Hindawi
International Journal of Polymer Science
Volume 2019, Article ID 4362395, 8 pages
https://doi.org/10.1155/2019/4362395

*Research Article*

# Fabrication and Characterization of Chitosan–Vitamin C–Lactic Acid Composite Membrane for Potential Skin Tissue Engineering

**Ahmed Madni,[1] Romana Khan,[2] Muhammad Ikram,[3] Syeda Sohaila Naz,[4] Taous Khan,[3] and Fazli Wahid[1]**

[1]*Biotechnology Program, Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus 22060, Pakistan*
[2]*Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus 22060, Pakistan*
[3]*Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus 22060, Pakistan*
[4]*Nanoscience and Technology Department, National Centre for Physics, Quaid–i–Azam University Campus, Islamabad 44000, Pakistan*

Correspondence should be addressed to Taous Khan; taouskhan@ciit.net.pk and Fazli Wahid; fazliwahid@ciit.net.pk

Received 31 May 2018; Revised 11 December 2018; Accepted 18 December 2018; Published 27 January 2019

Academic Editor: Matthias Schnabelrauch



Recent advances in tissue engineering have potential for the development of improved substitutes for damaged skin tissues. Vitamin C and lactic acid are well-known wound healing accelerators while chitosan is an important biomaterial having wound healing capabilities. However, addition of vitamin C induces fragility to the chitosan–lactic acid membranes. Therefore, the current study was designed to fabricate an intact chitosan–vitamin C–lactic acid composite membrane that may synergize the critical properties of every individual component for potential skin tissue engineering. For this purpose, different concentrations of glycerol and polyethylene glycol (PEG) were added to strengthen the chitosan–vitamin C–lactic acid membranes. The prepared membranes were characterized by Fourier transform infrared spectroscopy, X–ray diffraction, and field emission scanning electron microscopy. Moreover, the biocompatibility of the prepared membranes was evaluated with fibroblast NIH 3T3 cells. The results showed that addition of glycerol and PEG has improved the strength of chitosan–vitamin C–lactic acid composite membrane. Characterization studies revealed the successful synthesis of chitosan–vitamin C–lactic acid composite membrane. Moreover, the prepared membranes showed excellent biocompatibility with NIH 3T3 cells. However, it is important to note that cells showed more attachment and spreading on porous chitosan composites membranes as compared to nonporous membranes. This study provided a base for the development of an intact chitosan–vitamin C–lactic acid composite membrane for skin tissue engineering. However, further preclinical and clinical studies are required for its practical applications in skin tissue engineering.

## 1. Introduction

In recent past, tissue engineering emerges as an alternative to the conventional treatment of skin burns, which have a lot of associated problems and limited efficacy. Skin tissue engineering involves the replacement of damaged or injured tissues to restore the normal anatomy and physiology of the skin tissues [1]. It involves the development of three–dimensional scaffold consisting of any biomaterials, nanoparticles, or chemical transducers loaded with cells which mimic the native tissues to be repaired. Skin tissue engineering is used to treat skin injuries including extensive burns and deep wounds. The traditional treatments are unable to cure such cases; therefore, clinical practices are heading to utilize the skin tissue engineering platform to treat severe burn and deep wound by speeding up epithelialization and collagen formation [2].

Chitosan is the second most abundant natural polymer after cellulose. It possesses physical properties like high surface area, tensile strength, porous structure, and conductivity. It can also be easily designed into various forms such as membranes, films, fibres, sponges, beads, powder, gel, and

solutions. Chitosan has a wide range of bioactivities and biocompatibility along with nontoxic, biodegradable, and nonallergic nature. Due to these properties, chitosan and its derivatives find many potential applications in the biomedical field. Chitosan–based biomaterials are used in tissue engineering as they offer structural and mechanical properties to the damaged tissue. It is used as an excipient for the delivery of active biomolecules or ingredients, while chitosan microspheres are used for the control release of drug. Chitosan acts as a dietary supplement for lowering the serum cholesterol which results in controlling obesity. Similarly, chitosan is used in the treatment of cardiovascular and age–related diseases. Moreover, it also finds applications in the water purification and food industry [3].

Vitamins are organic substances, which are important for various body functions and are used as cofactors for certain enzymes [4]. Vitamin C, also known as ascorbic acid, is water-soluble. It acts as a cofactor for collagen synthesis and other organic constituents of the intracellular matrix of skin, bone, and other connective tissues [5]. Ascorbic acid is essential in hydroxylation of proline and lysine residues, which results in collagen formation. Hydroxyproline maintains the triple–helix structure of collagen [6]. It is involved in all phases of wound healing; in the inflammatory phase, it assists in neutrophil clearance while in the proliferative phase it helps in synthesis and maturation of collagen [7].

Lactic acid is the most abundant hydroxycarboxylic acid. It helps in wound healing by increasing collagen deposition and endothelial cell migration and stimulates angiogenesis [8]. It has antibacterial activity against *Salmonella typhimurium* and *Escherichia coli* O157:H7 and thus may act as a potential antimicrobial compound [9, 10]. Previously, lactic acid was used as solvent in the preparation of the chitosan membrane as it improves the quality and thickness of the membrane [11].

