Antimicrobial and Wound-Healing Activities of Graphene-Reinforced Electrospun Chitosan/Gelatin Nanofibrous Nanocomposite Scaffolds

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ABSTRACT: This study aims at preparing electrospun chitosan/gelatin nanofiber scaffolds reinforced with different amounts of graphene nanosheets to be used as antibacterial and wound-healing scaffolds. Full characterization was carried out for the different fabricated scaffolds before being assessed for their antimicrobial activity against Escherichia coli and Staphylococcus aureus, cytotoxicity, and cell migration capacity. Raman and transmission electron microscopies confirmed the successful reinforcement of nanofibers with graphene nanosheets. Scanning electron microscopy and porosity revealed that nanofibers reinforced with 0.15% graphene nanosheets produced the least diameter (106 ± 30 nm) and the highest porosity (90%), in addition to their good biodegradability and swellability. However, the excessive increase in graphene nanosheet amount produced beaded nanofibers with decreased porosity, swellability, and biodegradability. Interestingly, nanofibers reinforced with 0.15% graphene nanosheets showed E. coli and S. aureus growth inhibition percents of 50 and 80%, respectively. The cell viability assay showed no cytotoxicity on human fibroblasts when cultured with either unreinforced or reinforced nanofibers. The cell migration was higher in the case of reinforced nanofibers when compared to the unreinforced nanofibers after 24 and 48 h, which is substantially associated with the great effect of the graphene nanosheets on the cell migration capability. Unreinforced and reinforced nanofibers showed cell migration results up to 93.69 and 97%, respectively, after 48 h.

1. INTRODUCTION

Due to the abuse of using antibiotics, the world has started facing a great problem of running out of drugs owing to drug-resistant bacterial infections. Therefore, the field of drug-resistant bacteria has attracted many scientists’ attention recently to overcome such growing crisis worldwide. Skin is considered to be the first defensive and protective organ for the human body against many environmental or external hazards.

Skin is a soft organ possessing the highest surface area within the human body as it represents more than 8% of the human body surface area. Its main role is acting as a physical barrier to protect the anterior parts of the human body from any microbial infections or foreign particles. Skin is highly susceptible to damage or loss due to some skin diseases, injuries, and accidents. Skin can heal normally through the natural skin restoration phenomenon. However, many challenges overcome the proper and rapid wound-healing. These include possibility of microbial infection, moisture loss from wounds, and scar formation. Consequently, the healing time increases, leading to increasing the risk of more
complications of wounds, such as microbial contamination, gangrene, and sepsis, especially in diabetic patients.\textsuperscript{3,4} Therefore, it is highly recommended to always develop and use a proper wound dressing that can act immediately to prevent microbial infection and protect the wound during the different healing stages. For instance, a proper wound dressing should be highly antimicrobial to protect wound healing from infection. In addition, it should act as a scaffold to stimulate cell growth and regeneration to ensure adequate healing. Finally, it should also be able to absorb wound exudates to reduce inflammation within the wound area.\textsuperscript{1,5,6}

The traditionally followed approaches failed to achieve well-controlled rapid and proper wound healing. For instance, skin grafts failed to stop scar formation, in addition to their high cost, high morbidity rate, and high possibility of immune-rejection by the patient. Furthermore, traditional wound dressings failed to provide high porosity to guarantee appropriate oxygen perfusion within the wound and prevent water loss from the wound area.\textsuperscript{7,8}

Hence, developing highly porous wound dressings that can mimic the extracellular matrices of skin layers, provide a high-porosity scaffolding structure, and act as an antimicrobial protectant during wound treatment has become a mandatory step.\textsuperscript{9−12}

Scaffolds can be fabricated via numerous techniques such as self-assembly, phase separation, gas foaming, solvent casting, drawing, freeze-drying, solid-free forming, template synthesis, particulate leaching, and melt molding. However, these previously mentioned techniques failed to produce well-controlled nanostructures with controlled porosity and other physical properties.\textsuperscript{13−16}

Fabrication of nanofibrous scaffolds has recently attracted many researchers’ attention due to the unique properties of nanofibers such as their increased aspect ratio, “surface-to-volume ratio”, compared to their corresponding materials in their bulk form. Nanofibers have been fabricated previously through phase separation and self-assembly. However, these techniques were not efficient to develop scaffolds required to transport oxygen and mandatory nutrients to cells within the body due to their failure to control the pore size, dimensions, geometry, and the spatial orientation of the produced nanofibers.\textsuperscript{16}

