Antibacterial multicomponent electrospun nanofibrous mat through the synergistic effect of biopolymers

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Abstract
The endeavor was to adopt a facile bi-layered approach to fabricate a novel PVA-chitosan-collagen-licorice nanofibrous mat (PCCLNM) with maintaining the spinning parameters and conditions to assess the synergistic antibacterial action of two biopolymers and having properties for repairing tissues. Bonding behavior, morphological orientation, antibacterial activity, and moisture management features of the electrospun nanofibrous mat were investigated using various characterization techniques. The FTIR analysis of the manufactured nanofibrous mat revealed characteristic peaks of licorice, chitosan, collagen, and PVA polymer, confirming the presence of all polymers in the sample. Additionally, a scanning electron microscopy (SEM) image attributes the development of nanofibers with an average diameter for top and bottom sides were 219 and 188 nm respectively. Furthermore, moisture management tests (MMT) confirm PCCLNM's slow absorption and drying capabilities. Apart from that, a disk diffusion method was used to investigate antibacterial activity against the bacteria Staphylococcus aureus (S. aureus), which revealed a strong presence of antibacterial activity with a 20 mm zone of inhibition due to the chemical constituents of licorice and chitosan compound. The developed bio-nanocomposite could have a potential application as wound healing material.

Keywords
Electrospun nanofibrous mat, PVA, chitosan, collagen, licorice, bacterial resistance

Introduction
In the contemporary world, bio-derived materials are a promising candidate for mitigation of detrimental effects on the environment, that is why various industrial fields like nanotechnology are interested in biopolymers to develop electrospun nanofibrous membranes for their eco-friendly, sustainable, renewable nature, biodegradability, biocompatibility and most importantly they do not have any deleterious effect on human health and our habitat universe.¹ For the production of semi-microns or nano-sized fibers of polymer, electrospinning is a viable technique. It has ushered in a new era in the field of nanotechnology which is one of the most flexible and cost-effective techniques in allowing the formation of long and continuous nanofiber under the influence of high electrical voltage.²,³ Nanofibers due to having large surface area and their pores structure suitable for wound healing material, scaffolds for tissue engineering, adsorption layers, nano-composites, fibrous catalyst substrates filter medium
and in protective clothing and nanowire, etc.\textsuperscript{4,5} Particularly in the biomedical application such as feasible wound healing material should possess some properties like moist maintaining condition to the injured area, absorption of the exudates that come out from the maimed surface and provides an excellent moist environment and offers the facility for prevention of microorganism growth. For enhancing wound recuperation function and augment the activities of wound healing, incorporation of a bioactive compound such as licorice and chitosan shows outstanding properties toward healing the wounded portion. It is well known that metal particles also exhibit good antibacterial properties but due to their harmful effects on the human body, the formation of electrospun nanofibrous mats from natural polymers have achieved much attention and these are being extensively used due to their availability, superfluity, biocompatibility, sustainability and most importantly renewable nature. For instance, cellulose and its derivatives have been used for the development of nanostructure membranes that could be used in different fields like biomedical application which includes artificial organs, contact lenses, and implants, reverse osmosis, dialysis and ultrafiltration.\textsuperscript{6} Nanofibrous mat fabricated from chitin can be utilized in tissue scaffolding or wound healings. Silk with chitin is known for its biocompatibility, biodegradability, and minimal inflammatory reaction,\textsuperscript{7} while PVA-sericin holds the promise for tissue engineering application.\textsuperscript{8} Moreover, developed nanofibrous mat from PVA-\textit{Nigella sativa} (\textit{N. sativa}) for wound healing,\textsuperscript{9} wound healing nanofibrous mats with antibacterial efficacy from honey and curcumin longa,\textsuperscript{10} PVA-\textit{Azadirachta indica} (neem) for biomedical application\textsuperscript{11,12} and mikania micrantha nanofibrous mat with augmented moisture management properties.\textsuperscript{13} Apart from these, garlic, aloe vera, honey, gelatin, alginate, fish scale, eggshells membrane, plant extract and herbs have also been used for developing nanofibrous mat.\textsuperscript{14,15}

