Antimicrobial Films based on Chitosan, Collagen, and ZnO for Skin Tissue Regeneration

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Abstract: Bacterial infections represent a health issue worldwide. Over the past years, major interest has been given to developing new antibacterial and regenerative materials due to the increasing number of infections with pathogenic strains and the alarming antibiotic resistance. Polymer films and membranes with protective or even anti-infectious activity were developed. Some of them were based on nanoparticles with the main advantage that the resistance's development only seldom appears. Considering the Collagenic nature of the skin and the beneficial properties of Chitosan, the two polymers were proposed to be used in developing nanostructured wound dressing loaded with ZnO nanoparticles. These nanostructured materials confer promising characteristics to be used as anti-infectious wound dressing being biocompatible, antimicrobial against C. albicans and S. aureus, and highly hydrophilic able to absorb over 2300% water, which confer the premises of maintaining proper humidity and exudate absorption during wound healing. Fibrillar structures with Chitosan, Collagen, and Zinc Oxide can be an alternative for tissue regeneration. Electrospinning was used to fabricate fibrillar structures consisting of doing Chitosan, Collagen, and Zinc Oxide. The Zinc Oxide was used to defend the wound against infections and the beneficial role of Zn²⁺ in enhancing cell activity. The morphology of the fibrillar structures was studied by scanning electron microscopy while Collagen integrity by FT-IR spectroscopy.

Keywords: electrospinning; antimicrobial activity; wound dressing; Collagen; Chitosan; ZnO nanoparticles.

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1. Introduction

The skin represents a barrier against microorganisms and external factors, having a receptor surface that ensures sensitivity. If small wounds appear, the skin can self-repair without further scarring and without any external intervention but, larger/deeper wounds,
caused, for example, by burns or injuries, can cause significant physiological disorder, leaving the body exposed to infections, in this case, medical interventions are required. Autografts, allograft, or synthetic skin substituents can be used, each of them with advantages and disadvantages [1].

Electrospinning is a simple method to develop fibrillar materials (fibers or mats) but usually with nano- / micrometric thickness [2]. This is why electrospinning is suitable for developing membranes and films. Over the past years, electrospun nanofibers have been studied widely due to big potential in a field like a wound dressing, drug release, and tissue engineering. These fibers have a large surface area-volume ratio and can have good porosity to remove the excess of exudates and promote fast healing [3].

The electrospinning technique is based on the electric field’s interaction with an electrically charged polymer (melted or dissolved), poured from a syringe (with a specific diameter of the needle) and recovered on the rotating collector. Due to the electric field, the charged polymers/polymer blends are spined or sprayed onto the collector depending on several factors related to the solution: pH and viscosity, conductivity, surface tension, molecular weight, etc., as well as some electrospinning parameters such as electric power and the distance between collector and needle (electric field), collector - needle geometry, scanning rate and spinning rate, needle size, and the environmental parameters, especially temperature and humidity [4]. The fibers' diameter can be from tens of nanometers to few microns depending on the above-mentioned factors as well as the nature of the polymer or polymer blends [5-7].

Chitosan can be applied in the medical field due to its natural origin and properties such as good biocompatibility, antioxidant activity, non-toxicity, antimicrobial activity, and proper biodegradation rate, highly water and exudate absorption capacity, etc. [8].

Collagen is the most important fibrous of ECM, representing 20-30% of total body protein and has a major role in regulating cell function, and can be found in many parts of the body. According to Myllyharju and Kivirikko [9], Collagen can be separated into 8 groups. However, the fibrillar Collagen (especially Collagen type I and III) is the most used in medical applications [10]. According to the literature, just Collagen type I, II, III, and IV has been electrospun, according to the literature [10]. Chitosan and Collagen can form a complex and are anticipated to mimic the constituent of ECM [11]. The interactions between Collagen and Chitosan have the potential to produce biomaterials with new or improved properties.

The Chitosan chain can wind around the Collagen triple helix. The entanglement of Chitosan and Collagen molecules can form a complex. More than that, Chitosan-Collagen can be bonded ionically. The two molecules can be able to form complexes with oppositely charged ionic polymers. Chitosan can modify Collagen's biological properties, forming a polyanion-polycation complex as well as new hydrogen bonds; interactions were observed at the macroscopic scale. The Chitosan-Collagen complex can be used to mimic the extracellular matrix components [12].