On the other hand, electrospinning has proven to be the most convenient method for production of well-controlled nanofibers when compared to nanofibers produced by other traditional techniques. This is due to the high capability of the electrospinner to produce nanofibrous mats with well-control- lable interconnected structures, shape, geometry, nanofiber diameter, pore size, and volume.\textsuperscript{17,18}

Several studies showed that morphological features of electrospun nanofibers can be manipulated through controlling various electrospinning parameters that can be classified into: (a) solution parameters (viscosity, conductivity, surface tension), (b) processing parameters (flow rate, applied voltage, collector types, spinneret tip design and position), and (c) ambient or uncontrolled parameters (temperature, air velocity, humidity).\textsuperscript{1,6,19}

Electrospinning has been used in fabrication of either nanofiber made of one type of polymers, blended polymers or polymers reinforced with different nanofillers.\textsuperscript{20} Nanofiller reinforcements enhance the produced electrospun nanofibers’ properties such as porosity, antibacterial activity, and electrical and mechanical properties. Nanofillers can be metals and metal
oxides, e.g., silver nanoparticles and titanium dioxide or carbon-based materials, e.g., graphene nanosheets, carbon nanotubes, and cellulose nanocrystals. Among these nanofillers, graphene, a two-dimensional (2D) conformational crystalline structure with one-atom-layer thickness, has been reported to be a biocompatible material in several biomedical applications such as drug delivery, imaging, and biosensors, in addition to exhibiting antimicrobial activity with *Escherichia coli*.21,22

Chitosan (CS) is the second abundant polysaccharide produced by partial deacetylation of chitin in the presence of either NaOH or the deacetylase enzyme. It is a promising natural polymer for biomedical applications due to its high biocompatibility, good biodegradability, high swellability, and antimicrobial activities.10,23,24

Gelatin (GL) is another naturally derived FDA-approved polymer that possesses high hydrophilicity, biocompatibility, and biodegradability and low irritability, immunogenicity, and antigenicity. In addition, it has no carcinogenicity or toxicity. GL is produced through either alkaline or acid hydrolysis of collagen. Due to the presence of several functional groups on its surface, GL is easily chemically modified or cross-linked to other molecules or ligands. Finally, it enhances fluid diffusion and cell proliferation within its structure.25,26

Previous studies demonstrated that mixing CS and GL together led to formation of a promising blend composite material for biomedical engineering applications. This is mainly because of the electrostatic interactions between the positive moieties on the CS surface and the negative functionalities on the GL surface that prevent the interaction of CS with the negative moieties present on the cellular membrane surfaces. Thus, the cellular migration capacity is enhanced within the biomaterial surface. Moreover, the presence of hydrophilic GL increases the hydrophilicity of CS-containing materials, resulting in enhancing the cell adhesion and spreading on the fabricated biomaterial surface.27,28

Another study showed that reinforcement of nanofibers using nanofillers such as graphene nanosheets (GNSs) resulted in the formation of highly oriented structures and networks within the polymer matrices, which would change the electrical, mechanical, and thermal properties of the whole composite.29

### Table 1. CS/GL Composite Solution Reinforced with GNS at Different Concentrations and Their Physical Properties

| Electrospinning Solution | CS Solution Concentration | GL Solution Concentration | CS/GL Volumetric Ratio | Amount of GNS | Viscosity (cP) | Conductivity (mS/cm) |
|--------------------------|----------------------------|---------------------------|------------------------|---------------|---------------|----------------------|
| CS/GL 0.10% GNS-CS/GL | 3% w/v in 2% aqueous acetic acid solution | 25% w/v in 40% aqueous acetic acid solution | 3:7 (v/v) | N/A | 851.0 ± 3.17 | 2.22 ± 0.03 |
|                          | 0.10% | 1771 ± 3.97<sup>b</sup> | 2.33 ± 0.03<sup>b</sup> |
| CS/GL 0.15% GNS-CS/GL | 0.15% | 1880 ± 5.22<sup>b</sup> | 2.51 ± 0.02<sup>b</sup> |
| CS/GL 0.20% GNS-CS/GL | 0.20% | 1985 ± 5.23<sup>b</sup> | 2.73 ± 0.04<sup>b</sup> |

<sup>a</sup>Amount of GNS corresponding to the total amount of both CS and GL in the electrospinning solution.

<sup>b</sup>Significant difference between the unreinforced and reinforced CS/GL electrospinning solutions.
In the present study, as shown in Scheme 1, CS and GL were used as the main polymeric matrices to produce CS/GL nano fibers (CS/GL NFs) by an electrospinner. GNS was used as a reinforcement material for CS/GL solutions to enhance the different properties of the produced electrospun CS/GL NFs including their porosity and antimicrobial activity. The produced electrospun GNS-CS/GL NFs antimicrobial properties were then tested to determine the effectiveness of the electrospun NFs with two strains: *E. coli* and *Staphylococcus aureus*. In addition, the 4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to evaluate the biocompatibility of the created nano fibers. Similarly, the *in vitro* wound-healing assay was carried out in this study to evaluate the capacity of fibroblast cells to initiate migration in the presence of the fabricated scaffolds once a scratched area is induced.

