



Wound-dressing materials with antibacterial activity from electrospun polyurethane–dextran nanofiber mats containing ciprofloxacin HCl

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ABSTRACT

Dextran is a versatile biomacromolecule for preparing electrospun nanofibrous membranes by blending with either water-soluble bioactive agents or hydrophobic biodegradable polymers for biomedical applications. In this study, an antibacterial electrospun scaffold was prepared by electrospinning of a solution composed of dextran, polyurethane (PU) and ciprofloxacin HCl (CipHCl) drug. The obtained nanofiber mats have good morphology. The mats were characterized by various analytical techniques. The interaction parameters between fibroblasts and the PU–dextran and PU–dextran–drug scaffolds such as viability, proliferation, and attachment were investigated. The results indicated that the cells interacted favorably with the scaffolds especially the drug-containing one. Moreover, the composite mat showed good bactericidal activity against both of Gram-positive and Gram-negative bacteria. Overall, our results conclude that the introduced scaffold might be an ideal biomaterial for wound dressing applications.

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1. Introduction

In the management of certain types of open wounds, wound dressing plays a major role (e.g. traumatic, thermal, or chronic wounds). Ideal condition for microbial growth is provided by the moist, warm and nutritious environment of wound beds (Wright, Lam, Buret, Olson, & Burrell, 2002). Various substances such as toxins, proteases and pro-inflammatory molecules, which may cause an excessive and prolonged inflammatory response of the host tissues by the bacterial colonization and subsequent infection, this can seriously interfere with the wound healing process (Jones, Bowler, Walker, & Parsons, 2004) and (Leaper, 2006). Ideal antimicrobial dressings should have a number of features such as provision of a moist environment to enhance healing (Kerstein, 1994), and broad-spectrum antimicrobial activity, including activity against

antibiotic-resistant bacteria. Therefore, immediate care of skin wounds is important for prevention of microbial infection and trans-epidermal water loss leading to acceleration of wound regeneration (Sikareepaisan, Ruktanonchai, & Supaphol, 2011). Thus, restoration of skin barrier is crucial importance in the treatment of injuries.

The latest advances in the field of nanotechnology have enabled to fabricate nanofibrous constructs having architectural features and morphological similarities matching the natural extracellular matrix (ECM) (Jayakumar, Prabakaran, Nair, & Tamura, 2010). Electrospinning is a simple, versatile, cost-effective, and scalable system using high voltage electrical field to generate aligned or random nanofibers from several synthetic and natural polymers (Barakat, Kanjwal, Sheikh, & Kim, 2009; Barakat, Kim, et al., 2009). Wound dressing from electrospun nanofibers potentially offers many advantages over conventional processes (Supaphol et al., 2012). Generally, the ultimate goal of the nanofiber design is to provide an ideal structure that can replace the natural extra cellular matrix until the host cells can grow and synthesize a new natural cellular matrix. Due to the huge surface area and micro-porous structure of the electrospun nanofiber mats, they could quickly start signaling pathway and attract fibroblasts to the derma

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layer, which can excrete important extracellular matrix components, such as collagen and several cytokines (e.g. growth factors and angiogenic factors), to repair the damaged in the tissue. In addition, the unique electrospinning process can be invoked to impregnate the nanofiber membranes with antibacterial and therapeutic agents (Bölgen, Vargel, Korkusuz, Menceloglu, & Piskin, 2007; Hong, 2007; Prabakaran, Jayakumar, & Nair, 2012).

Dextran is a bacterial polysaccharide that consists of R-1, 6 linked D-glucopyranose residues with some R-1, 2-, R-1, 3-, or R-1, 4 linked side chains. Due to its biodegradability and biocompatibility, it has been used for various biomedical applications (Hennink & Van Nostrum, 2002). Compared with other biodegradable polymers, dextran is inexpensive and readily available. Most importantly, dextran is soluble in both water and some organic solvents. This unique solubility characteristic of dextran ventures forth the possibility of directly blending it with biodegradable hydrophobic polymers such as polyurethane (PU) to prepare composite nanofibrous membranes by electrospinning. Biodegradable hydrophobic polymers generally have good mechanical strength but lack cell affinity, however most of the hydrophilic polymers have high cell affinities; but they have low mechanical strength. So blending of biodegradable hydrophobic and hydrophilic polymers will overtake the shortcomings of the individual materials. PU is a commonly used candidate for wound dressing applications. PU is frequently used in wound dressings because of its good barrier properties and oxygen permeability (Lakshmi, Shalumon, Sreeja, Jayakumar, & Nair, 2010). Research has reported that semi-permeable dressings, many of which are PU, enhance wound healing (Woodley et al., 1993). The permeability to water is also important so that fluid from the wound does not build up between the wound and the dressing and that wound desiccation does not occur and the pore sizes of electrospun mats are well suited to this.

Ciprofloxacin HCl (CipHCl), a fluoroquinolone antibiotic, is one of the most widely used antibiotics in wound healing because of its low minimal inhibitory concentration for both Gram-positive and Gram-negative bacteria that cause wound infections (Tsou et al., 2005; Yu, Xu, Chen, Hao, & Jing, 2006) and the frequency of spontaneous resistance to ciprofloxacin is very low (Dillen, Vandervoort, Van den Mooter, Verheyden, & Ludwig, 2004).

