the outer membrane of the cell bacteria, which results in decreased functionality of the cell and leads to detergent-induced lysis [35].

To further improve anti-bacterial activity in the spun chitosan fibers, cinnamaldehyde and AgNPs were added. CA is an essential oil derived from cinnamon bark which has been reported to be effective against gram negative *Pseudomonas aeuroginosa (P. aeruginosa)* and gram positive *Staphylococcus aureus* bacteria [36], with minimum inhibitory concentration (MIC) ranging from 75- 600 µg/mL. Essential oils such as CA contain hydrophobic components that can permeate through the bacteria cell membrane to the mitochondria, leading to proton depletion and disruption of adenosine triphosphate synthesis [37]. In one study by Rieger et. al., electrospun CA- chitosan /PEO fiber mats were shown to have anti-microbial activity against *E-coli* [38]. In the latter study, after only 30 minutes of fiber-bacteria contact, a high-level of inactivation against *E-coli* was seen. In the case of silver, the anti-bacterial activity has been well studied, silver has become a viable treatment option for infections found in burns and chronic wounds [39-41] In particular, silver nanoparticles (AgNPs) have shown increased activity against a broad range of microbes and parasites, and it has been determined that in the presence of oxygen, the Ag^+ state will have the best antibacterial effect [42, 43]. Silver embedded in the fiber dressing can therefore be expected to provide a controlled and long lasting anti-bacterial activity. For example, An et al., reported on electrospun chitosan/PEO membranes containing silver nanoparticles showing improved resistance to bacteria such as gram-positive *Staphylococcus aureus* and gram-negative *E. coli* [44].

Here, the binary composites, chitosan/ CA and chitosan/AgNPs were also exposed to *Staphylococcus aureus* and the anti-microbial activity was investigated. Fig 4 indicates an antibacterial effect for both composites, evident by the inhibition zone around the fiber mats. This effect was evaluated using a viable cell counting method and further compared to a control without the fibers. The results indicate that there was complete inhibition of *Staphylococcus aureus* growth, indicating that the composite effectively promotes antimicrobial activity. Fig 4a shows a brownish color in the center of the mat that is intrinsic to this sample, not to be confused with bacteria outside the inhibition zone. The inhibition zone was found to range from5 to 10 mm.

Cell attachment

Cell attachment to the chitosan fibers are expected given the similar dimensions for both structures. For instance, silk fibrin/ collagen nanofibers have been shown to promote cell adhesion due to the high surface to volume ratio and 3D network, which allows for cells to attach to the fibers more readily [45]. It was also reported that chitosan nanofibers can promote cell attachment and facilitate the spreading of normal human keratinocytes and fibroblasts [46]. With an attempt to improve the cultivation of cells, hybrid chitosan nanofibres, composed of 10% PEO, and 90% chitosan, have demonstrated structural integrity in water and cellular compatibility [47]. In the latter study, it was further shown that human osteoblasts / chondrocytes cells could adhere to and spread on the chitosan/PEO membranes. Hybrid scaffold composites such as PLGA (poly(lactic-co-glycolic acid) nanofiber / chitin nanoparticle (80/20, w/w), containing collagen, have also demonstrated cell attachment of fibroblasts and human keratinocytes [48].

The composited produced herein were also tested for their ability to support cell adhesion. As shown in Fig 5, 3T3 mouse embryonic fibroblasts were seeded on the binary composite fiber mat (this particular image corresponding to the chitosan-0.2 wt/wt %AgNPs samples) and incubated overnight, followed by staining with MitoTracker Red and DAPI to visualize cells using confocal fluorescence microscopy. MitoTracker is a cell-permeable dye that is taken up by mitochondria into the matrix in response to the transmembrane potential across the inner mitochondrial membrane [49]. DAPI, which binds to double-stranded DNA was utilized