Glycerol or glycerin is a polyol compound, which is a colourless, viscous, and odourless liquid. It is nontoxic and sweet in taste. It possesses three hydroxyl groups which makes it soluble in water. Glycerol is widely used in the food and pharmaceutical industries [12]. Glycerol is used as a vehicle for drug delivery and exhibits limited antimicrobial activities [13]. Polyethylene glycol (PEG) is a water–soluble polymer. It exists in different molecular weights, namely, PEG 40, PEG 1000, PEG 10000, and PEG 20000. PEG is widely used in drug delivery and can play a useful role in the medical field because of its biocompatibility, minimum toxicity, and solubility in water. It can be copolymerized with a linear aliphatic compound such as polylactic acid to improve the biocompatibility of the polymer. The resultant polymer can be used in tissue engineering and in drug delivery systems. Moreover, PEGs improve protein adsorption, cell adhesion, and proliferation [14, 15].

The main purpose of this study was to synthesize an innovative intact chitosan–vitamin C–lactic acid composite as an advanced material for skin tissue engineering. Glycerol and PEG were added to increase the strength of the membranes. The prepared membranes showed excellent biocompatibility with fibroblast cells. These results suggested that intact chitosan–vitamin C–lactic acid composite membranes can be prepared with the addition of glycerol and PEG which could be used for skin tissue engineering.

## 2. Materials and Methods

*2.1. Materials.* Chitosan powder was purchased from MP Biomedicals, Illkirch, France. Lactic acid, vitamin C, and sodium hydroxide (NaOH) were obtained from Daejung, Korea. Glycerol was purchased from BDH Laboratory Supplies, England, and PEG 400 and ethanol were acquired from Merck, USA. 4′,6-Diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Burlingame, CA, USA. NIH-3T3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was acquired from HyClone, GE Healthcare Life Sciences, Logan, UT, USA. Fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were purchased from Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA.

*2.2. Preparation of Membranes*

*2.2.1. Preparation of Simple Chitosan and Chitosan Composite Membranes.* The simple chitosan membrane was prepared by a previously reported method with some modifications [16]. In brief, for the 100 ml solution, 2 g of chitosan powder was added to 1% lactic acid solution and was mixed in a shaking incubator at 30°C, 200 rpm, for 24 h. After complete dissolution, the solution was filtered through a nylon cloth to separate undissolved chitosan particles. Then, the filtrate was casted on petri plates (100 × 15 mm) and placed in an oven at 80°C for 2 h. The neutralization of membranes was carried out by treating these with 10 ml of 10% NaOH for 15–20 min. Afterwards, the membranes were washed with distilled water thrice to remove alkali followed by storing in distilled water for future use. Chitosan composite membranes were synthesized by following the same method as described above with slight modification. For the 100 ml solution, vitamin C (1 g), glycerol (5 ml), and PEG (5 ml) were added to the chitosan–lactic acid solution under continuous stirring for 5 min and the rest of the procedure was the same.

*2.2.2. Preparation of Porous Chitosan and Porous Chitosan Composite Membranes.* Porous chitosan membranes were prepared with the freeze–gelation method with few modifications [17]. Briefly, for the 100 ml solution, 2% chitosan solution was prepared in 1% lactic acid solution and placed in a shaking incubator at 30°C, 200 rpm, for 24 h. Afterwards, the solution was casted on petri plates and placed at –80°C for 12 h. The frozen membranes were treated with 100 ml mix solution of NaOH (30 ml) and ethanol (70 ml). After this, the membrane containing the same solution was placed at –20°C for a further 12 h. The resulting membrane was washed with ethanol followed by phosphate-buffered saline. Finally, the membranes were lyophilized and preserved at 4°C for further use. For synthesis of 100 ml porous chitosan composites, the abovementioned procedure was used with modifications. In this, vitamin C (1 g), glycerol (5 ml), and PEG (5 ml) were added at the time of stirring of the

Table 1: Formulations used to make different composite membranes. The calculation is for the 100 ml solution.

| Membranes | Chitosan (C) | Vitamin C (VC) | Lactic acid (LA) | Glycerol (G) | PEG |
|---|---|---|---|---|---|
| C–LA | 2 g | — | 1 ml | — | — |
| C–VC–LA | 2 g | 1 g | 1 ml | | |
| C–VC–LA | 3 g | 1 g | 1 ml | — | — |
| C–VC–LA–G | 2 g | 1 g | 1 ml | 5 ml | — |
| C–VC–LA–PEG | 2 g | 1 g | 1 ml | — | 5 ml |
| C–VC–LA–G–PEG | 2 g | 1 g | 1 ml | 5 ml | 5 ml |

chitosan–lactic acid solution. Different formulations for making the composite membranes are summarized in Table 1.

*2.3. Characterization of Membranes.* The resulting membranes were characterized by Fourier transform infrared spectroscopy (FTIR), X–ray diffraction (XRD), and field emission scanning electron microscopy (FE–SEM). FTIR spectrometer model JASCO FTIR–6600 was used to record FTIR spectra in a range of 500–4000 $cm^{-1}$. The sample was kept in powder form and directly analyzed in ATR mode. The XRD technique was used for structural and phase characterization of synthesized materials. This analysis was carried out by using Bruker (D8 Advanced) at the scan rate of 1.2/min in the $2\theta$ range of 10–80°. Cu $K_\alpha$ ($\lambda = 1.54056$ Å) was used as radiation source and operated at 40 kV and 40 mA.

The surface morphology of freeze–dried membranes was determined using FE–SEM (MIRA3 TESCAN). Prior to FE–SEM analysis, the samples were fixed in a brass holder, coated with gold particles using a sputter coating instrument (Q150T Plus, Quorumtech). Micrographs were taken from different locations of the membrane surface at various resolutions.