Extensive studies have been conducted to develop biocompatible electrospun nanofibrous scaffolds for wound dressing applications. An electrospun nanofiber membrane containing antibiotic agents has been used as a barrier to prevent the post wound infections. The combination of both of these properties can result in a perfect wound dressing material. So in this work, a composite nanofibrous wound dressing material of PU–dextran loaded with CipHCl was obtained through electrospinning. This study involves the characterization of these nanofibers and analysis of cell growth and proliferation to determine the efficiency of tissue regeneration on these biocomposite polymer nanofibrous scaffolds along with the simultaneous antibacterial activity of CipHCl.

2. Experimental

2.1. Materials and methods

Polyurethane (PU ($M_w = 110,000$) Cardio Tech.Intern., Japan,) and dextran (from *Leconostoc mesenteroides*, average $M_w = 8500$ – $11,500$, Sigma–Aldrich) were used in making the solution. CipHCl (drug) was supplied from LKT laboratories, Inc., USA. PU (10 wt%) solution with 20 wt% concentration of dextran to PU and 10 wt% of drug to PU solution were used to prepare the composite nanofiber mats. A mixed solvent, DMF:THF (1:1) was used to prepare the composite polymer solution. CipHCl powder was added to the polymer solutions 2 h before electrospinning. The

concentration of dextran was 20% to the polymer (polyurethane). A high voltage power supply (CPS-60 K02V1, Chungpa EMT, South Korea) of 22 kV to the syringe micro-tip was supplied to electrospin the nanofibers, whereas a ground iron drum covered by polyethylene sheet served as counter electrode. In our study we used the conventional electrospinning setup, where the syringe has been kept inclined to flow the spinning solution. The tip-to-collector distance was kept at 15 cm. Polymer solution was fed to the 5 mL syringe with plastic micro-tip. A horizontal electrospinning set up was used. The addition of CipHCl significantly decreased the solution viscosity, so the inclination of the syringe was adjusted to protect the dropping of the solution and also prevent beads formation in the electrospun mat. Moreover, addition of CipHCl did not affect solution conductivity, so the electrospinning process was not affected. Finally, the PU–dextran–drug nanofiber mats were vacuum dried in an oven at 30 °C for 24 h to remove the residual solvent and this sample was used for further characterizations.

2.2. Characterizations

The morphology of the electrospun PU, PU–dextran and PU–dextran–drug loaded composite nanofibers was observed by using scanning electron microscopy (SEM, S-7400, Hitachi, Japan). The XRD patterns of pristine PU nanofibers and PU–dextran nanofiber mats with and without drug were determined by XRD (Rigaku, Japan) operated with Cu-K α radiation ($\lambda = 1.540 \text{ \AA}$). The bonding configurations of the samples were characterized by means of Fourier-transform infrared (FT-IR) spectroscopy. Thermogravimetric analysis (TGA, Perkin-Elmer, USA) was carried out for the samples using a platinum pan from 30 to 800 °C at a rate of 10 °C/min.

2.3. Cell culture

3T3-L1 fibroblasts (preadipocytes, Korean Cell Line Bank, Korea) were initially maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 $\mu\text{g/mL}$ penicillin, and 100 $\mu\text{g/mL}$ streptomycin in a humidified atmosphere of 95% air/5% CO₂ at 37 °C. The electrospun scaffolds on cover slips were ultraviolet (UV) sterilized, rinsed in phosphate-buffered saline (PBS) and soaked in cell culture medium overnight prior to cell seeding to facilitate protein adsorption and cell attachment. The fibroblasts were separated by trypsinization, centrifuged, counted using a hemocytometer and seeded on the scaffolds at a cell density of 2×10^4 cells/well and incubated at conditions suitable for cell growth. In order to observe cell attachment manner on composite nanofibers, chemical fixation of cells was carried out in each sample. After 1, 3 and 5 days of incubation, the scaffolds were rinsed twice with PBS and subsequently fixed in 2.5% glutaraldehyde for 1 h. After that, a sample was rinsed with distilled water and then dehydrated with graded concentration of ethanol, i.e., 20, 30, 50, 70 and 100% ethanol for 10 min each. Finally, the samples were kept in a vacuum oven and then sputter coated with gold for the cell morphology observation by using SEM.

2.4. MTT test

The viability of cultured fibroblasts was monitored on the third, sixth and ninth day of culture using the colorimetric MTT assay (Sigma, USA). The scaffolds were washed twice with PBS and were then treated with approximately 50 μL of the MTT solution (DMEM); the scaffolds, after mixing of the contents by side-tapping, were incubated at 37 °C for 2 h. The scaffolds containing MTT–cell mixtures were gently rocked to deposit the cells. The supernatant MTT solution was pipette out and then acid–isopropanol (95 mL isopropanol with 5 mL 3 N HCl) was added to the colored cell