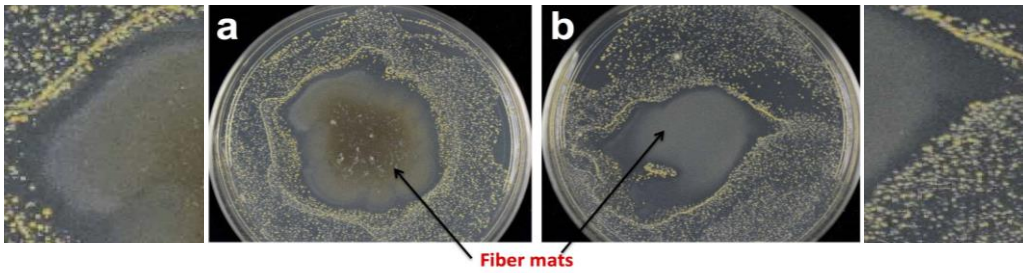

Fig 4. Antibacterial effect (a) Chitosan-0.2 wt %AgNPs with Staphylococcus bacteria and a (b) Chitosan- 0.8 wt % CA sample with Staphylococcus bacteria. The whitish and yellowish colors surrounding the mats indicate the presence of the Staphylococcus bacteria. The images shown to the right and left of (a) and (b) show a magnified section of the inhibition zone. The bacteria does not penetrate the mats

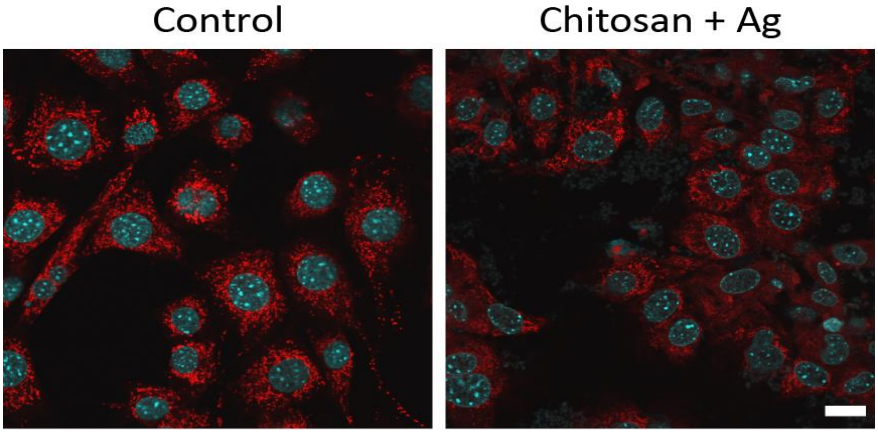

Fig 5. Confocal microscopy of 3T3 Mouse embryonic fibroblast cells. DAPI (blue) signaled for cell nuclei, while Mito-Tracker Red (MTR) signaled for mitochondria. Samples were composed of 3T3 cells seeded to glass coverslips (control) and 3T3 cells seeded to chitosan composite fine fiber mats. Size bar = 20 m

to ascertain cell viability and nuclear morphology. Normal cells typically show smooth and oval nuclei, whereas cells undergoing cell death (via apoptosis or other mechanisms) show blebbing or fragmentation of the nucleus [50]. In control cells grown on glass coverslips, cell nuclei are apparent and the mitochondria, shown by MitoTracker staining, are clearly apparent, indicating that the cells are fully viable. The fibroblasts are observed to grow with normal morphology, within the chitosan mats. Interestingly, the chitosan composite material appears blue via confocal imaging, either due to intrinsic blue fluorescence or through binding DAPI. These images demonstrate that 3T3 fibroblasts are viable on chitosan fibers, and suggest that the chitosan/ composite fibers may serve as an effective 3D environment that effectively mimics the extracellular matrix of skin while providing protection against bacterial infection.

CONCLUSION

Long, continuous and homogeneous chitosan fine fibers were successfully produced using the Forcespinning® technology. Binary composites were developed by introducing antibacterial agents to the chitosan solutions. AgNPs and cinnamaldehyde were added and the antibacterial effect against *Staphylococcus aureus* and cytotoxicity of the developed composite fiber mats was evaluated. However, preparation of these chitosan composite fibers (The chitosan/AgNP and chitosan/CA) showed loss in structural integrity when exposed to acidic environments. To maintain the structural integrity of the dressing, TA was incorporated into the chitosan fiber membranes, followed by alkaline treatment with a strong base. The morphology of the chitosan/ TA membranes showed a ribbon like network due to partial crosslinking, and demonstrated structural stability.