*2.4. Biocompatibility of Membranes with NIH 3T3 Cells.* The assay was applied according to Khan et al., with some modifications [18]. Briefly, the synthesized membranes were placed inside 6-well plates and sterilized with 70% ethanol under UV radiation for 1-2 h in a clean bench. Next, membranes were washed three times with Dulbecco's phosphate-buffered saline (DPBS) for 5 min. Following sterilization, membranes were incubated with DMEM cell culture media at 37°C overnight. Mouse fibroblast NIH 3T3 cells were harvested with trypsin-EDTA from a previously cultured plate, and $3 \times 10^5$ cells of single cell suspension were placed on a membrane in culture media for 5 days. Images were obtained under a phase-contrast microscope to observe cell growth and spreading on the membrane.

*2.5. Immunofluorescence Staining.* In the current study, DAPI staining was performed using a previously reported method with modifications [19]. Briefly, cells were cultured on the prepared membranes for 5 days and then composite membranes were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde (in phosphate–buffered saline) for 5–10 min at room temperature. Fixatives were washed out and blocked with 0.3% Triton X–100/10% normal goat serum/PBS for 30 min. Cells were stained using Vectashield containing DAPI for 10 min in the dark. Then, the membranes were transferred to glass slides and analyzed using a fluorescent microscope (Olympus BX50). Photomicrographs were acquired digitally at 1360 × 1024 pixel resolution with an Olympus DP70 digital camera.

## 3. Results and Discussion

*3.1. Improvement in Membrane's Formation with Addition of Glycerol and PEG*

*3.1.1. Chitosan–Vitamin C–Lactic Acid Composite.* Chitosan membranes have the potential to be used as a substitute of the conventional wound dressing system with addition of other wound healing and antimicrobial components. The membrane formation is an essential factor for skin tissue engineering application and wound dressing system to avoid mechanical breakage during clinical practice [20, 21]. Vitamin C is a wound healing accelerator as it plays a vital role in proline and lysine hydroxylation which results in collagen synthesis and is thus involved in wound healing [6]. However, it has been observed in the current study that addition of vitamin C to chitosan shows a negative impact on the formation of the membrane. The synthesized simple chitosan (2%) membrane possessed good strength, while the chitosan–vitamin C–lactic acid membrane resulted in formation of fragile membranes. These observations were in accordance to a previous report where vitamin C was used as a solvent system for preparing the chitosan membrane; however, it resulted in the formation of fragile membranes [22]. The increasing concentration of chitosan (3%) added some strength to the membrane as compared to (2%) chitosan; however, the formation of the membrane gets reduced upon the neutralization of the chitosan–vitamin C–lactic acid membrane. The results indicated that the increasing chitosan concentration added no significant integrity to chitosan–vitamin C–lactic acid membrane formation. Therefore, further experiments were conducted to increase the integrity of chitosan–vitamin C–lactic acid membranes.

*3.1.2. Chitosan–Vitamin C–Lactic Acid–Glycerol–PEG Composite.* It was previously reported that addition of polymers like glycerol and PEG can add strength to the fragile membranes [23–25]. The amount of glycerol and PEG can affect the overall formation of membranes [14, 21]. Therefore, initially different concentrations (2 and 5%) of glycerol were added to the chitosan–vitamin C–lactic acid solution

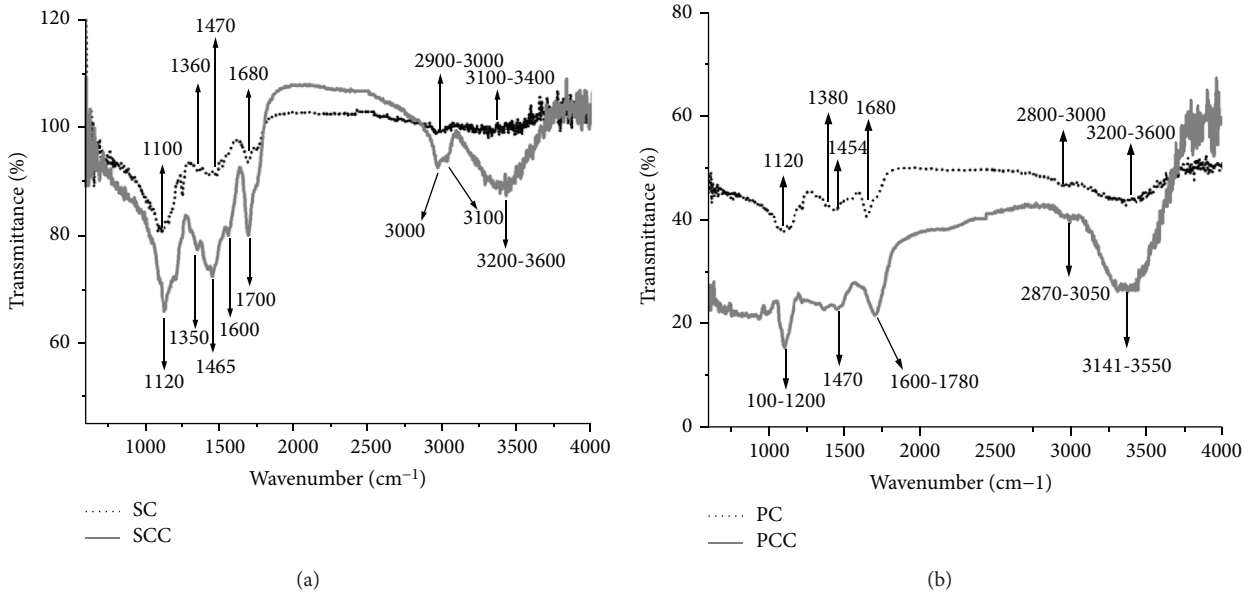


Figure 1: Comparative FTIR spectra of (a) simple chitosan (SC) and simple chitosan composite (SCC) membranes; (b) porous chitosan (PC) and porous chitosan composite (PCC) membranes.