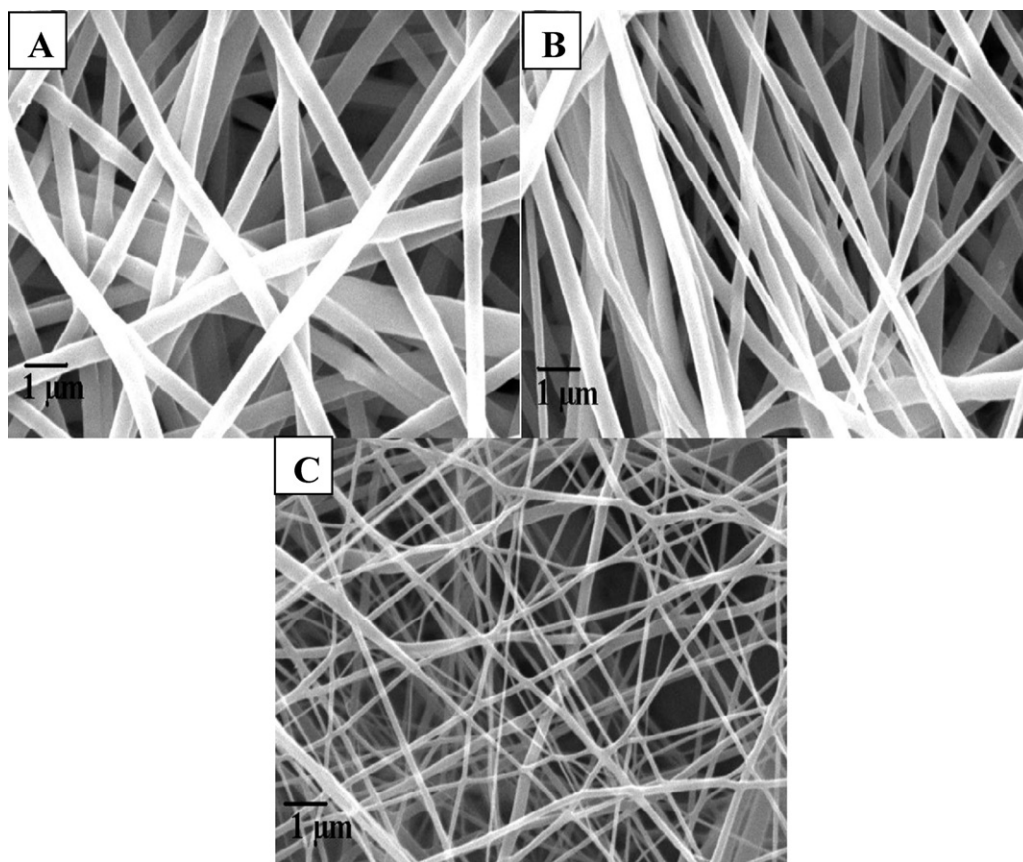


Fig. 1. SEM images of electrospun (A) PU, (B) PU-dextran and (C) PU-dextran-drug nanofibrous mat.

deposit. After gently mixing the acid–alcohol-treated scaffolds was then allowed to react for 5 min. One hundred microliter of the purple–blue colored supernatant that contained the solubilized formazan in each sample was added to a well in a 96-well plate for spectrophotometric analysis at 580 nm in an ELISA reader. The cytotoxicity of PU–dextran–drug loaded nanofibers was evaluated in comparison with pristine PU nanofibers. The cell viability was obtained by comparing the absorbance of cells cultured on the nanofibers scaffold to that of control well containing cells. The tissue culture coverslips were used as control for the study. The results were expressed as the mean \pm standard error of the mean. The data were analyzed via the Student's *t*-test and repeated measures of analyses of variance (ANOVA) test. A probability of less than 0.01 was considered to be statistically significant.

2.5. Antibacterial assessment

2.5.1. Microbial strains and culturing conditions

The following micro-organisms were used for antibacterial assessment study: *Escherichia coli* K12-MG1655, *Salmonella typhimurium*, *Vibrio vulnificus* CMCP6, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*. Preparation of bacterial inoculum as follows: The Gram-negative *E. coli*, *S. typhimurium* and *V. vulnificus* and Gram-positive bacteria *S. aureus* and *B. subtilis* were precultured in Luria–Bertani Broth (LB) overnight in a rotary shaker at 37 °C, centrifuged at 13,000 rpm for 2 min, pellet was suspended in sterile water and the cell density was standardized spectrophotometrically (A570 nm). The 100 μ L of diluted bacterial suspension (10^8 cells/mL) was seeded into respective medium in duplicate by spread plate method. The nutrient agar was used for *S. aureus* and *B. subtilis* and MacConkey agar used for *E. coli*. The thiosulfate citrate

bile salts sucrose agar (TCBS) was selective for *Vibrio* and *Salmonella* Shigella agar (SS) for *Salmonella*.

2.5.2. Antibacterial activity measurement

An inhibitory test of PU–dextran nanofibers containing drug, PU–dextran nanofibers and PU was tested by the disc diffusion method (Bauer, Kirby, Sherris, & Turck, 1996). The nanofibrous mats, PU–dextran–drug, PU–dextran and pristine PU, were cut into small circular discs of diameter around 5 mm each and denoted as A, B and C respectively. These discs were put on the surface of the Petri dishes. The inhibition zones were estimated after 0–240 min. PU–dextran loaded discs and PU only discs were placed as control discs on tested organism seeded plates. The antibacterial activity plates were incubated at 37 °C. The diameters of the inhibition zones were measured in diameter with transparent ruler.

3. Results and discussion

3.1. Morphological structure studies

Fig. 1A–C shows the SEM images of electrospun PU, PU–dextran and PU–dextran–drug loaded composite nanofibers respectively. These as-spun nanofibers exhibited a smooth surface and uniform diameters along their lengths. As seen in Fig. 1C, no drug crystals were detected by the electron microscopy on the surface or outside the fibers loaded with the drug, showing that CipHCl was loaded inside the nanofibers, and demonstrating a good compatibility of drug–polymer–solvent. According to the morphology obtained in Fig. 1, one can claim that the obtained nanofibers are beads-free. The resulting nanofibers also showed that the incorporation of the drug into the nanofibers not only dramatically decreased their