Both composite systems showed anti-bacterial activity, inhibition zones fluctuating between 5 to 10 mm were observed depending on the size of the fiber mat and no bacteria was found within the mats. The systems were also shown to be noncytotoxic, NIH 3T3 cells were able to grow normally on top / near the vicinity of the fibers. The results of this study indicate that produced chitosan/ composite membranes can serve as potential wound dressing materials given its antimicrobial activity and similarity to the extracellular matrix which promotes cell adhesion/growth.

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CONFLICT OF INTEREST

Author has no received research grants. The author declares that he has no conflict of interest.

REFERENCES

1. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: A major and snowballing threat to public health and the economy. Wound Repair Regen. 2009; 17(6): 763-771.
2. Sarhan WA, Azzazy HME, El-Sherbiny IM. Honey/Chitosan Nanofiber Wound Dressing Enriched with Allium sativum and Cleome droserifolia: Enhanced Antimicrobial and Wound Healing Activity. ACS Appl Mater Interfaces, 2016; 8(10): 6379-6390.
3. Transparency Market Research 2016. http://www.transparencymarketresearch.com/wound-dressing-market.html
4. Greiner A, Wendorff JH. Electrospinning: A Fascinating Method for the Preparation of Ultrathin Fibers. Angew Chem Int Ed. 2007; 46(30): 5670-5703.
5. Abrigo M, McArthur SL, Kingshott P. Electrospun Nanofibers as Dressings for Chronic Wound Care: Advances, Challenges, and Future Prospects. Macromol Biosci. 2014; 14(6): 772-792.
6. Min B-M, Lee SW, Lim JN, You Y, Lee TS, Kang PH, Park WH. Chitin and chitosan nanofibers: electrospinning of chitin and deacetylation of chitin nanofibers. Polymer. 2004; 45(21): 7137-7142.
7. Le HR, Qu S, Mackay RE, Rothwell R. Fabrication and mechanical properties of chitosan composite membrane containing hydroxyapatite particles. J Adv Ceram. 2012; 1(1): 66
8. Pillai CKS, Paul W, Sharma CP. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. Prog Polym Sci. 2009; 34(7): 641-678.
9. Rinaudo M. Chitin and chitosan: Properties and applications. Prog Polym Sci. 2006; 31(7): 603-632.
10. Ahmad M, Ahmed S, Swami BL, Ikram S. Adsorption of Heavy Metal Ions: Role of Chitosan and Cellulose For Water Treatment. Int J Pharmacognosy. 2015; 2(6): 280-289.
11. Simsek EB, Saloglu D, Ozcan N, Novak I, Berek D. Carbon fiber embedded chitosan/PVA composites for decontamination of endocrine disruptor bisphenol-A from water. J Taiwan Inst Chem Eng. 2017; 70: 291-301.
12. El Salmawi KM. Gamma Radiation-Induced Crosslinked PVA/Chitosan Blends for Wound Dressing. J Macromol Sci. Part A. 2007; 44(5): 541-545.