Due to its physical, chemical, and biological properties, zinc oxide can be considered a versatile material because of the high physical, chemical, and photo-stability, high electrochemical coupling coefficient, absorption of a wide spectrum of radiation, and high photo-activity in bare or especially in the doped form [13] [14]. Although ZnO's action mechanism is not fully understood, it has been successfully used as an antibacterial agent in wound dressings, tissue engineering, and food packaging [15-18].
Electrospun nanofiber-based on Chitosan, Collagen, and zinc oxide can be desirable for wound healing due to their specific therapeutic properties [19]. The ternary CS-Col-ZnO wound dressing was proposed and developed by electrospinning from the existent literature data. ZnO has good antibacterial properties and activities and present low toxicity. Using ZnO, Chitosan, and Collagen for skin injuries, it is possible to improve the wound dressing's biocompatibility and antibacterial activity and promote fast healing of the wound [20].

2. Materials and Methods

Chemicals - Chitosan (medium molecular weight) was purchased from Sigma-Aldrich; bovine Collagen gel, MW = 300 000Da was obtained in the National Institute of Leather and Footwear, Bucharest Romania, Zinc Nitrate dihydrate, 99% purity, was purchased from Sigma - Aldrich. All reagents were used without any purification. Double-distilled water was used.

2.1. Preparation of solutions.

All the solutions were prepared to assure a final Chitosan: Collagen: ZnO molar ratio of 1:1:1. First, Chitosan solution of 3 wt.% was prepared using aqueous acetic acid glacial solution 2% (AA) in the water at room temperature using magnetic stirring for 24 h. The pH of the Chitosan solution was acidic, around 3 [21].

Collagen gel of 3.47% was used and was added to the Chitosan solution and mixed well until complete homogenization, under mechanical stirring conditions. As a zinc oxide precursor, zinc nitrate hexahydrate was used and was added to the CS-Coll gel.

ZnO was prepared by a precipitation method. Two solutions were made: the first solution was prepared by dissolving Zinc Nitrate hexahydrate in water (or aqueous polymer solution). The second solution (A) was prepared by dissolving NaOH in water. The second solution (B) was added to the first solution dropwise under magnetic stirring [22].

In Figure 1, the technological flow of the preparation of the films is presented.

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**Figure 1.** The technological process flowchart for manufacturing Chit/Coll/ZnO membranes.
Two samples of Chitosan solution 3% with aqueous acetic acid glacial solution 2%, named P1 and P2 were obtained. The crosslinking process represents the difference between these 2 samples. P1 was obtained without crosslinking, while P2 was obtained by crosslinking with GA 1% after the electrospinning process was done. P3 represents the sample obtained from Chitosan+Collagen, crosslinking after the electrospinning process with GA 1%. For P4 ZnO (initially obtained by a precipitation method), the Chitosan solution was added under stirring mechanical conditions; after the electrospinning process, the sample for P4 was not crosslinking.

The solution of the P5 sample was made with Chitosan, Collagen, and ZnO. For this solution, we made 2 samples under the same conditions. One of the samples was crosslinking with Ammoniac, and the other was not. For the same investigations, we have used the sample without crosslinking and for other samples with crosslinking.

The obtained gels were loaded in a syringe of 15 mL, having a metallic needle of 20, 21, 22, and 23G. For the film deposition, the collector was coated with aluminum foil or baking paper. The best results were obtained on baking paper. All the results will be presented using the baking paper as support. The collector and the needle were connected to a power supply. Different electric fields were applied between the collector and the needle by keeping constant the distance between the needle and collector at 100, 110, or 120mm while the applied potential was 10, 16, 22, or 23kV, these fields being optimized in order to obtain a Taylor cone. To obtain electrospun fibers, several attempts have been made, which are listed in Table 1.

Due to high viscosity and low electric charge, Collagen itself is hard to electrospun, so nanofibers based on Chitosan (P1 and P2), Chitosan and Collagen (P3), Chitosan, and ZnO (P4) and Chitosan/Collagen/ZnO (P5) samples were obtained. For the Chitosan samples, we tried to obtain nanofibers without the crosslinking process (P2) and with the crosslinking process after the electrospinning process (P1). For all these attempts, we have used different process parameters, as we can see in table 1.

| Sample type and number | Abbreviation | Type of needle | Flow, ml/h | Applied voltage, keV | Collector speed, rpm | Time, min | Collection distance, mm |
|------------------------|--------------|----------------|------------|----------------------|---------------------|-----------|-------------------------|
| P1. Chitosan 3%         | Chit         | G23            | 2.5        | 16                   | 400                 | 60        | 110                     |
| P2. Chitosan 3% (crosslinking with Glutaraldehyde) | Chit (GTA) | G23            | 6          | 10                   | 380                 | 80        | 100                     |
| P3. Chitosan/Collagen   | Chit/Coll    | G23            | 2.5        | 22                   | 300                 | 100       | 120                     |
| P4. Chitosan/Zinc Oxide | Chit/ZnO    | G23            | 1.5        | 23                   | 350                 | 120       | 110                     |
| P5. Chitosan/Collagen/Zinc Oxide | Chit/Coll/ZnO | G20            | 8          | 16                   | 400                 | 45        | 110                     |