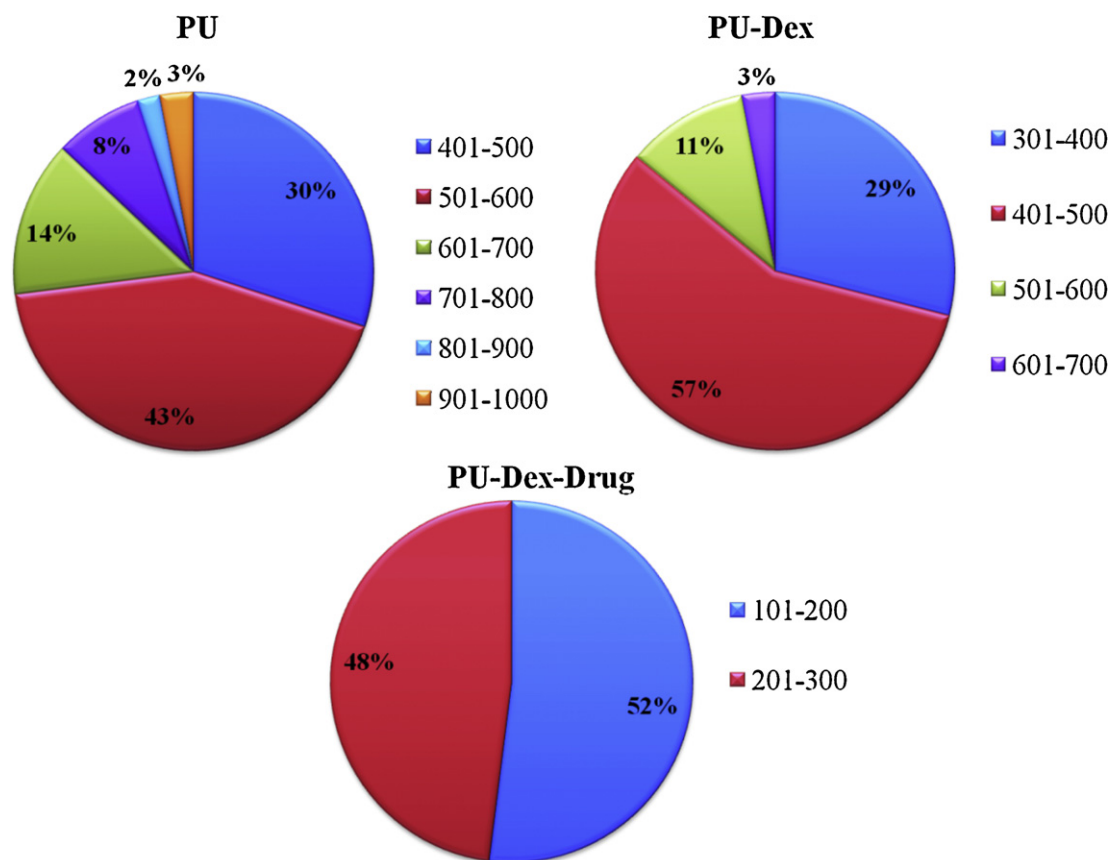


Fig. 2. Diameter distribution of the PU nanofiber, PU-dextran mat and PU-dextran-drug mat.

average diameter but also reduced the diameter distribution of electrospun nanofibers (Fig. 2). The addition of CipHCl can significantly decrease the solution viscosity; however, it did not affect solution conductivity. Solution viscosity influences the morphological structure and average size of resulting fibers (Thompson, Chase, Yarin, & Reneker, 2007). A possible explanation for the decreasing viscosity of the polymeric solution by the addition of CipHCl is that the drug could be trapped between polymeric chains, and thus acts as a plasticizer (Zamani, Morshed, Varshosaz, & Jannesari, 2010). The composite electrospun nanofiber mat produced in this study was desirably smooth and flexible. This flexibility, in addition to hydrophobicity provides easy handling during implantation and a comfortable texture for use.

3.2. Phase study

Pure electrospun PU, PU-dextran and drug-loaded PU/dextran nanofibers were studied by XRD in order to further illustrate the physical structure and distribution of the drug in the nanofibers. XRD patterns of pure PU nanofibers and PU-dextran nanofibers are shown in Fig. 3. Typical broad peaks were appeared around $2\theta = 20^\circ$ since they are amorphous polymeric in nature. Blended nanofiber mats loaded with 10% (w/w) CipHCl also have the same peaks at $2\theta = 20^\circ$ along with the CipHCl characteristic peaks at $2\theta = 19.04, 24.8, 26.6$ and 29.4° because of its regular crystallization (Marziyeh, Jaleh, Mohammad, & Maedeh, 2011), proving that loading the drug did not change the nature of the PU/dextran blend nanofibers.

FT-IR spectroscopy was used to investigate the changes of the functional groups that occur during the blending of drug and dextran with PU nanofibers. Fig. 4 illustrates the FTIR spectra of PU, PU-dextran and PU-dextran-drug loaded nanofibers.