13. Ahmad M, Jayachandran M, Qureshi MA, Ikram S. Chitosan Based Dressings for Wound Care. Immunochem Immunopathol. 2015; 1: 1-6.
14. Ohshima Y, Nishino K, Yonekura Y, Kishimoto S, Wakabayashi S. Clinical application of chitin non-woven fabric as wound dressing. Eur J Plast Surg. 1987, 10(2): 66-69.
15. Okamoto Y, Kawakami K, Miyatake K, Morimoto M, Shigemasa Y, Minami S. Analgesic effects of chitin and chitosan. Carbohydr Polym. 2002; 49(3): 249-252.
16. Huang Z-M, Zhang YZ, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. Compos Sci Technol. 2003; 63(15): 2223-2253.
17. Chen Z, Mo X, Qing F. Electrospinning of collagen–chitosan complex. Mater Lett. 2007, 61(16), 3490-3494.
18. Chen J-P, Chang G-Y, Chen J-K. Electrospun collagen/chitosan nanofibrous membrane as wound dressing. Colloids Surf. A. 2008; 313-314: 183-188.
19. Jiang H, Fang D, Hsiao B, Chu B, Chen W. Preparation and characterization of ibuprofen-loaded poly(lactide-co-glycolide)/poly(ethylene glycol)-g-chitosan electrospun membranes. J Biomater Sci., Polym. 2004; 15(3): 279-296.
20. Ramakrishna S, Fujihara K, Teo W-E, Lim T-C, Ma Z. An Introduction to Electrospinning and Nanofibers; World Scientific Publishing Co. Pte. Ltd.: Hackensack, NJ, USA, 2005.
21. Sarkar K, Gomez C, Zambrano S, Ramirez M, Hoyos ED, Vasquez H, Lozano K. Electrospinning to Forcespinning™. Materials today. 2010; 13(11): 12-14.
22. Padron S, Fuentes A, Caruntu D, Lozano K. Experimental study of nanofiber production through forcespinning. J Appl Phys. 2013; 113(2): 024318
23. Weng B, Xu F, Salinas A, Lozano K. Mass production of carbon nanotube reinforced poly(methyl methacrylate) nonwoven nanofiber mats. Carbon. 2014; 75: 217-226.
24. Weng B, Xu F, Lozano K. Mass production of carbon nanotube-reinforced polyacrylonitrile fine composite fibers. J Appl Polym Sci. 2014; 131(11): 40302.
25. Rane Y, Altecor A, Nelson BS, Lozano K. Preparation of Superhydrophobic Teflon® AF 1600 Sub-Micron Fibers and Yarns Using the Forcespinningtm Technique. J Eng Fiber Fabr. 2013; 8(4): 88-95.
26. Xu F, Weng B, Gilkerson R, Materon LA, Lozano K. Development of tannic acid/chitosan/pullulan composite nanofibers from aqueous solution for potential applications as wound dressing. Carbohydr Polym. 2015; 115: 16-24.
27. Xu F, Weng B, Materon LA, Gilkerson R, Lozano K. Large-scale production of a ternary composite nanofiber membrane for wound dressing applications. J Bioact Compat Pol. 2014; 29(6): 646-660.
28. Xu F, Weng B, Materon LA, Kuang A, Trujillo JA, Lozano K. Fabrication of cellulose fine fiber based membranes embedded with silver nanoparticles via Forcespinning. J Polym Eng. 2016; 36(3): 269.
29. Ooi LS, Li Y, Kam SL, Wang H, Wong EY, Ooi VE. Antimicrobial Activities of Cinnamon Oil and Cinnamaldehyde from the