### 2. RESULTS AND DISCUSSION

#### 2.1. Viscosity and Conductivity of Electrospinning CS/GL and GNS-CS/GL Composite Solutions

The viscosity and conductivity results of the electrospinning solutions summarized in Table 1 showed that both the viscosity and conductivity increased on increasing the amount of GNS incorporated within the CS/GL solution. The effect of GNS on increasing a solution’s conductivity has been previously reported with other composite solutions. Increasing the electrical conductivity of a material is an advantageous property for a material to be used as a scaffold for regeneration of certain types of cells, *e.g.*, neural cells. This returns back to the ability of a conductive material to provoke cell proliferation and propagation. Furthermore, since the main approach in tissue engineering is mimicking the target tissue for regeneration, developing a conductive material is essential for neural tissue regeneration.

#### 2.2. Morphological Examination

**2.2.1. Scanning Electron Microscope (SEM)**

SEM images showed that CS/GL films possess few irregular voids (pores), as shown in Figure 1a, unlike images of electrospun CS/GL NFs that possessed more regular void spaces (high porosity), as shown in Figure 1b, due to formation of regular uniform nano fiber interconnected meshes. Using the aforementioned electrospinning parameters, formation of more uniform free-beaded nano fibers was successfully achieved compared to what has been previously reported. This was justifiable by the decrease in the amount of the polymer solution ejecting from the spinneret tip in the case of the electrospinning solution loaded with nano fillers compared to the pure electrospinning solution. However, upon increasing the amount of GNS beyond 0.20%, beads started to appear among the produced electrospun nano fibers, as shown in Figure 1e. Moreover, the nano fiber diameter and distribution started to increase again to 163 ± 48 nm and from 72 to 258 nm, respectively, as shown in Figure 1e. Electrospinning was totally hindered upon increasing the GNS amount beyond 0.20% GNS. This could be attributed either to increasing the electrospinning solution conductivity, as illustrated in Table 1, or to the nonhomogeneous

![Figure 2](https://doi.org/10.1021/acsomega.1c05095)
distribution of the hydrophobic GNS among the hydrophilic matrix of CS and GL. This may also be the reason for the appearance of beads in the electrospun nanofibers containing 0.20% GNS, as shown in Figure 1e. This is quite similar to what has been previously reported about graphene oxide when incorporated previously in the hydrophilic matrix of poly(vinyl alcohol) nanofibers.29

The external morphologies of CS/GL NFs and different GNS-CS/GL NFs were examined by SEM after being cross-linked using glutaraldehyde to reveal the morphological changes that occurred during the cross-linking process. The images of the different nanofibers confirmed the successful cross-linking step, as shown in Figure 2a–d, as the nanofibers were able to form interconnected meshes or networks. In addition, it was found that the cross-linking process did not affect the porous structure or the morphological features of the prepared nanofibers. However, cross-linking increased their durability and feasibility in handling during further experiments and applications.

2.2.2. Transmission Electron Microscope (TEM). The TEM image of 0.15% GNS-CS/GL NFs showed the internal morphology of the nanofibers. These nanofibers were chosen for TEM imaging as they are the nanofibers containing the highest concentration of GNS that have been electrospun successfully into uniform nonbeaded meshes. TEM scanning revealed the successful reinforcement of CS/GL NFs with GNS nanofillers. This could be observed through Figure 2e where the yellow arrows point to the GNS flakes within the nanofibers. It could also be observed that the GNS are evenly and homogeneously dispersed within the nanofibers.

2.3. Fourier Transform Infrared (FTIR) Spectroscopy. FTIR confirmed the formation of a polycationic–polyanionic complex between CS and GL through hydrogen bonds within the nanofiber matrices, as shown in Figure 3. This can be illustrated by shifting of the amide and amino bands to 1690 and 3381 cm\(^{-1}\), respectively, due to the possible interaction among \(-\text{OH}, -\text{NH}_2\), and C=O groups abundant in the gelatin structure and \(-\text{OH}\) and \(-\text{NH}_2\) abundant on the chitosan structure, respectively.