Among many plants, licorice (\textit{Glycyrrhiza glabra}) is a plant of ancient origin. Moreover, it possesses a sweet taste and its height is approximately\textsuperscript{2} m.\textsuperscript{16} The roots are multi branched and thick cylindrical shape. Since ancient times, licorice has been extensively used as folk medicine. The roots of licorice contains commercially important chemical constituents such as lupiwinghitenone, glabrene glycyrrhizin, or glycyrrhizinic acid\textsuperscript{17}glabridin, glabrol, licoflavonol, 3-methoxy glabridin, glycerol, licoricone, formononetin, hispaglabridin\textsuperscript{18,19}glabranin isomer and narigenin.\textsuperscript{20} Extracts from licorice root may be used for the treatment of throat infections, peptic ulcers, joint diseases, immunodeficiency,\textsuperscript{21} tuberculosis, cancer, cough, respiratory diseases, endocrine disorders,\textsuperscript{22} diseases relating to the kidney,\textsuperscript{23} asthma, psoriasis, hemorrhoids and bronchitis,\textsuperscript{24} heart diseases, chronic hepatitis, ordinal diseases, epilepsy.\textsuperscript{25}

Chitosan is a natural polysaccharide biopolymer and deacetylation of chitin is required for achieving this bio-compound. As a natural polymer and having biocompatibility, non-toxicity, biodegradability, antibacterial activity, chitosan-based nanofibrous mats have been used in biomedical applications\textsuperscript{26,27} and tissue engineering fields.\textsuperscript{28,29} Additionally, it actives macrophages and promotes wound healing.\textsuperscript{30} Moreover, when chitosan sticks to a cell wall of gram-negative or positive bacteria, it circumvents the growth of cells due to penetration into the cells and thereby impedes the transformation of DNA into RNA. Therefore, the metabolism of bacteria can be suppressed. Moreover, it assists in natural blood clotting and helps reduce pain by blocking nerve endings. In the contemporary research field, chitosan-based materials have been produced as membranes, sponges fibers, hydrogels and scaffolds for healing wounds.\textsuperscript{31,32} Chitosan is a natural polymer and having activities against gram-negative and positive bacteria,\textsuperscript{33} it has been employed with various bio-derived and synthetic materials like neem, honey, silk fibroin, sericin, henna, starch, PVA, PEO among many others.\textsuperscript{34,35} Furthermore, specifically, some studied have been reported by different researchers such as a chitosan-based nanofibrous mat for antibacterial and antioxidative activity,\textsuperscript{36} chitosan composites for artificial skin,\textsuperscript{37} chlorhexidine in gel form and the addition of chitosan for recuperating wound,\textsuperscript{38} application on burn wounds by fabricated nanomot with glycerol plasticized chitosan/PVA blends,\textsuperscript{39} PVA/chitosan/starch electrospin mat for wound healing,\textsuperscript{40} chitosan-alkali lignin-based electrospun mat for wound healing application.\textsuperscript{41} However, those are disadvantages of the chitosan including difficulties in electrospinning, fast degradation rate and weak mechanical properties that could be modified by blending and crosslinking with several polymers such as PVA and PEO.

Collagen is an abundant natural protein and finds in mammals having structural integrity and being an important protein for connective tissues of mammals.\textsuperscript{42} From the viewpoint of structural contributions, it provides whole-body protein contents of almost 35\%. Furthermore, collagen is the vital component for different tissues of a body part such as bone, ligaments, tendons, cartilage. Due to its structure of triplehelical and having glycine amino acid content, it enhances the proliferation and attachment of cells and emulates the activity of the native cells.\textsuperscript{43,44} Eventually, in biomedical fabrication, collagen-based materials have become a thing of interest and promising candidates.\textsuperscript{45,46} Moreover, collagen possesses excellent biocompatibility,\textsuperscript{47} noninflammatory\textsuperscript{48} and low antigenicity\textsuperscript{49} characteristics that is why it can effectively be used for application in tissue engineering.\textsuperscript{50,51} Because of its good cell permeability, collagen incorporated nanofibrous mat can be employed in wound areas for repairing and regeneration of damaged tissue.\textsuperscript{52,53} Collagen is a protein polymer that exhibits good properties that enable the culture of muscle and lung cells, fibroblasts, spinal ganglion cell line. If repair, restoration of the structure of disrupted injured tissues is needed, collagen acts as an active bio-compound.\textsuperscript{54,55} Therefore, the Incorporation of collagen in the medicinal plant (e.g. licorice),\textsuperscript{56} chitosan could enhance bioactive sites in electrospun mats leading to increased migration.
Materials and methods