Chinese Medicinal Herb Cinnamomum cassia Blume. Am J Chin Med. 2006; 34(3): 511.
30. Geng X, Kwon O-H, Jang J.Electrospinning of chitosan dissolved in concentrated acetic acid solution. J Jang. Biomaterials. 2005; 26(27): 5427-5432.
31. Duan B, Dong C, Yuan X, Yao K. Electrospinning of chitosan solutions in acetic acid with poly(ethylene oxide). J Biomater Sci Polym. Ed. 2004; 15(6): 797-811.
32. Ohkawa K, Cha D, Kim H, Nishida A, Yamamoto H. Electrospinning of Chitosan. Macromol Rapid Commun. 2004; 25(18): 1600-1605.
33. Ignatova M, Starbova K, Markova N, Manolova N, Rashkov I. Electrospun nano-fibre mats with antibacterial properties from quaternised chitosan and poly(vinyl alcohol). Carbohydr Res. 2006; 341(12): 2098-2107.
34. Zhou Y, Yang H, Liu X, Mao J, Gu S, Xu W. Potential of quaternization-functionalized chitosan fiber for wound dressing. Int J Biol Macromol. 2013; 52: 327-332.
35. Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol. 2001; 71(2-3): 235-244.
36. Ooi LSM, Li Y, Kam S-L, Wang H, Wong EYL, Ooi VEC. Antimicrobial Activities of Cinnamon Oil and Cinnamaldehyde from the Chinese Medicinal Herb Cinnamomum cassia Blume. Am J Chin Med. 2006; 34(3): 511-522.
37. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol. 2004; 94(3): 223-253.
38. Rieger KA, Schiffman JD. Electrospinning an essential oil: Cinnamaldehyde enhances the antimicrobial efficacy of chitosan/poly(ethylene oxide) nanofibers. Carbohydr Polym. 2014; 113: 561-568.
39. Wakshlak RB-K, Pedahzur R, Avnir D. Antibacterial activity of silver-killed bacteria: the "zombies" effect. Sci Rep. 2015; 5: 9555.
40. Clement JL, Jarrett PS. Antibacterial Silver. Met. Based Drugs 1994; 1(5-6): 467-482.
41. Atiyeh BS, Costagliola M, Hayek SN, Dibo SA. Effect of silver on burn wound infection control and healing: Review of the literature. Burns. 2007; 33(2): 139-148.
42. Le Ouay B, Stellacci F. Antibacterial activity of silver nanoparticles: A surface science insight. Nano Today 2015; 10(3): 339-354.
43. Swathy JR, Sankar MU, Chaudhary A, Aigal S, Anshup, Pradeep T. Antimicrobial silver: An unprecedented anion effect. Sci Rep. 2014; 4: 7161.
44. An J, Zhang H, Zhang J, Zhao Y, Yuan X. Preparation and antibacterial activity of electrospun chitosan/poly(ethylene oxide) membranes containing silver nanoparticles. Colloid Polym Sci. 2009; 287(12): 1425-1434.
45. Min B-M, Lee G, Kim SH, Nam YS, Lee TS, Park WH. Electrospinning of silk fibroin nanofibers and its effect on the adhesion and spreading of normal human keratinocytes and fibroblasts in vitro. Biomaterials. 2004; 25(7-8): 1289-1297.
46. Noh HK, Lee SW, Kim J-M, Oh J-E, Kim K-H, Chung C-P, Choi S-C, Park W-H, Min B-M. Electrospinning of chitin nanofibers: Degradation behavior and cellular response to normal human keratinocytes and fibroblasts. Biomaterials. 2006; 27(21): 3934-3944.
47. Bhattarai N, Edmondson D, Veiseh O, Matsen FA, Zhang M. Electrospun chitosan-based nanofibers and their cellular compatibility. Biomaterials. 2005; 26(31): 6176-6184.
48. Min B-M, You Y, Kim J-M, Lee SJ, Park WH. Formation of nanostructured poly(lactic-co-glycolic acid)/chitin matrix and its cellular response to normal human keratinocytes and fibroblasts. Carbohydr Polym. 2004; 57(3): 285-292.
49. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol. 2010; 12: 119-131.
50. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, Bredesen DE, Brenner C, Castedo M, Cidlowski JA, Ciechanover A, Cohen GM, De Laurenzi V, De Maria R, Deshmukh M, Dynlacht BD, El-Deiry WS, Flavell RA, Fulda S, Garrido C, Golstein P, Gougeon ML, Green DR, Gronemeyer H, Hajnóczky G, Hardwick JM, Hengartner MO, Ichijo H, Jäättelä M, Kepp O, Kimchi A, Klionsky DJ, Knight RA, Kornbluth S, Kumar S, Levine B, Lipton SA, Lugli E, Madeo F, Malomi W, Marine JC, Martin SJ, Medema JP, Mehlen P, Melino G, Moll UM, Morselli E, Nagata S, Nicholson DW, Nicotera P, Nuñez G, Oren M, Penninger J, Pervaiz S, Peter ME, Piacentini M, Prehn JH, Puthalakath H, Rabinovich GA, Rizzuto R, Rodrigues CM, Rubinsztein DC, Rudel T, Scorrano L, Simon HU, Steller H, Tschopp J, Tsujimoto Y, Vandenabeele P, Vitale I, Vousden KH, Youle RJ, Yuan J, Zhivotovsky B, Kroemer G . Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 2009; 16(8): 1093-1107.
51. Bauer AW, Kirby WM, Sherris JC, Turck M . Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; 45(4): 493-496
52. Aguirre-Chagala YE, Pavon-Perez LB, Altuzar V, Dominguuez-Chavez JG, Munoz-Aguirre S, Mendoza-Barrera C. Comparative Study of One-Step Cross-Linked Electrospun Chitosan-Based Membranes. J Nanomat. 2017; 1980714.