2.4. Raman Spectroscopy. Appearance of the D-band, G-band, and 2D-band of graphene in the Raman spectrum of GNS-CS/GL NFs at 1469, 1696, and 2952 cm\(^{-1}\), respectively, confirmed the reinforcement of CS/GL NFs with GNS, as shown in Figure 4. However, the three bands are slightly shifted away from their normal location, which should have been observed at 1340, 1600, and 2700 cm\(^{-1}\), respectively. This could be attributed to the stress occurring due to reinforcement of hydrophobic graphene sheets within the hydrophilic CS/GL matrix. This is quite similar to what has been observed previously upon reinforcement of the poly(vinyl alcohol) matrix, which is also a hydrophilic polymer with graphene.29

2.5. Porosity. Porosities of the casted film and the different electrospun nanofibrous membranes were estimated, as shown in Figure 5. Porosity was increased around threefold (from 23% ± to 60) upon electrospinning the same material with the same amount instead of casting the film. This is due to the increased surface area of nanofibers compared to films. Furthermore, generating interconnected meshes of electrospun nanofibers led to the formation of numerous regular void spaces (pores) within the fabricated material, as shown in SEM images (Figure 1). Porosities of nanofibers kept increasing upon their reinforcement with GNS in 0.10% GNS-CS/GL NFs and 0.15% GNS-CS/GL NFs to reach 77 and 90%, respectively. This can be attributed to the decrease in the nanofiber diameter leading to an increase in the pore volume.
among them. The 0.15% GNS-CS/GL NF can be considered to be a promising material to be used as a scaffold material for biomedical applications according to previously published reports, which suggest that a material should possess a porosity range between 87 and 97% to be used as an ideal scaffold. However, upon increasing the GNS concentration to 0.20% in GNS-CS/GL NFs, the porosity started to drop down again to around 70%. This could return back to the formation of beads and the increase in the nanofiber diameter that was previously observed in the SEM images of 0.20% GNS-CS/GL NFs in Figure 1e. It can be concluded from the figure that the unreinforced and reinforced materials possessed a significantly higher porosity than casted films (marked with asterisks).

2.6. Swelling Percentage. Swelling percent of the CS/GL film reached 60% within the first 2 h and then reached a plateau until the end of the first 3 h. Interestingly, CS/GL NFs demonstrated up to 150% swelling capacity during the first 3 h. This proves that fabrication of the same material as nanofiber meshes rather than a casted film led to a noticeable increase (more than twice) in the swelling property. This can be attributed to increasing the surface area of the exposed material to PBS in the case of nanofibers compared to the exposed area of the casted film. Consequently, this allows the exposure of more amino groups of both CS and GL to PBS, thus forming more hydrogen bonds with water molecules, leading to increasing swellability. However, upon addition of 0.10, 0.15, and 0.20% GNS within the nanofiber matrices, the swelling percent started to decrease gradually to 135, 90, and 60%, respectively, during the first 3 h. The reasons may be as follows: (a) GNS’s inability to form hydrogen bonds with water molecules since its H atoms have no partial positive charges due to their linkage to C atoms, unlike positively charged H atoms of amino groups in CS and GL that are linked to N atoms, and (b) GNS’s presence within the CS/GL matrix may act as an obstacle for the exposure of amino groups of CS and GL to water molecules, thus hindering the formation of hydrogen bonding. The swelling profiles of all of the tested samples during the first 3 h were plotted to show the difference between their capacity to absorb PBS and swell. Furthermore, the swelling profile of 2 h was selected and plotted again to highlight the difference in performance of the different samples during the swelling experiment. The point of 2 h was selected to be replotted again as it is the point at which almost all samples showed a steady state for their swelling ability, illustrating their swelling maximum achieved during the experiment. Swelling percent values of both 0.10% GNS-CS/GL NFs and 0.15% GNS-CS/GL NFs are still acceptable since they exceed 100%, a considerable value for a biomaterial as previously reported. The significant increase in swellability of nanofibers indicates their hydrophilicity, which would help the materials as a scaffold to absorb any wound exudates to decrease inflammation.

Figure 6. (a) Swelling profile of the different fabricated scaffolds along 3 h and (b) swelling maximum after 2 h (ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

Figure 7. (a) Biodegradability of the different fabricated scaffolds over 21 days and (b) weight remaining percentage after the first, second, and third weeks (ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).
2.7. In Vitro Biodegradability. The results in Figure 7 show the in vitro biodegradability of the different fabricated scaffolds over 21 days. CS/GL NFs showed more rapid biodegradability than the CS/GL casted film due to the increase in surface area and porosity of the nanofibers allowing more PBS perfusion within the nanofibers. However, upon addition of increasing concentrations of GNS within CS/GL NF matrices, the biodegradability decreased, since GNS is a nonbiodegradable material when compared to CS and GL. Furthermore, the hydrophobicity of GNS would even prevent PBS perfusion within the nanofibers. It is concluded from the plot that the in vitro biodegradability of the same material increased significantly when fabricated in the form of nanofibers rather than a casted film. However, GNS reinforcement retained it again. It is interesting to slightly retain the in vitro biodegradability of the scaffold so it could withstand a longer duration of wound treatment.