to increase the integrity of membranes. The resulting membranes possessed strength but were not of the quality to be used in skin tissue engineering applications as they were easily breakable. So, in another set of experiment, 5% PEG was added to the chitosan–vitamin C–lactic acid solution. The resulting membranes had better integrity than chitosan–vitamin C–lactic acid membranes but are much fragile in comparison to chitosan–vitamin C–lactic acid–glycerol membranes. Finally, the combination of 5% glycerol and 5% PEG was added to the chitosan–vitamin C–lactic acid solution in an attempt to form membranes having excellent strength. The prepared membranes were of such a quality that they could be used in skin tissue engineering applications.

*3.1.3. Chitosan–Vitamin C–Lactic Acid–Glycerol–PEG Porous Membrane.* The porosity of the membrane facilitates cell growth, attachment, and spreading for cells [18]. Therefore, attempts were made to prepare the chitosan–vitamin C–lactic acid–glycerol–PEG porous membrane. The porosity was induced through a freeze-gelation method. The resulting porous chitosan composite membranes showed good integrity and porosity which is in accordance to previous reports [17, 20, 21, 26]. The preparation of simple chitosan, simple chitosan composite, and porous chitosan membranes was easy and simple, though in the case of the porous chitosan composite membrane, it was a little time-consuming. Still, it is an appreciable method because the resulting membrane possesses porosity that helps in cell growth. In the current study, the simple chitosan composite and porous chitosan composite membranes were compared with simple chitosan and porous chitosan membranes, respectively, in *in vitro* evaluation.

*3.2. FTIR Analysis.* A comparative FTIR spectrum of simple chitosan and simple chitosan composite membranes is given in Figure 1(a). The spectrum of simple chitosan showed characteristic peaks of chitosan at 1100, 1360, 1470, and 1680 cm$^{-1}$ due to C–O ether linkage, bending vibration of $CH_3$ and $CH_2$, and stretching vibration of amide carbonyl groups, respectively [27, 28]. Stretching vibrations of OH, NH, and CH groups appeared at 3100–3400 and 2900–3000 cm$^{-1}$, respectively. Encapsulation of vitamin C in the simple chitosan composite was indicated by appearance of its characteristics peaks at 1120, 1465, 1600, and 1700 cm$^{-1}$ due to C–O ester linkage, bending vibration of $CH_2$, and stretching vibration of ester carbonyl and C=C groups, respectively. Stretching vibration of CH and OH groups was observed at 3000 and 3200–3600 cm$^{-1}$, respectively [29, 30]. Peaks that appeared at 1350 and 3100 cm$^{-1}$ are due to bending vibration of $CH_3$ and stretching vibration of NH groups of chitosan. Figure 1(b) shows the comparative FTIR spectra of porous chitosan and porous chitosan composite membranes. In the spectrum of the porous chitosan composite, the characteristic peaks of chitosan due to C–O ether linkage, bending vibration of $CH_3$ and $CH_2$, and stretching vibration of amide carbonyl functional groups appeared at 1120, 1380, 1454, and 1680 cm$^{-1}$, respectively [27, 28]. Peaks appearing at 3200–3600 and 2800–3000 cm$^{-1}$ indicated the stretching vibration of OH, NH, and CH groups, respectively. In the spectrum of the porous chitosan composite, the characteristics peaks appeared at 1000–1200, 1470, and 1600–1780 cm$^{-1}$ due to C–O ester linkage, bending vibration of $CH_2$, and stretching vibration of ester carbonyl and C=C groups, respectively. The stretching vibration of CH and OH groups appeared at 2870–3050 and 3141–3550 cm$^{-1}$, respectively [29, 30]. The presence of these characteristic peaks showed the encapsulation of vitamin C in the porous chitosan composite.

*3.3. XRD Analysis.* The XRD patterns of simple chitosan and simple chitosan composite membranes are shown in