Materials

Licorice roots were collected from Gazipur, Bangladesh; being cleansed carefully using distilled water and then being cut into small pieces to ensure better penetration. Chitosan of medium molecular weight (75%–85% degree of deacetylation) was collected from Sigma Aldrich, USA; cationizer was collected from Fortune Top Pte Ltd. Collagen was purchased from Baoding Faithfull Industry, China. Acetic acid (99.7%) with reagent grade and methanol of 99% purity were collected from Merk, Germany. PVA was collected from Loba Chemical, India with 99% hydrolyzed granules form and its degree of polymerization, molecular weight and viscosity were 1700–1800, 115,000 and 26–32 cps respectively. All these chemicals were used without any further purification.

Preparation of licorice extracts

First of all, the dried licorice roots were made of fine powder by a grinder machine. Then 10 g of licorice powder was immersed into 125 mL of absolute methanol at the ratio of 1:12.5 and kept for 24 h. Next, for achieving clear extract suspended licorice was filtered by four-layer nylon mesh and the process was repeated twice to ensure better extraction.

Preparation of solutions

Firstly, adding 40 g collagen powder with 100 mL distilled water and stirred 12 h for preparing 40 weight % collagen solutions. Then, for making chitosan solution, 50 weight % acetic acid was taken and chitosan powder was added to make 3 weight % chitosan solutions. Additionally, with a magnetic stirrer and by maintaining the pH of 4, the solution of chitosan was stirred almost for 24 h to get a better solution. Since chitosan solution has surface tension that is why 1 mL cationizer was added to mitigate the surface tension and then stirred for a further 1 h. Next, the final spinning solution was made with the ratio of three components where licorice extract, chitosan, and collagen were 40 weight %, 40 weight %, and 20 weight % respectively. Therefore, the prepared solution contained 80 weight % of antibacterial components and 20 weight % of proteins. Basically, the ratio was used in order to develop a nanofibrous mat with better bacterial resistance and the ability to mend damaged tissues. Then, to get a homogeneous mixture the prepared solution was stirred for 12 h with a magnetic stirrer. Apart from this, 20 g PVA powder was dissolved in 200 mL distilled water and 70°C was maintained for 2 h for preparing the PVA solution.

Process optimization

An electrospinning setup having syringe pump of TL F6, Tong Li Tech, China; high voltage supply of –20 kV and +50 kV; heater of 0.50 kW; rotary drum diameter of 158 mm with 500 rpm; a syringe (30 mL); needles (20 gauge) and needle to collector distance being 15 cm as well as five needles were used for the experiments. A rotated collector was used to get the uniform alignment of the nanofibrous mat. Fabrication of the nanofibrous mat was accomplished using the bi-layered technique. This technique has been applied because of the adhesive characteristics of collagen which cannot be separated from the foil paper. Hence, PVA and licorice/collagen/chitosan layers were deposited separately on the same foil paper sequentially. An electrospinning machine was run for 20 h to fabricate the nanofibrous mat. This duration was divided into two segments first 10 h for the PVA layer and the next 10 h for the licorice/collagen/chitosan layer. The trial-and-error method was followed to obtain the optimum spinning conditions. The optimum spinning process was recorded at −12.75 kV, +24 kV, and 0.31 kW with a flow rate of 3–3.5 mL per hour under a relative humidity of 66% at room temperature 28°C. After completing the development process by depositing all solutions on the foil paper, the fabricated nanofibrous mat was scrupulously separated from the foil paper using forceps followed by removal of the foil paper from the collector drum.

Characterization

Structural analysis

Scanning electronic microscope (SEM), model: SU 1510, Hitachi, Japan was used to observe the diameter and orientation of nanofibers in fabricated PCCNLNM with a 6 k and 8 k times magnification with a voltage of 10 kV. Moreover,
the chemical structure of the developed PCCLNM was characterized using spectroscopy (IR Prestige21, Shimadzu, Japan) operating in the range of 400–4000 cm\(^{-1}\) while spectra were recorded with a resolution of 4 cm\(^{-1}\).