2.3. Characterization.

2.3.1. SEM analysis.

The morphology and diameter of the electrospun nanofiber were observed via a QUANTA INSPECT F50 scanning electron microscope equipped with field emission gun electron-FEG (field emission gun) with 1.2 nm resolution and an energy dispersive X-ray spectrometer (EDS) with an MnK resolution of 133 eV.

2.3.2. FTIR analysis.

The electrospun fibers were characterized by FTIR using a Nicolet iS50FT-IR (Nicolet, MA, USA) spectrometer equipped with a DTGS detector, which provides information with...
high sensitivity in the range of 4000 and 400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). All spectra were obtained by co-adding 32 scans, with the scanning time being 47 s.

2.3.3. Water absorption.

The fibers samples’ water absorption was evaluated by measuring the fibers sample's weight change during the adsorption if distilled water within 72 hours.

2.3.4. Antibacterial activity.

The antibacterial activity was evaluated against Staphylococcus aureus ATCC 6538 and Candida albicans ATCC 10231. An adapted growth inhibition protocol was used. Briefly, 0.5 Mc Farland suspensions (1.5x10\(^{8}\)CFU (colony forming units)/mL) were obtained form overnight cultures. The prepared suspensions were swab inoculated on Mueller Hinton agar and the obtained samples (previously sterilized by UV exposure for 30 min) were aseptically placed on the inoculated Petri dishes. Samples were incubated for 24h at 37\(^{o}\)C to allow microbial development. After the incubation period, diameter of inhibition zones were measured.

2.3.5. Biocompatibility study of the obtained surfaces.

UV sterilized samples were placed in 24-well plates, and 50,000 HEp2 cells in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum were added. The plates were maintained at 37\(^{o}\)C, 5%CO\(_2\), and humid atmospheres to allow cellular development. After 24 hours of incubation in the presence of the prepared polymeric nanostructured samples, the cells were fixed in cold ethanol, stained with propidium iodide, and analyzed under the Leica DFC 450C fluorescence microscope.

2.3.6. Enzymatic degradation.

It was evaluated by monitoring the samples' mass loss according to the time of exposure to the Collagenase solution. For this reason, the samples used for water absorption, after reaching the steady-state regarding the swelling, were exposed to Collagenase solution at 37±1\(^{o}\)C and acidic conditions (pH=1.5). The following relation determined the percent of hydrogel degradation:

\[
\text{% weight loss} = \frac{(W_i - W_t)}{W_i} \times 100
\]

where, \(W_i\) is the initial weight, and \(W_t\) is the weight after time t;

3. Results and Discussion

3.1. FT-IR analysis.

The characteristic spectra of Chitosan, Chitosan/Collagen, Chitosan/ZnO, and Chitosan/Collagen/ZnO are given in Figure 2. According to these spectra, one can identify the most important characteristic bands of the polymers as well as the broadband associated with the hydrogen bonds developed between the hydroxyl and amino groups of the polymers as well as due to the presence of humidity. The FT-IR analysis for Chitosan shows a peak around 1377.78 cm\(^{-1}\) for CH\(_3\) stretching and another peak around 1022.85 cm\(^{-1}\), which can be attributed to stretching vibrations of C-O in Chitosan [20]. With ZnO's addition into the polymer matrix, new peaks around 797.47, 661.34 cm\(^{-1}\) appeared, and other peaks are changed due to the
interaction between polymer and ZnO nanoparticles [23]. Band for free acetic acid, around 1700 cm⁻¹, was not identified, indicating adequate purification of this material [16].

Figure 2. FT-IR spectrum for P1 (Chit), P3 (Chit/Coll), P4 (Chit/ZnO) and P5 (Chit/Coll/ZnO) matrix.

3.2 Scanning Electron Microscopy (SEM).

In Figure 3, image A, A’ and A’’ the morphology of the P1 - Chitosan 3% sample at different magnification is presented. The sample P1 was not crosslinking; the working parameters can be found in table 1. One can observe smooth and homogeneous fibers but also droplets attached to these fibers. The diameters of the fibers are ranging between 25 and 66nm, while the droplets can reach diameters of hundreds of nanometers, usually having a fusiform shape. Such fusiform morphologies are usually because of the viscous nature of the Chitosan.