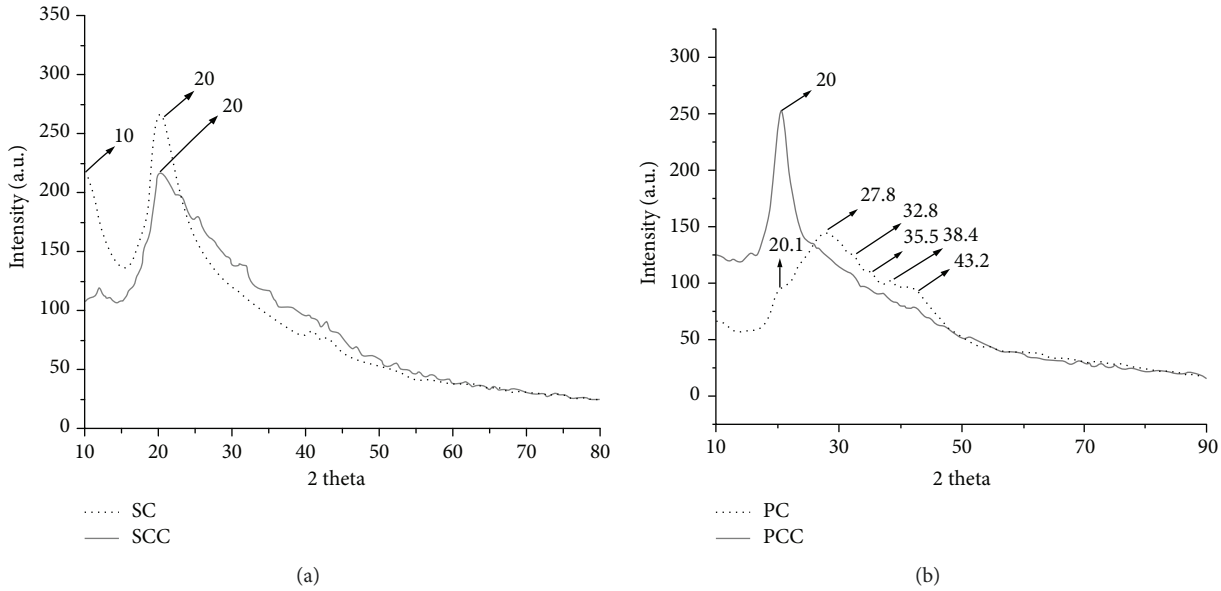


Figure 2: XRD patterns of (a) simple chitosan (SC) and simple chitosan composite (SCC) membranes; (b) porous chitosan (PC) and porous chitosan composite (PCC) membranes.

Figure 2(a). Simple chitosan membrane patterns showed two characteristic broad diffraction peaks at $(2\theta) = 10^\circ$ and $20^\circ$ which are typical fingerprints for semicrystalline chitosan [28]. Literature showed that vitamin C exhibits characteristic diffraction peaks at $2\theta$ of 10.3, 14.09, 17.3, 25.24, 40.29, 48.19, and 54.3° due to its crystalline nature. These peaks of vitamin C did not appear in simple chitosan composite membranes. This indicated that vitamin C is dispersed in amorphous form in chitosan membranes [29]. The simple chitosan composite showed only one broad hump at $\sim 2\theta = 20^\circ$. This result revealed that the crystallinity of the simple chitosan membrane was reduced after encapsulation of vitamin C. Figure 2(b) shows the comparative XRD patterns of porous chitosan and porous chitosan composite membranes. The porous chitosan XRD pattern showed a prominent broad diffraction peak at $(2\theta) = 20^\circ$ which is a characteristic fingerprint for chitosan. The porous chitosan composite membrane showed new peaks at $(2\theta) = 20.1$, 27.8, 32.8, 35.5, 38.4, and 43.2°. This indicated that vitamin C is encapsulated in the porous chitosan membrane [29].

*3.4. FE-SEM Analysis.* FE-SEM analysis was performed to present the morphology of the prepared membranes. Figure 3 depicts the microphotographs of simple chitosan, simple chitosan composites, porous chitosan, and porous chitosan composite membranes. The surface of the simple chitosan membrane appeared in the form of a nonporous, smooth membrane comprising of microfibrils and crystallites as shown in Figure 3(a). It can be clearly seen in images as shown in Figures 3(b) and 3(c) that the surface morphology of the composite membrane is different from the simple chitosan membrane. In the composite membrane, vitamin C is incorporated in chitosan as spherical nanoparticles which are nonuniformly dispersed on its surface. The background nonporous smooth membrane with microfibrils and crystallite is also visible in the composite membrane.

The surface of porous chitosan appeared in lamellar phase form and possesses a number of pores in lamellar structure as shown in Figure 3(d). On the other hand, in porous chitosan composite membranes, the appearance of a lamellar phase form is more prominent and vitamin C is present as embedded round nanoparticles as shown in Figure 3(e).

*3.5. Biocompatibility of Membranes with Fibroblast NIH 3T3 Cells.* The prepared simple chitosan, simple chitosan composite, porous chitosan, and porous chitosan composite membranes were evaluated for potential skin tissue engineering applications. For this purpose, fibroblast NIH 3T3 cell attachment, growth, and spreading on membranes were investigated. Cells need some essential elements for its proliferation so it was expected that the prepared chitosan composite membrane possesses the required essential factors for the growth of cells. For instance, vitamin C is a candidate for the healing process by collagen synthesis and mechanical strength is provided by chitosan, glycerol, and PEG polymers [7, 23, 24]. On the other hand, the presence of the porous structure in the membrane plays an important role in cell growth and attachment [18]. The results indicate the successful attachment and proliferation of cells on different membranes. Low attachment of cells was observed on the simple chitosan membrane in comparison to the simple chitosan composite membrane on day 1 as shown in Figure 4. Similarly, the porous chitosan membrane showed lower cell adhesion and spreading compared to porous chitosan composite membranes. These images clearly showed that cell adhesion on the surface of simple chitosan composite and porous chitosan composite membranes enhanced with prolonged duration of culture in comparison to the simple chitosan membrane and porous chitosan membrane on day 5 as shown in Figure 4. The five-day-cultured composite membranes were stained by DAPI (Figure 5), which confirmed