**Measurement of moisture management properties**

Moisture management properties were assessed by MMT machine, model of M290 following the principle of AATCC 195–2009. MMT was carried out to evaluate absorption rate: moisture absorbing time of the mat’s inner and outer surfaces; one-way transportation capability: liquid moisture one-way transfer from the inner surface to the outer surface and spreading/drying rate: speed of liquid moisture spreading on inner and outer surfaces of the fabricated nanofibrous mat.

**Antibacterial assay**

To assess the antibacterial properties and measuring the zone of inhibition of the fabricated nanofibrous mat disk diffusion method was employed against the bacteria namely *S. aureus*. To carry out the study, cultured bacteria (1000 CFU/mL of the *S. aureus*) were streaked on the agar plate. Then, an 11 × 11 mm diameter disk accompanied by nanofibrous mats was placed on the plate. Next, the agar plate was put into the incubator maintaining a temperature of 37°C for 18 h. The growth of the bacteria and the inhibition zone were observed. Next, the zone of inhibition was measured with calipers. Amoxicillin (10 µg) was used as the positive control in the antibacterial assay.

**Results and discussion**

**Structural analysis**

To obtain the nanofiber from the prepared solution, the parameters of electrospinning must be maintained, otherwise, the required nanostructure would not be achieved. For instance, when the voltage is less than 12 kV, no fiber is formed due to the incapability of overcoming the surface tension of the solution. Therefore, optimum electrospinning conditions for voltage, flow rate, needle orifice diameter, distance between needle to collector, heater power and ambient condition were set by several trails at −12.75 kV, 24.84 kV, 0.3 kW with flow rate of 3–3.5 mL/h. Since the collector distance affects the fiber diameter, 15 cm distance was maintained during the fabrication of nanofiber. The image of PCCLNM is given in Figure 1. To assess the nanostructure of the developed mat, the diameter of fibers for both sides of the nanofibrous mat have been measured and the average diameter have been found 219 ± 33 nm from the top side and 188 ± 30 nm from the bottom side of fabricated nanofibrous mat. Here, top side has higher average diameter than the bottom side. The differences due to different materials used for top and bottom layer of the bi-layered nanofibrous mat. Average diameters ensure the formation of a nanofibrous mat. Because, previous studies regarding natural polymer based nanofibrous mat showed average diameter range 175–245 nm and so on.\(^9,12,60\)

Functional groups of licorice, chitosan, collagen and PVA polymer in PCCLNM sample have been identified by infrared spectroscopy. From Figure 2 it is observed that the range of functional group region is 4000–698 cm\(^{-1}\) and the fingerprint region belongs to the range of 1000–698 cm\(^{-1}\). Here, the characteristic peaks have been observed in the PCCLNM sample at 1030 cm\(^{-1}\) (O-H bending), 1077 cm\(^{-1}\) (C-H stretching), 1662 cm\(^{-1}\) (C=O stretching) that indicate the presence of licorice components while 3412 cm\(^{-1}\) (O-H stretching), 2862 cm\(^{-1}\) (C-H stretching) and 1095 cm\(^{-1}\) (C-O-C stretching) peaks attributed to the presence of PVA polymer in the same sample. Moreover, a significant peak observed at 1530 cm\(^{-1}\) which exhibit the presence of amine band of chitosan in PCLNM sample. Besides, the peaks at 1642 cm\(^{-1}\) represents stretching vibration of the peptide carbonyl group (C=O) which indicated the presence of collagen constituents in the developed sample These are following the characteristics peaks of licorice, chitosan, collagen and PVA polymer as mentioned in the previous findings.\(^61–64\)