The spectroscopy of electrospun polyurethane has characteristic absorption bands at $3320, 2960, 1710, 1530, 1220, 1110$ and 777 cm^{-1} , which represents $\nu_{(\text{N-H})}, \nu_{(\text{C-H})}, \nu_{(\text{C-O})}, \nu_{(\text{C-C})}, \nu_{(\text{C-C})}, \nu_{(\text{C-O})}, \nu_{(\text{C-H})}$, respectively (Jiang, Yuan, Li, & Chow, 2006). In addition to that several characteristic bands of dextran are located at 759 and 849 cm^{-1} (CH bend); 1272 cm^{-1} (C–O stretch); 1435 cm^{-1} (CH₃ bend); 2922 cm^{-1} (CH stretch); 3117 cm^{-1} (CH₃ stretch); 3335 cm^{-1} (OH stretch, end group), $1140, 1122$ and 1033 cm^{-1}

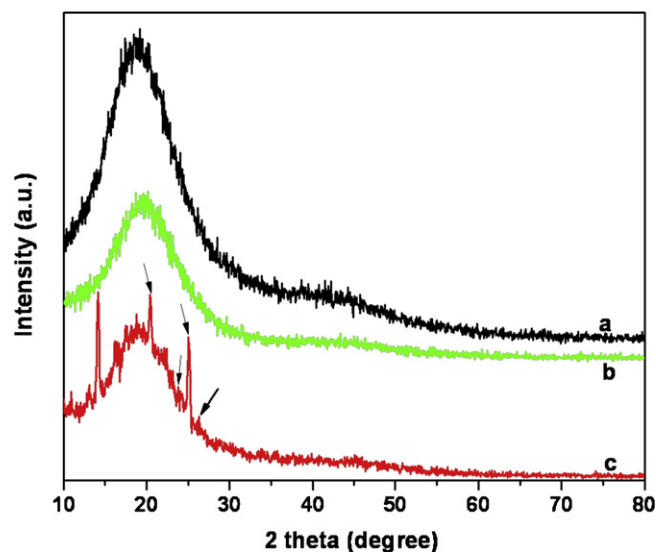


Fig. 3. X-ray diffraction of electrospun (a) PU nanofiber, (b) PU-dextran and (c) PU-dextran-drug (arrow denotes the peaks belongs to drug).

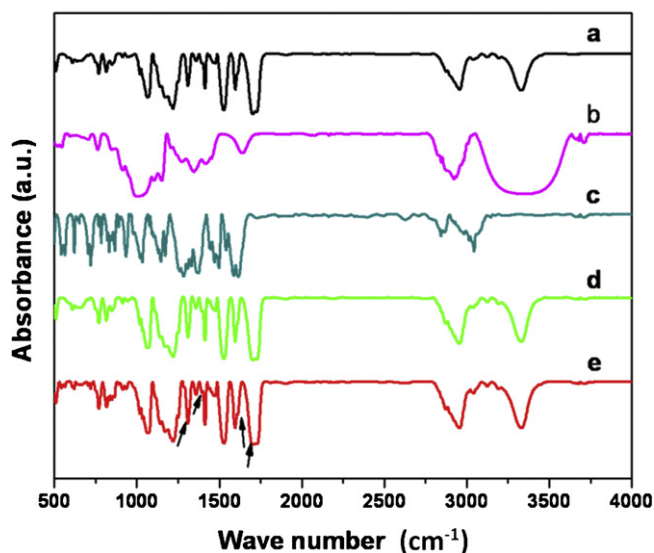


Fig. 4. FTIR spectra of electrospun (a) PU nanofiber, (b) dextran (c) drug, (d) PU-dextran and (e) PU-dextran-drug (arrow denotes the peaks belongs to drug).

(saccharide structure) (Shawki, Hereba, & Abeer, 2010). It can be seen that all the characteristic peaks of PU and dextran were visible in PU-dextran nanofibers and some peaks were being overlapped. The FTIR spectra of CipHCl-loaded PU/dextran nanofibers showed well resolved vibration bands (Fig. 4C). Within 1250–1850 cm^{-1} , the band positions of pure CipHCl were assigned to the C=O stretching vibration of carboxylic acid at 1702 cm^{-1} , the ketone C=O stretching vibration at 1616 cm^{-1} , and the coupling of the carboxylic acid C–O stretching and O–H deformation vibration at 1282 cm^{-1} (Trivedi & Vasudevan, 2007). The absorption band at

1374 cm^{-1} was due to the protonation of the amine group of the piperazine moiety (Gu & Karthikeyan, 2005). Some shifts were found in the FTIR spectra of CipHCl-loaded PU-dextran nanofibers. The shift of the band of the protonated amine group to 1393 cm^{-1} is suggesting the presence of electrostatic attraction between the protonated amine group and the polymer. In addition, the ketone stretching band shifted to a lower frequency of 1614 cm^{-1} , indicating reinforcement of the ketone C=O bond due to the release of intramolecular hydrogen bonding between the ketone group and the carboxylic group. Thus, a new hydrogen bond between the hydrogen of the carboxylic acid groups and the basal oxygen of the polymer could be formed and it shifts the peak to higher frequency of 1288 cm^{-1} . Most of the peaks of PU, dextran and drug have been overlapped in the PU-dextran-drug loaded nanofibers due to the relative similarity and shifting. When compared with pristine PU the peaks intensities were observed to be decreased with dextran and drug content. This may be due to the formation of hydrogen bonds among the components and the inter-hydrogen bonds formed between two different macromolecules were stronger than those formed between the molecules of the same polymer (Gonzalez, Guerrero, & Ortliz, 2000). Therefore, the inter-hydrogen bonds between PU, dextran and drug were prone to formation. So the blending of drug and dextran with PU has been confirmed with FTIR.