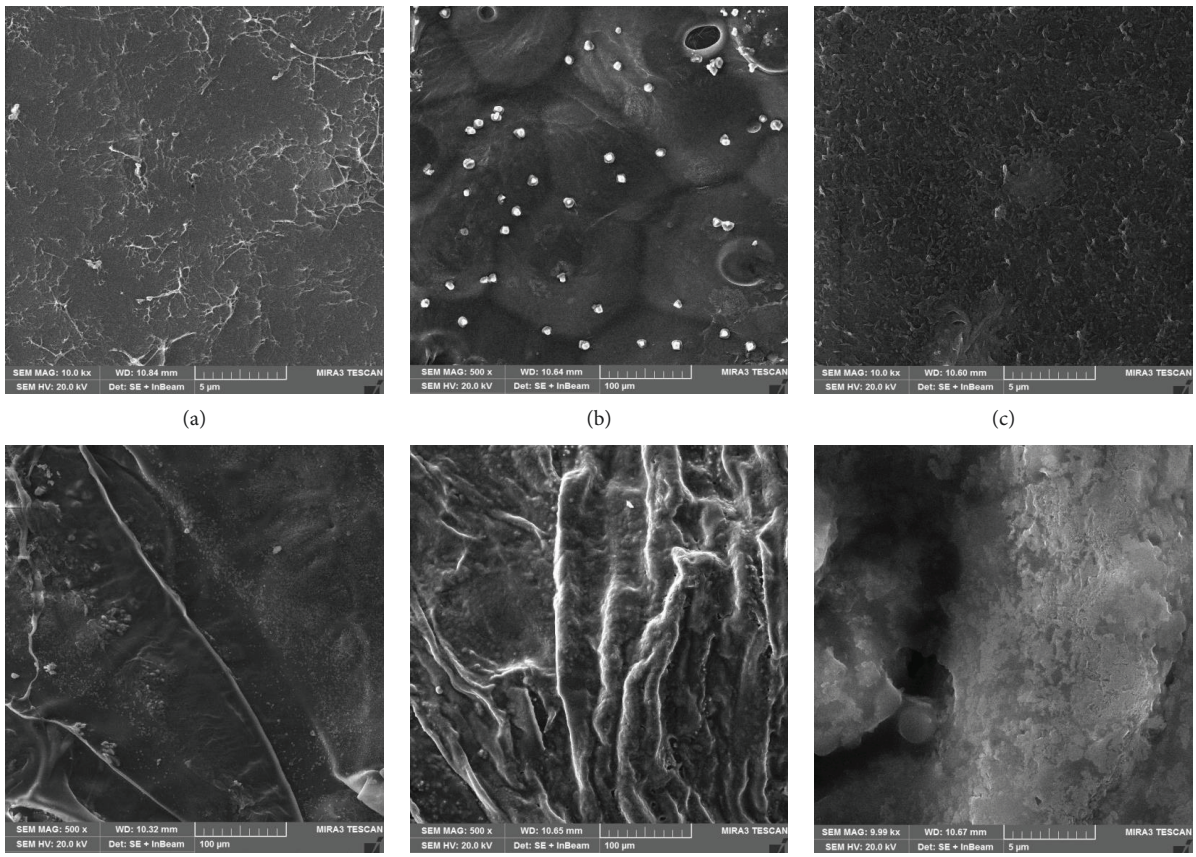


FIGURE 3: Surface morphology of the prepared membranes. (a) Simple chitosan (5 $\mu$m), (b) simple chitosan composite (low resolution, 100 $\mu$m), (c) simple chitosan composite (high resolution, 5 $\mu$m), (d) porous chitosan (100 $\mu$m), (e) porous chitosan composite (low resolution, 100 $\mu$m), and (f) porous chitosan composite (high resolution, 5 $\mu$m).