2.8. Antibacterial Assessment. The 0.15% GNS-CS/GL NFs were selected among the three reinforced nanofibers for the antibacterial assessment due to their uniformity, narrowest diameter, least diameter distribution, and highest porosity.

Both bacterial strains’ growth (CFU enumeration) and growth inhibition percent are shown in Figure 8. As expected, the pure CS film (+ve control) showed the least number of *E. coli* and *S. aureus* colonies (Figure 8a and 8b, respectively) and consequently the highest growth inhibition percent after only 5 h, which continued until 24 h (Figure 8c and 8d, respectively). This is due to the proven ability of CS to possess several antibacterial mechanisms: (a) due to the presence of positively charged amino groups on the CS surface, it can interact with the negative moieties on the bacterial membrane surface, leading to changes in membrane permeability, releasing lactate dehydrogenase and glucose penetrate that finally leads to bacterial death; (b) CS can chelate metal traces and other negatively charged constituents inside the bacterial cells, leading to its death. 34,39

On the other hand, the pure GL film (−ve control) promoted both bacterial strains’ growth. This is in good agreement with some reported studies that confirmed the utilization of GL by some bacterial strains as a nutrient for their growth.40

Interestingly, upon blending CS and GL together in CS/GL NF membranes, there is a significant decrease in bacterial colonies (Figure 8a,b) and consequently a significant increase in bacterial growth inhibition (Figure 8c,d). This proves that the high antimicrobial activity of chitosan was sufficient to overcome the bacterial growth that has been noticed due to the abundance of gelatin. CS/GL NF membranes showed enhanced antibacterial activity against both strains when compared to the CS/GL casted film starting after the 5 h time and continued until the end of 24 h. The larger surface area of the CS/GL NF surface area exposed to the bacteria permits more interaction between the positive moieties on the CS surface and negative moieties on bacterial membranes.15 This led to a noticeable decrease in bacterial growth as shown in Figure 8a,b. GNS-CS/GL NFs showed a higher antibacterial activity than CS/GL NFs. This proves that the presence of GNS within the nanofibers enhanced the antibacterial activity of the fabricated nanofiber membranes. This is in good agreement with previously reported results that proved that GNS has good antibacterial activity owing to its structural sharp edges that are capable of damaging the bacterial membranes, resulting in bacterial death.41,42 This is considered a great advantage to prevent any possibility of microbial contamination of wound and consequently gangrene, sepsis, and even death.

Figure 8. (a) *E. coli* growth and CFU enumeration, (b) *S. aureus* growth and CFU, (c) *E. coli* growth inhibition percent, and (d) *S. aureus* growth inhibition percent. (ns *P* > 0.05, *P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001, ****P** ≤ 0.0001).
2.9. In Vitro Cell Viability and Adhesion. The MTT assay was done to confirm the biocompatibility of the fabricated scaffolds. As illustrated in Figure 9a, both CS/GL NFs and GNS-CS/GL NFs have no cytotoxicity when...
chitosan. Their synergistic effect would render the developed material to act as a promising antibacterial wound dressing that could protect the wound from any contamination or wound complications. Furthermore, cell migrations were found to be around 94 and 97% in the presence of unreinforced and reinforced nanofibers, respectively, within only 48 h, which confirms the rapid wound-healing activity of the developed material. Finally, it is concluded that the GNS-reinforced electrospun CS/GL NFs could be used as promising base materials for fabrication of antibacterial scaffolding materials for proper and rapid wound-healing purposes.

4. MATERIALS AND METHODS

4.1. Materials. Chitosan “medium molecular weight and 75–85% deacetylation”, gelatin “type A obtained from porcine skin”, and glutaraldehyde solution [grade I, 25% in H₂O] were purchased from Sigma Aldrich. Glacial acetic acid, 99.7% [ACS Reagent; M = 60.05 g/mol], and absolute ethanol [95%] were purchased from Sigma Aldrich, Germany. Phosphate-buffered saline (PBS) pH 7 was purchased from Oxford, India.

Both Difco LB broth medium and agar were purchased from Beckman Dickinson Company. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin EDTA, penicillin/streptomycin, and phosphate-buffered solution (PBS) were obtained from Lonza, Switzerland. S. aureus (ATCC 6538), “a Gram-positive bacteria”, and E. coli (ATCC 8739), “a Gram-negative bacteria”, were used to test antibacterial properties of some selected CS/GL nanocomposite NFs. Both strains were obtained from the Biotechnology laboratories at The American University in Cairo.