The TGA results (Fig. 5) provide quantitative information on the PU-dextran-drug and PU-dextran composite nanofibers functionalization. The TGA of PU-dextran-drug loaded nanofibers decomposed in a single step. However, the onset decomposition temperature for each sample was observed to be different as shown in Fig. 5. Pristine PU nanofibers had higher onset temperature ($\sim 300^\circ\text{C}$) among all samples, while the blended nanofibers were in the range between 250 and 290 $^\circ\text{C}$. The onset decomposition temperature decreased with the addition of dextran in the PU-dextran

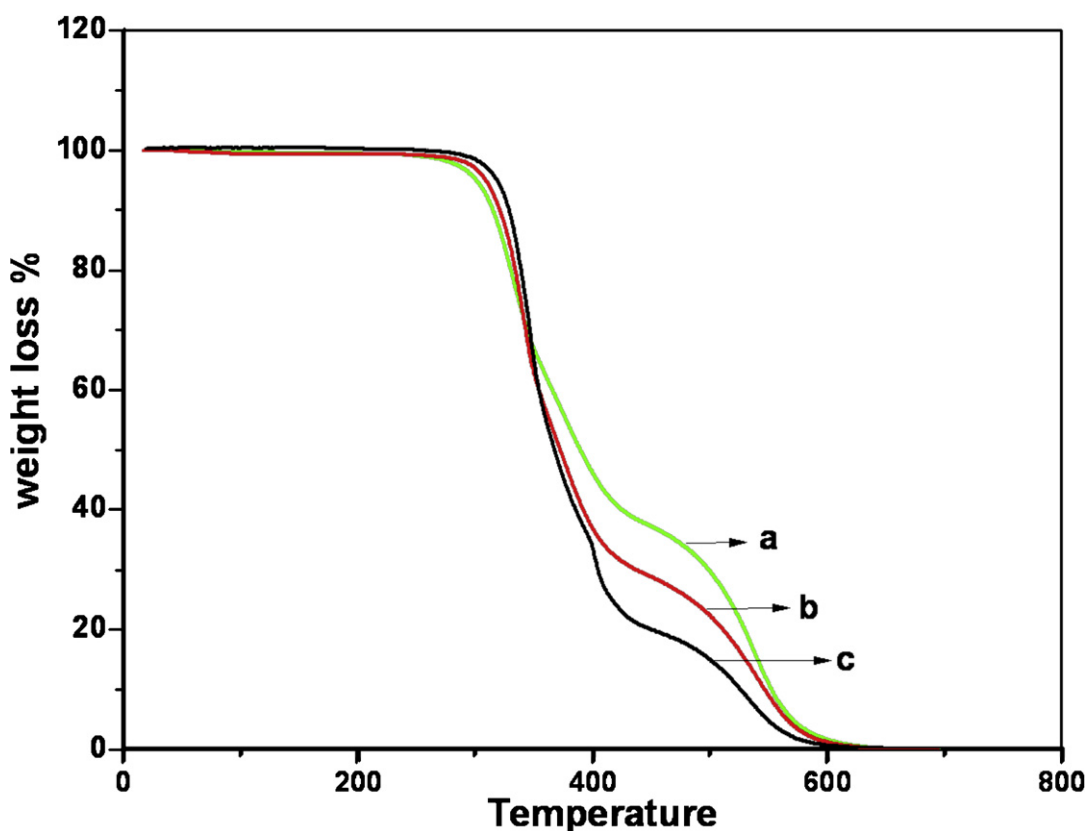


Fig. 5. TGA graphs of electrospun (a) PU-dextran, (b) PU-dextran-drug nanofibrous mat and (c) pristine PU mat.

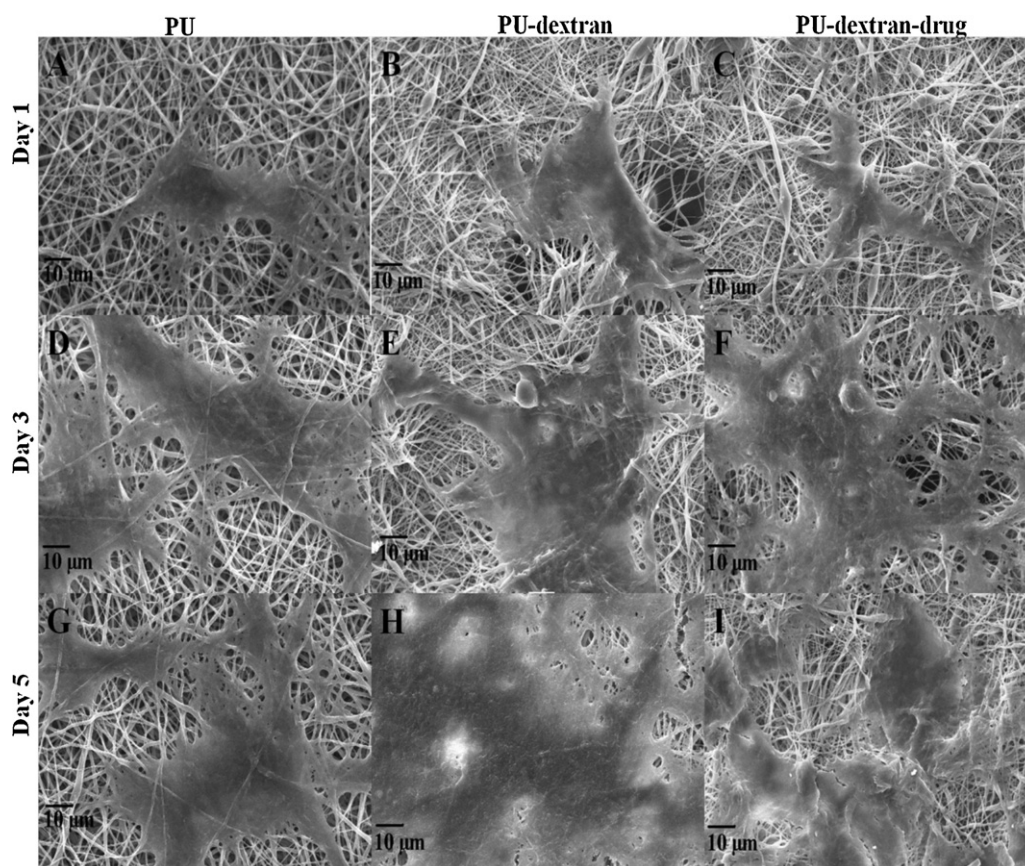


Fig. 6. SEM images showing the cell attachment on PU, PU-dextran and PU-dextran-drug after day 1 (A, D, G), day 3 (B, E, H) and day 5 (C, F, I) respectively.