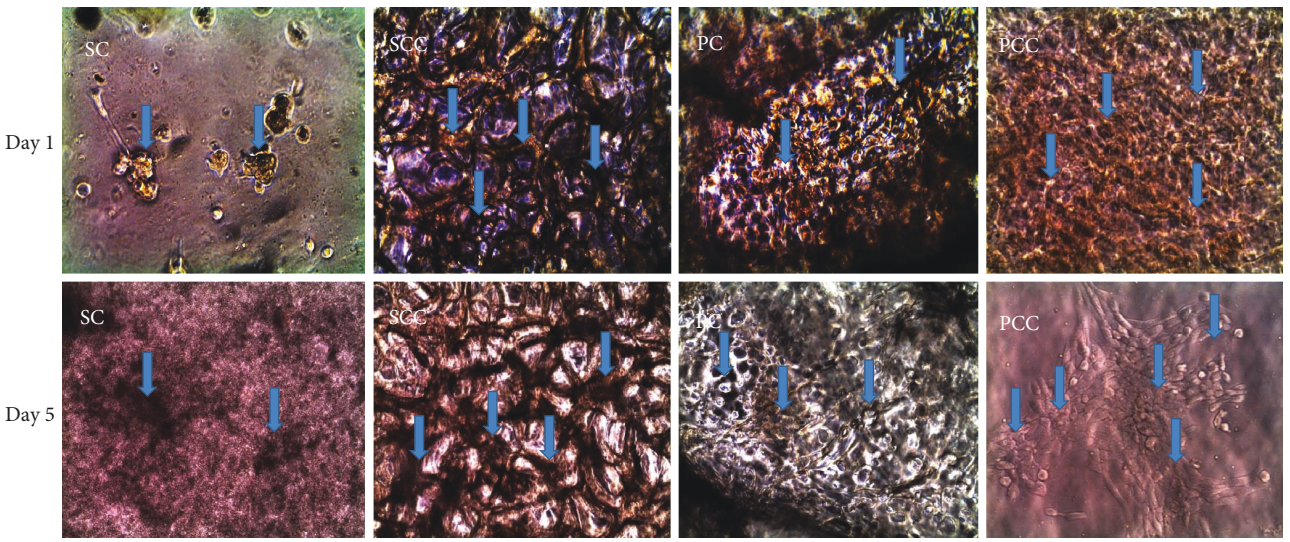


FIGURE 4: Cell adhesion and proliferation of fibroblast cells, cultured on simple chitosan (SC), simple chitosan composite (SCC), porous chitosan (PC), and porous chitosan composite (PCC) membranes on day 1 and day 5 (the cells are indicated by arrows). Day 5 image shows increased cell growth and adhesion on SCC compared to SC membrane and remarkable cell attachment and spreading on PCC composites in comparison to PC membranes. Images were taken at 100x.

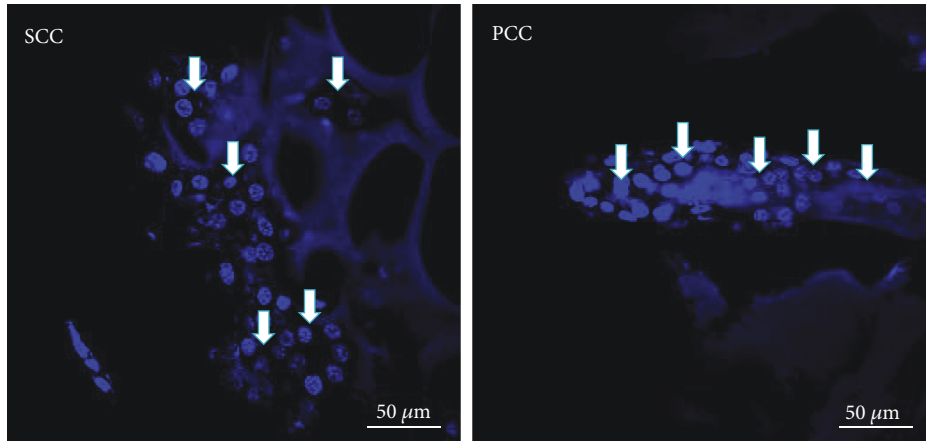


Figure 5: Cells cultured for 5 days on composite membranes were stained with DAPI. As shown in the images, cell number (the cells are indicated by arrows) enhanced in porous chitosan composite (PCC) membranes compared to that in simple chitosan composite (SCC).

that cells can grow and attach very well in both types of composite membranes. These results showed that the composite membrane may be used for skin tissue engineering applications.

## 4. Conclusion

Based on the results obtained, it is concluded that glycerol and PEG add strength to the chitosan–vitamin C–lactic acid membrane. The addition of glycerol and PEG enables to prepare chitosan–vitamin C–lactic acid membranes that may be of such quality to be used in skin tissue engineering. It is also concluded that the composites provided optimum environment for skin cell (fibroblast NIH 3T3 cell–line) attachment, growth, and spreading. Further preclinical and clinical experiments are suggested to fully exploit the potential of these membranes in skin tissue engineering application.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

This study was partially supported by the COMSATS Research Grant Program (CRGP) (Grant No. 16–27/CRGP/CIIT/ATD/16/1125).

## References

[1] S. Böttcher-Haberzeth, T. Biedermann, and E. Reichmann, "Tissue engineering of skin," *Burns*, vol. 36, no. 4, pp. 450–460, 2010.

[2] A. W. C. Chua, Y. C. Khoo, B. K. Tan, K. C. Tan, C. L. Foo, and S. J. Chong, "Skin tissue engineering advances in severe burns: review and therapeutic applications," *Burns and Trauma*, vol. 4, no. 1, p. 3, 2016.

[3] R. C. F. Cheung, T. B. Ng, J. H. Wong, and W. Y. Chan, "Chitosan: an update on potential biomedical and pharmaceutical applications," *Marine Drugs*, vol. 13, no. 8, pp. 5156–5186, 2015.

[4] D. J. MacKay and A. L. Miller, "Nutritional support for wound healing," *Alternative Medicine Review*, vol. 8, no. 4, pp. 359–377, 2003.

[5] R. P. da Rocha, D. P. Lucio, T. de Lima Souza, S. T. Pereira, and G. J. M. Fernandes, "Effects of a vitamin pool (vitamins A, E, and C) on the tissue necrosis process: experimental study on rats," *Aesthetic Plastic Surgery*, vol. 26, no. 3, pp. 197–202, 2002.

[6] R. L. Gross, "The effect of ascorbate on wound healing," *International Ophthalmology Clinics*, vol. 40, no. 4, pp. 51–57, 2000.

[7] J. Moores, "Vitamin C: a wound healing perspective," *British Journal of Community Nursing*, vol. 18, no. Sup12, pp. S6–S11, 2013.

[8] P. E. Porporato, V. L. Payen, C. J. de Saedeleer et al., "Lactate stimulates angiogenesis and accelerates the healing of superficial and ischemic wounds in mice," *Angiogenesis*, vol. 15, no. 4, pp. 581–592, 2012.

[9] M. Tajkarimi and S. A. Ibrahim, "Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on *Escherichia coli* O157: H7 in laboratory medium and carrot juice," *Food Control*, vol. 22, no. 6, pp. 801–804, 2011.