Sterile 24-well plates, tissue culture pipettes (5 and 10 mL), T 25 cm², T 75 cm², and pipette tips all were purchased from Grenier Bio-One, Austria.

4.2. Nanofiber Preparations. 4.2.1. Graphene Nanosheet (GNS) Suspension and Polymer Composite Solutions. GNS suspension and CS/GL composite solution have been prepared according to previously reported protocols. First, GNS was prepared through Hummer’s method by preoxidizing graphite powered using a concentrated solution of H₂SO₄, K₂S₂O₇, and P₂O₅. Then, the graphite was reoxidized using conc. H₂SO₄ and KMNO₄ solution to end up with graphene oxide nanosheets. Finally, graphene oxide was reduced to graphene nanosheets through the thermal reduction process. Afterward, a series of CS/GL composite solutions were prepared containing three different increasing concentrations of GNS nanofillers of 0.10, 0.15, and 0.2% (w/w) relative to the total weight of both CS and GL in the solution (Table 1). The final solutions were left on a magnetic stirrer at 500 rpm for at least 2 h to obtain homogenous viscous (GNS-CS/GL) solutions.

4.2.2. Electrospinning Setup and Experiments. Each solution has been fed in the syringe of an in-house-designed electrosprinner. The electrosprinner was composed of a high-voltage generator, a syringe pump, and a plate collector to obtain the corresponding nanofibers. The fed syringe (13.1 mm diameter) was placed in a horizontal position in front of a stationary metal collector. Then, the produced nanofibers were collected on aluminum foil sheets placed on top of the stationary plate collector for further characterization and application experiments.

Different electrospinning parameters (solution flow rate, applied voltage, distance between the spinneret tip and the mobile collector to collect the electrospun fibers, spin diameter, and feed rate) were applied to prepare the matrices.
stationary plate collector) were carefully adjusted to obtain uniform free-headed nanofiber matrices.

4.2.3. CS/GL Composite Nanofibers (CS/GL NFs). The previously prepared CS/GL composite electrospinning solutions were fed each separately into the syringe pump of the electrospinning setup. After manipulation of the electrospinning parameters to obtain a stable uniform jet of nanofibers, the recorded and adjusted parameters had a flow rate of 0.4–0.6 mL/h, applied voltage of 20–23 kV, and 18–22 cm distance between the spinneret tip and the stationary collector. Electrospinning was carried out at room temperature, where the humidity ranged from 30% to 53%.

4.2.4. GNS-CS/GL Nanocomposite Nanofibers (GNS-CS/GL NFs). Each of the CS/GL solutions containing increasing concentrations of GNS (0.10, 0.15, and 0.20) was fed into the syringe pump separately, and each was processed separately using the same aforementioned parameters to obtain the corresponding electrospun nanofibers.

4.2.5. CS/GL Composite Bulk Film by Solution Casting. The CS/GL composite film was prepared by casting on an aluminum foil sheet and left to dry inside a fume hood before being put inside a freeze-dryer to ensure complete dryness. The properties of the resulting CS/GL film were compared with their corresponding electrospun nanofibers.

4.2.6. Scaffold Chemical Cross-Linking, Preparation, and Punching. The CS/GL bulk film, CS/GL NFs, and GNS-CS/GL NFs were chemically cross-linked to decrease their water solubility so they could be further incorporated in characterization and antibacterial experiments. The cross-linking step was done through subjecting the fabricated films and nanofibrous matrices to the vapors of a 25% glutaraldehyde aqueous solution, as reported previously. However, in the present work, the cross-linking time was reduced to 12–16 h instead of 3 days. Afterward, all of the films were left inside the fume hood for 2 h and then immersed in deionized water for at least another 2 h to check for their water stability. Finally, they were left inside the freeze-dryer for 3 days to get rid of any solvent residuals. After complete dryness, scaffolds from each film were prepared using a cork-porer puncher of 12 mm diameter to be used in further characterization and in vitro testing.

4.3. Physical Properties of Electrospun CS/GL Composite and GNS-CS/GL Nanocomposite Solutions. Viscosity and conductivity of CS/GL and GNS-CS/GL solution series were measured using a rheometer (Brookfield Programmable) and a conductivity meter (ino Lab “WTW series, Cond 720”), respectively.