Figure 3. SEM micrographs of P1- Chitosan 3%.

In figure 4 the morphology of P3- Chitosan/Collagen sample is presented. It can see that the presence of Collagen leads to a great change of the morphology even if the fibrillar structure is maintained. The ternary samples, P5 - CS/Coll/ZnO, are quite similar to the sample P3, but important changes appear. These samples are decorated with agglomerates of nanoparticles with diameters of about 50-100nm.

3.3 Water absorption.

The water absorption data for Collagen, Chitosan(P1), Collagen-Chitosan (P3), Chitosan-ZnO (P4), and Collagen-Chitosan-ZnO (P5) are presented in figure 5. These data show comparable absorptions for all materials. It can observe a small regression of the water
absorption for the samples loaded with ZnO but remain higher than 2300%, which means that these materials will be efficient in water and exudate absorption and maintain the proper humidity of the wound during healing. It can notice that the highest absorption is for the Collagen-Chitosan sample. The water absorption is even higher than that of the components, which means that the electrospinning parameters are influencing the water absorption.

| Figure 4. SEM micrographs of P2 (Chit crosslinking), P3 (Chit/Coll) and P5 (Chit/Coll/ZnO). |
| P3. Chit/Coll crosslinking 500X | P3. Chit/Coll crosslinking 1000X | P3. Chit/Coll crosslinking 2000X |
| P5. Chit/Coll/ZnO crosslinking 500X | P5. Chit/Coll/ZnO crosslinking 40000X | P5. Chit/Coll/ZnO crosslinking 2000X |

**Figure 5.** Water Absorption for different samples.

The Chitosan/ZnO system has the lowest absorption, regardless of the time interval. Practically, all the samples reach a steady state at about 24h because the mass gain between 24 and 72h is between 0.12 for Chitosan (P1) and 2.54% for Chitosan/ZnO (P4).
3.4. Enzymatic degradation.

Enzymatic degradation can be useful in assessing the biodegradation ability of the membranes. Based on the data from Figure 6, the enzymatic degradation of Collagen occurs in less than 3 days. The samples with Chitosan show a loss of ~50% because Chitosan is not enzymatically degraded, and the COLL: Chit ratio is 1:1. The samples' degradation rate can be suitable for wound dressing applications. The samples being also able to provide Collagen for healing.

3.5. Biological assessments.

The biocompatibility was assessed in vitro by analyzing the interaction between the samples obtained with Hep-2 cells cultured for 24 hours on the obtained substrates. Staining of the 24-hour cell monolayer obtained on the analyzed substrates with propidium iodide allowed highlighting some morphology details of the adhered cells, which confirms their normal appearance and active proliferation on the investigated surfaces (Figure 7). Considering that the total number of cells did not differ between samples in the quantification with Trypan Blue (data not shown), we can say that all the samples are biocompatible.

The evaluation of the nanoparticle suspensions' antimicrobial activity (Figure 8) was performed by the adapted diffusimetric method, measuring the diameters of the zones of inhibition, noting that all three polymer samples used (5mm in diameter) had an inhibitory effect on microbial growth with slightly increased efficiency of ZnO samples. According to these data, it can conclude that all the samples loaded with ZnO have antimicrobial activity against both S. aureus and C. albicans. The best antimicrobial activity against S. aureus was obtained for the CS-Coll-ZnO, a significant improvement being observed for the materials obtained by precipitation in the presence of NaOH. The C. albicans strain was more sensitive to the antimicrobial membranes compared to S. aureus.
Figure 7. Fluorescence microscopy images of the Hep-2 cell monolayer developed on the tested materials’ surfaces (Coll gel; Chit-Coll P3; Chit/Coll/ZnO P5; and Chit/Coll/ZnO crosslinking P5”).

Figure 8. Diameters of inhibition zones, in mm, following qualitative testing of the antimicrobial activity.

4. Conclusions

Chitosan-based membranes were obtained by electrospinning at different spinning conditions. These formulations were enriched by Zn$^{2+}$, and finally, after spinning, Zn$^{2+}$ was converted into ZnO by precipitation in the presence of NaOH or NH$_3$. These membranes have good biocompatibility and good water absorption properties being able to maintain proper humidity at the wound area as well as to absorb a high amount of exudates. The enzymatic stability was also suitable for these materials for wound dressing applications. Most probably Collagen degradation is beneficial in healing. The obtained membranes were proved to induce...
a bacteriostatic activity against *S. aureus* and even bactericide *C. albicans* being promising materials for antimicrobial wound dressing, especially to protect against infections. Further works will be necessary to assess ZnO’s role in healing, and in vivo tests will be necessary.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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