blended nanofibers and a little enhanced in the PU-dextran-drug loaded nanofibers. This may be occurred due to the crystallinity of the drug molecules. The data obtained in this study demonstrated a significant difference in the thermal stabilities between the PU-dextran and PU-dextran-drug nanofibers in comparison to electrospun PU nanofibers. It is noteworthy mentioning that DSC analysis was carried out (data are not shown) for all formulations, there is no observable difference in the polymers behavior could be obtained which means that crystallinities of the polymers were almost not affected by drug incorporation.

3.3. Cell study

To confirm the cell viability, the morphological appearances of cells on composite nanofiber mats were obtained after 3 days of culture. Fig. 6 shows the SEM images of cell attachment manner on PU and PU-dextran blended nanofibers. The SEM micrographs of fibroblasts on PU and PU-dextran scaffolds obtained on 1, 3 and 5 days of culture showed a normal morphology of cell growth on the nanofibers (Fig. 6). Cell growth was higher on PU-dextran and drug loaded PU-dextran composite nanofibrous scaffolds than on PU nanofibers. Blending dextran with PU provides the scaffold high bioactivity and cell affinity for skin tissue regeneration. The cells spread over the scaffold fibers, linked with fibers by cytoplasmic extensions. It was observed that the cells were well incorporated into the composite nanofibers compared to the pristine PU nanofibers. The hydrophilic nature of the PU-dextran scaffolds is another reason for better adhesion and proliferation of fibroblasts. From this data, one can clearly confirm the cell attachment and cell spreading in the nanofiber matrix.

PU-dextran nanofibrous scaffolds were more suitable for growth of fibroblasts as compared to PU scaffolds, attaining a

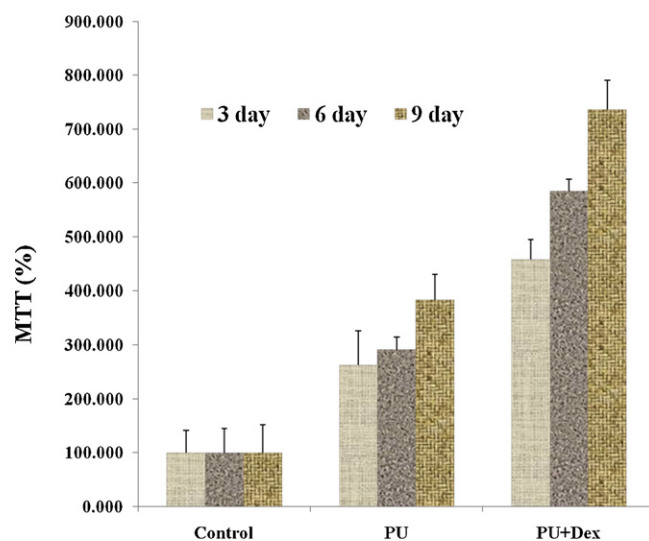


Fig. 7. MTT cell growth measurement assay. The viability of control cells was set at 100%, and viability relative to the control was expressed.

significant level of increase in cell proliferation after day 6 and day 9 of culture. PU-dextran nanofibrous scaffolds were more suitable for growth of fibroblasts as compared to PU scaffolds, attaining a significant level ($P < 0.01$) of increase in cell proliferation after day 6 and day 9 day of culturing and a percent level of increase up to 40% after day 9 of culture (Fig. 7). The percentages of cell proliferation increase on PU were only 10%. A large number of interconnected pores and the rough surface of the nanofibrous membrane support the proliferation and quicker regeneration of cells. In addition to