4.4. Morphology. External morphologies of the CS/GL composite film, CS/GL NFs, and GNS-CS/GL NFs were investigated using SEM (FESEM, Leo Supra 55—Zeiss Inc., Germany), where a sample of each of the fabricated materials was gold-coated and then examined on a SEM grid under a low vacuum. The diameter and polydispersity index of the formed nanofibers were estimated using Image J analysis software. On the other hand, the internal morphology of GNS-CS/GL was investigated using a transmission electron microscope (TEM, Tecnai G2 F20 U-Twin), where the nanofibers were electrospun directly on a TEM grid and then investigated in the presence of reduced vacuum.

4.5. Chemical Characterization. 4.5.1. Fourier Transform Infrared (FTIR) and Raman Spectroscopies. Fourier transform infrared spectroscopy “FTIR” (Avatar 360) was used first to examine the chemical structure of raw CS and GL samples. Each sample of the powders used (chitosan and gelatin) was individually mixed and then compressed with KBr into very slim discs to be examined in the range of 600–4000 cm\(^{-1}\). Afterward, small samples of each of unreinforced and reinforced nanofiber matrices were examined for their chemical composition using FTIR in the range of 600–4000 cm\(^{-1}\). A high-performance Raman analyzer (ProRaman—L, Model B) was used to examine 12 mm-diameter discs. This was done for both CS/GL NFs and GNS-CS/GL NFs. Raman bands at 352 nm laser exposure were used to confirm the successful reinforcement of CS/GL NFs with GNS.

4.6. Physical Characterization. 4.6.1. Porosity. Porosity is defined as the percentage of voids found inside the material solid matrix. The liquid displacement method was used to estimate the pore volume within the CS/GL film, CS/GL NF, and GNS-CS/GL NF matrices. In brief, a known amount of 12 mm-diameter discs of each material was added separately in a measuring cylinder. Then, a known volume of ethanol (\(V_e\)) was added inside the measuring cylinder. Afterward, the volume of ethanol was detected again (\(V_e')\). Finally, the immersed discs were removed (\(V_s\)) and the remaining amount of ethanol in the cylinder was recorded (\(V_e''\)). The experiment was conducted in triplicates.

The total volume of scaffolds (\(V_t\)) was estimated through

\[
V_T = V_2 - V_3
\]

\[\text{(1)}\]

The porosity (\(\chi\)) of each scaffold individually was obtained through

\[
\chi = \left(\frac{V_2 - V_1}{V_T}\right) \times 100
\]

\[\text{(2)}\]

4.6.2. Swelling Property. Hydrophilicity of a material is detected through detecting its swelling property. Briefly, a dry 12 mm disc of each type of scaffold was weighed individually (\(W_0\)) before being immersed in a well plate containing the PBS buffer solution (pH 7). After 1 h, the scaffolds were removed, left to dry on a filter paper in air, and then weighed again individually (\(W_s\)). This step was repeated every 1 h for three consecutive hours and then every 6 h until 24 h to guarantee reaching the swelling maximum. The experiment was carried out three times. Finally, the swelling percentage (%S) was calculated using

\[
S\% = \left(\frac{W_s - W_0}{W_0}\right) \times 100
\]

\[\text{(3)}\]

4.6.3. In Vitro Biodegradability. A dry 12 mm disc of each type of prepared scaffold was weighed individually to determine its initial weight (\(W_0\)). Then, they were kept inside a well plate containing a PBS buffer solution (pH 7) in a water bath for 21 days. Every 2 days, the scaffolds were removed, freeze-dried, and weighed again to estimate their final weight (\(W_f\)). The experiment was run three times, and then, the weight loss due to in vitro biodegradability was estimated using

weight percent loss = \(\left(\frac{W_0 - W_f}{W_0}\right) \times 100\)

\[\text{(4)}\]

4.7. Antibacterial Assessment. Two different bacterial strains—S. aureus (Gram-positive) and E. coli (Gram-negative)—were selected to test the antibacterial efficiency of the different fabricated scaffolds. The pure CS film was used
as a positive control since it was reported to show a high antibacterial activity,49−53 while the pure gelatin film was used as a negative control since it showed enhanced bacterial growth.46 The CS/GL casted film and the CS/GL NFs matrices were tested to compare the effect of fabrication of the material at the nanoscale form with its bulk form. Finally, 0.15% GNS-CS/GL NF matrices were selected among the three reinforced CS/GL NF matrices to be tested for their antibacterial activity since they showed the best structure and properties according to the characterization techniques carried out. For instance, they showed the best porosity, biodegradability, and swellability results, which suggested their capability to be promising materials for scaffold fabrication.