**Moisture properties**

The feasibility of electrospun nanofibrous mat as a wound-healing material demands that when it will be employed on wounded area, the mat ought to absorb the exudates from the wound surface and maintains an excellent moist environment and provides the ability for preventing microorganism growth. Additionally, it should exhibit better absorbing and quick-drying properties for providing thermal comfort to the human skin. To evaluate the moisture properties of PCCLNM sample has been shown in Figure 3. In this case, the wetting time of the top and bottom surface of PCCLNM sample was found as 5.242 and 5.523 respectively, which shows a little difference between the two surfaces. The bottom surface has taken a longer time than the top surface that may be due to the inferior wicking properties. Moreover, the absorption rate of the top surface is higher than the bottom surface, which is 9.7861 %/s for the top and 4.898 in the case of the bottom surface. This might be attributed to the presence of licorice/chitosan/collagen on the top surface that contributes to water absorption whereas a high concentration of PVA lessens the absorbency of the bottom surface. On the other hand, the wetted radius of both top surface and bottom surfaces are the same. Moreover, the spread-out speed is lower on the bottom than on the top surface. The poor spreading speed of the bottom surface is likely due to the higher
viscosity and adhesive nature of only the PVA-containing bottom layer. Hence, overall moisture management quality is poor based on a fair one-way transport index. The results of the moisture management test reveal that the licorice/chitosan/collagen-based nanofibrous mat possesses slow absorbing and slow drying properties.

**Antibacterial activity**

As far as the biomedical application is concerned, the antibacterial activity of the electrospun nanofibrous mat plays a crucial role. Hence, the antibacterial property of PCCLNM against the bacteria *S. aureus* has been elucidated in this segment. Figure 4 shows antibacterial property against gram-positive bacteria. The zone of inhibition has been observed 20 mm for PCCLNM which is excellent for any gram-positive bacteria. This finding is consistent with previous research on the antibacterial characteristics of licorice and chitosan reported by several researchers. Although electrospun nanofibrous mat from PVA/chitosan/neem extract showed ZOI 14.5 mm. But, the addition of licorice extract with PVA and chitosan enhances the ZOI against the bacteria *S. aureus* which indicates the contribution of licorice extract for enhancing bacterial resistance. Although the thick cell wall of *S. aureus* may hamper the action of the bioactive compound, the synergistic action of both licorice and collagen

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**Figure 1.** SEM image of the PCCLNM (a) Top side at 6k (b) distribution of fiber diameter of Top side (c) Bottom side at 6k (d) distribution of fiber diameter of bottom side.

**Figure 2.** ATR-FTIR spectra of collagen and PCCLNM respectively.
disrupts the resistance of bacteria cell walls. Antibacterial constituents of licorice have been shown in Figure 5. Licorice contains more than 20 triterpenoids and nearly 300 flavonoids which possess antiviral and antimicrobial activities. Therefore, these compounds prevent the activity of bacteria by disrupting the thick cell wall. Besides, chitosan also has some chemical constituents that act in the same manner. Apart from this, the nanostructure of fabricated nanofibrous mat hinders the intrusion of bacteria due to its mesh-like structure. Hence, the structure of nanofibrous mat together with synergistic actions of antibacterial chemical compounds of licorice and chitosan demonstrate a good antibacterial property. In addition, a previous study was carried out to assess the biocompatibility of PVA/licorice nanofibrous mat and the result showed good biocompatibility of the developed mat. To know the actual activities of the chemical compounds of licorice extracts and chitosan against both bacteria (gram-negative and gram-positive), further studies are required.

**Conclusion**

PCCLNM has been fabricated in this work following the bi-layer technique which is a unique technique for
developing nanofibrous mat. During the fabrication process, all relevant parameters were controlled meticulously and their optimum ranges were investigated as well. Characterization of the nanofibrous mat was done by SEM, FTIR, MMT, and disc diffusion method. Moreover, functional groups of licorice, chitosan, collagen and polymer have been confirmed in the PCCLNM sample by infrared spectra. In addition, the moisture management test result reveals the slow absorbing and slow drying properties which may lead to slow drying of wound liquid. Furthermore, the antibacterial activity test has exhibited a strong antibacterial activity as 20 mm zone of inhibition due to the synergistic effects of licorice and chitosan antibacterial constituents is formed. Since the findings of this study show the bacterial resistance, moderate moisture management properties and intrinsic biocompatibility of the bio-based nanofibrous mat. It can be concluded that the PCCLNM is a promising candidate to be used as a wound-healing material.

Figure 4. Formation of inhibition zone around developed PCCLNM.

Figure 5. Antibacterial constituents of licorice extract (glycyrrhizin (GL), liquiritigenin (LTG), glabridin (GLD), licochalcone A (LCA), licochalcone E (LCE)).
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