[10] S. C. J. De Keersmaecker, T. L. A. Verhoeven, J. Desair, K. Marchal, J. Vanderleyden, and I. Nagy, "Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid," *FEMS Microbiology Letters*, vol. 259, no. 1, pp. 89–96, 2006.

[11] T. Moe and T. Khaing, "Lactic acid–chitosan films' properties and their *in vivo* wound healing activity," *International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering*, vol. 8, no. 9, pp. 633–637, 2014.

[12] A. Behr, J. Eilting, K. Irawadi, J. Leschinski, and F. Lindner, "Improved utilisation of renewable resources: new important derivatives of glycerol," *Green Chemistry*, vol. 10, no. 1, pp. 13–30, 2008.

[13] T. M. Nalawade, K. Bhat, and S. H. P. Sogi, "Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms," *Journal of International Society of Preventive & Community Dentistry*, vol. 5, no. 2, pp. 114–119, 2015.

[14] M. Zhang, X. H. Li, Y. D. Gong, N. M. Zhao, and X. F. Zhang, "Properties and biocompatibility of chitosan films modified by blending with PEG," *Biomaterials*, vol. 23, no. 13, pp. 2641–2648, 2002.

[15] T. Chandy, D. L. Mooradian, and G. H. R. Rao, "Chitosan/-polyethylene glycol–alginate microcapsules for oral delivery of hirudin," *Journal of Applied Polymer Science*, vol. 70, no. 11, pp. 2143–2153, 1998.

[16] A. K. Azad, N. Sermsintham, S. Chandrkrachang, and W. F. Stevens, "Chitosan membrane as a wound-healing dressing: characterization and clinical application," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 69B, no. 2, pp. 216–222, 2004.

[17] C. Y. Hsieh, S. P. Tsai, D. M. Wang, Y. N. Chang, and H. J. Hsieh, "Preparation of $\gamma$-PGA/chitosan composite tissue engineering matrices," *Biomaterials*, vol. 26, no. 28, pp. 5617–5623, 2005.

[18] S. Khan, M. Ul-Islam, M. W. Ullah et al., "Engineered regenerated bacterial cellulose scaffolds for application in *in vitro* tissue regeneration," *RSC Advances*, vol. 5, no. 103, pp. 84565–84573, 2015.

[19] H. K. Kim, Y. W. Choi, E. N. Lee et al., "5-Hydroxymethylfurfural from black garlic extract prevents TNF$\alpha$-induced monocytic cell adhesion to HUVECs by suppression of vascular cell adhesion molecule-1 expression, reactive oxygen species generation and NF-$\kappa$B activation," *Phytotherapy Research*, vol. 25, no. 7, pp. 965–974, 2011.

[20] I. Y. Kim, S. J. Seo, H. S. Moon et al., "Chitosan and its derivatives for tissue engineering applications," *Biotechnology Advances*, vol. 26, no. 1, pp. 1–21, 2008.

[21] E. Szymańska and K. Winnicka, "Stability of chitosan–a challenge for pharmaceutical and biomedical applications," *Marine Drugs*, vol. 13, no. 4, pp. 1819–1846, 2015.

[22] P. K. Swain, M. Das, and P. Nayak, "Preparation and characterization of chitosan nanocomposite films in different solvent and its antimicrobial activity," *International Journal of Applied Biology and Pharmaceutical Technology*, vol. 5, no. 4, pp. 187–196, 2014.

[23] K. Hermans, D. van den Plas, S. Kerimova et al., "Development and characterization of mucoadhesive chitosan films for ophthalmic delivery of cyclosporine A," *International Journal of Pharmaceutics*, vol. 472, no. 1-2, pp. 10–19, 2014.

[24] M. Luangtana-anan, S. Limmatvapirat, J. Nunthanid, R. Chalongsuk, and K. Yamamoto, "Polyethylene glycol on stability of chitosan microparticulate carrier for protein," *AAPS PharmSciTech*, vol. 11, no. 3, pp. 1376–1382, 2010.

[25] Y. Ma, L. Xin, H. Tan et al., "Chitosan membrane dressings toughened by glycerol to load antibacterial drugs for wound healing," *Materials Science and Engineering: C*, vol. 81, pp. 522–531, 2017.

[26] C. Clasen, T. Wilhelms, and W. M. Kulicke, "Formation and characterization of chitosan membranes," *Biomacromolecules*, vol. 7, no. 11, pp. 3210–3222, 2006.

[27] G. Dennis, W. Harrison, K. Agnes, and G. Erastus, "Effect of biological control antagonists adsorbed on chitosan immobilized silica nanocomposite on *Ralstonia solanacearum* and growth of tomato seedlings," *Advances in Research*, vol. 6, no. 3, pp. 1–23, 2016.

[28] V. Mohanasrinivasan, M. Mishra, J. S. Paliwal et al., "Studies on heavy metal removal efficiency and antibacterial activity of chitosan prepared from shrimp shell waste," *3 Biotech*, vol. 4, no. 2, pp. 167–175, 2014.

[29] K. G. H. Desai and H. J. Park, "Encapsulation of vitamin C in tripolyphosphate cross–linked chitosan microspheres by spray drying," *Journal of Microencapsulation*, vol. 22, no. 2, pp. 179–192, 2005.

[30] M. M. Sk and C. Y. Yue, "Synthesis of polyaniline nanotubes using the self-assembly behavior of vitamin C: a mechanistic study and application in electrochemical supercapacitors," *Journal of Materials Chemistry A*, vol. 2, no. 8, pp. 2830–2838, 2014.