4.7.2. Bacterial Serial Dilution and Spread-Plating Method. Isolated colonies of S. aureus and E. coli were each added separately to 10 mL of a freshly prepared LB broth and then left in a shaking incubator at 37 °C for 18 h. The culture was then subcultured in fresh LB broth, and the colony-forming units (CFU) for S. aureus and E. coli were counted to be 53 and 550, respectively, when spread over an agar plate at zero time. Afterward, an amount of 2 mL of the bacterial suspension was added to each of the tested sterile materials in a 24-well plate and then left in the shaking incubator at 37 °C. The antibacterial activity of the tested materials was evaluated by the colony-forming unit (CFU) count method at 0, 5, 10, and 24 h. Serial dilutions in fresh LB broth for each sample were spread individually on an LB agar medium and incubated at 37 °C for 24 h. Finally, the viable count of the 10−5 dilution in each sample was used to calculate the bacterial growth reduction percent using eq 56

\[
\text{bacterial growth reduction percent} = \left(\frac{\text{viable count of control} - \text{viable count of test}}{\text{viable count of control}}\right) \times 100
\]

4.8. Cell Culture Assays. 4.8.1. Cell Line Maintenance. Normal human fibroblast cell line was grown as a monolayer in DMEM media supplemented with 4500 mg/L glucose, L-glutamine, sodium pyruvate, sodium bicarbonate, 10% fetal bovine serum (FBS), and 100 μg/mL streptomycin. The fibroblast cells were passaged in 75 cm2 tissue culture flasks and incubated in a 5% CO2 incubator at 37 °C. Trypsin (0.25%) containing 0.1% EDTA was used for cell detachment before passageing. Trypan blue was used in cell counting using a hemocytometer. All nanofiber samples were sterilized for 1 h using UV irradiation. Then, they were neutralized using PBS (pH 7.4) for 30 min to remove any acidic traces. A condition medium was developed by overnight incubation of each sample in DMEM at 37 °C, separately. The samples were investigated in triplicates, and the average of results was plotted as mean ± standard deviation.

4.8.2. Cell Viability and Adhesion. The previously reported ISO 10993-5 protocol using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was followed to determine the cell viability of normal human fibroblasts in the presence of CS/GL NFs and GNS-CS/GL NFs.55 In a 96-well plate, an amount of 200 μL of fibroblast suspension (1 × 104 count) was placed in each well to test the biocompatibility of both CS/GL NFs and GNS-CS/GL NFs each individually with the cells. The plate was left in a 5% CO2 incubator for 24 h. Afterward, the medium was withdrawn, and the wells were washed three times using DMEM (w/o) FCS before an amount of 200 μL of the MTT assay agent (5 mg/mL) was added in each well. The plate was left again in the same incubator for 6 h. After incubation, DMSO (100 μL) was added in each well and mixed well before detecting the purple color developed due to formation of formazan crystals. Cell viability and optical density were represented by the intensity of the developed purple color, which was assessed using a UV spectrophotometer at 595 nm wavelength. The percentage of cell viability was calculated using eq 6

\[
\text{cell viability} = \left(\frac{\text{mean optical density}}{\text{control optical density}}\right) \times 100
\]

For imaging, an amount of 1 mL of fibroblast suspension (1 × 104 count) was added on each of the scaffolds individually in a 24-well plate. The well plate was left in the CO2 incubator for 48 h. Afterward, the cells were fixed with 3.7% formaldehyde solution for 6 h, washed with ethanol 3 times, and then imaged for cell attachment.57

4.8.3. In Vitro 2D Wound-Healing Assay (Mechanical Wounding). The cell migration capability of seeded human fibroblasts in the presence of a fabricated nanofiber condition medium was determined using an in vitro scratch assay. A straight line scratch was made in the center of each sample's well using a p200 pipette tip. After wound induction, the cells were washed twice using PBS to get rid of any cell debris and replaced by 100 μL/well of each sample’s condition media. The induced wounds were examined at fixed interval time points (0 h, 24 h, and 48 h) using an Olympus IX70 Fluorescence Microscope. Image J analysis software was used to evaluate the wound closure manner of each produced scratch beginning from the 0 time point to the 48 time point. The wound recovery (%) was calculated according to eq 7

\[
\text{wound recovery} (%) = \frac{X_{T0} - X_{T_{\text{fix}}}}{X_{T0}} \times 100
\]

here, X_{T0} refers to the induced wound area at time 0 and X_{T_{fix}} is the wound area detected at a specific time interval point.

4.9. Statistical Analysis. All of the experiments were carried out in triplicates, and the results were expressed as mean ± standard deviation. The significant differences of all data were examined by one-way analysis of variance (ANOVA) and Student’s t-test. All of the calculations were done through GraphPad Prism software version 6.

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Notes

The authors declare no competing financial interest.

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