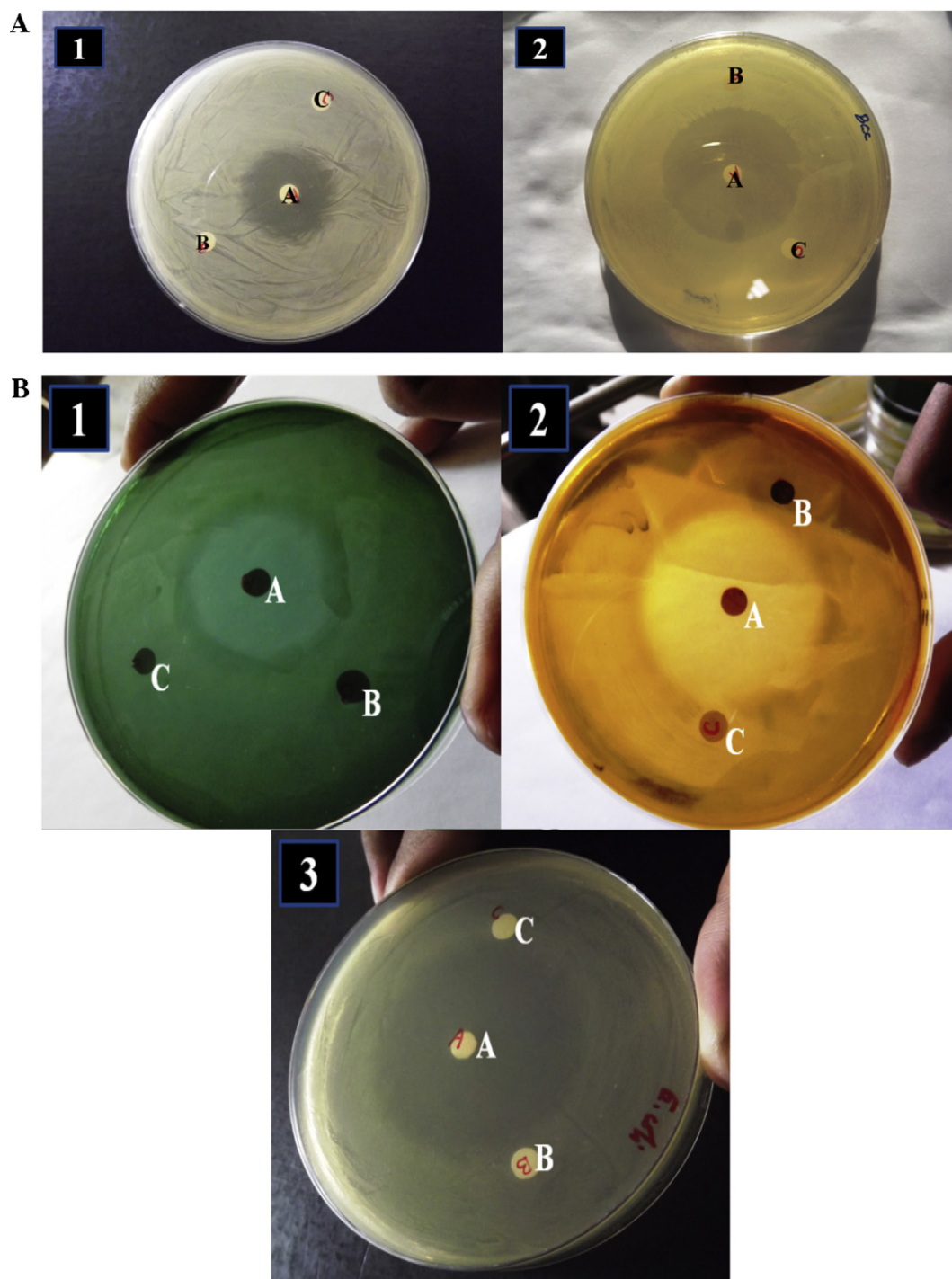


Fig. 8. (A) Bactericidal activity of PU-dextran-drug nanofibrous mat with Gram-positive *Staphylococcus aureus* (1), *Bacillus subtilis* (2), respectively. PU-dextran-drug, PU-dextran and pristine PU discs were denoted as A, B and C respectively in the Petri plates. (B) Bactericidal activity of PU-dextran-drug nanofibrous mat with Gram-negative *Escherichia coli* (1), *Salmonella typhimurium* (2) and *Vibrio vulnificus* (3), respectively. PU-dextran-drug, PU-dextran and pristine PU discs were denoted as A, B and C respectively in the Petri plates.

the porous nature and suitable mechanical properties, molecular signals from the nanofibers may also guide cells entering the cell substrate by their amoeboid movement (Breda, Jimenez-Kairuz, Manzo, & Olivera, 2009). PU is a biocompatible polymer and there are many available published reports about the cytocompatibility of PU. PU is a commonly used candidate for wound dressing applications because of its good barrier properties and oxygen permeability. These properties led to obtain good results for PU with

respect to control (Fig. 7). Because of these properties, PU was selected in our study. The error bars were based on three consecutive experiments.

3.4. Antimicrobial test of the composite nanofibers

The bactericidal activity indicated a clear zone of inhibition within and around the drug loaded nanofiber mat after an overnight

incubation of the agar plate at 37 °C. The growth inhibition rings of both Gram-positive and Gram-negative bacteria were measured. As shown in Fig. 8A(1, 2) for Gram-positive bacteria *S. aureus* and *B. subtilis* the mean diameter of inhibition ring of drug loaded PU–dextran composite nanofibers were around 15 mm and 20 mm respectively. In case of Gram-negative *E. coli*, *S. typhimurium* and *V. vulnificus* the diameters of the inhibition zones reached 20 mm as shown in Fig. 8B(1, 2, 3 respectively). No bactericidal activity was detected for pristine PU and PU–dextran nanofibrous mats. The drug loaded PU–dextran composite nanofibers showed excellent bactericidal activity against the wide range of bacteria, therefore avoiding exogenous infections effectively. It has been a known factor that the decontamination of exogenous organisms is a critical factor for a wound healing material; the antibacterial property plays a crucial role for the electrospun-based wound dressing membranes. As the interconnected nanofibers create perfect blocks and pores in nanofiber membrane, the nanofiber membrane should be able to prevent any bacteria from penetrating, therefore avoiding exogenous infections effectively. The results showed that our composite mat is a good antibacterial membrane and it can be applied as a perfect wound dressing material.

4. Conclusion

In this study continuous uniform nanofibers of PU–dextran and a blend of these polymers loaded with CipHCl were successfully electrospun. Addition of the drug reduced the size and narrowed the distribution of electrospun nanofiber diameters, which could be attributed to the decrease in solution viscosity. The addition of dextran into the PU increased the cell attachment and viability. These composite nanofiber mats showed very good antibacterial activity toward both Gram-positive and Gram-negative bacteria. This work provides a basic understanding of the design of efficient nanofiber-based antibacterial wound dressing material.

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