Nanostructured Lipid Carriers of Pioglitazone Loaded Collagen/Chitosan Composite Scaffold for Diabetic Wound Healing

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Diabetic wound is a major problem that often needs amputation of the concerned organ in patients suffering from diabetes. In diabetes, the prolonged phase of inflammation obstructs the further phases of healing which, in turn, lead to improper healing of the wounds in diabetes. Pioglitazone (Pio) hydrochloride is an antidiabetic drug with reported anti-inflammatory properties. The aim of this study was to develop a Pio-nanostructured lipid carrier (Pio-NLC)-loaded collagen/chitosan (COL-CS) scaffold and evaluate its healing ability in diabetic wounds. The results of characterization of composite scaffolds reveal that cross-linked scaffolds possess optimum porosity, low matrix degradation, and sustained drug release compared with noncross-linked scaffolds. The *in vitro* studies reveal that the Pio-NLC-COL-CS scaffold was biocompatible and enhanced cell growth compared with control and NLC-COL-CS. Using the streptozotocin-induced diabetic wound model, significantly ($p < 0.001$) higher rates of wound contraction in Pio-NLC-COL-CS scaffold-treated group were observed in comparison with that in control and NLC-COL-CS-treated group. The enzyme-linked immunosorbent assay results indicate a significant ($p < 0.001$) decrease of matrix metalloproteinases-9 levels in the Pio-NLC-COL-CS-treated group compared with those in control group. Use of nanostructured lipid carrier (Pio-NLC-COL-CS) scaffold can prove to be a promising strategy for local treatment for diabetic wounds.

Keywords: pioglitazone, nanostructured lipid carrier, collagen, chitosan, scaffold and diabetic wound

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INTRODUCTION

DIABETES MELLITUS (DM) is a condition in which inability of the body to produce insulin or respond to its results in anomalous metabolism of simple carbohydrates result in upsurge of blood glucose.¹ Most frequent and devastating complication of diabetes is the diabetic wound

that may end up in amputation. Approximately 25% of patients with DM have chance to develop a diabetic wound in their life time.¹ The impaired healing of diabetic wound is attributed to triopathy of diabetes, that is, immunopathy, vasculopathy, and neuropathy. If left untreated, these wounds may result in

the development of gangrene, subsequently leading to amputation.^{2,3}

A plethora of treatment strategies of diabetic wounds are in practice that include educating the patients, controlling their blood sugar, wound debridement, advanced dressings, offloading, surgery, and advanced therapies. These constitute the standard care therapies for treating diabetic wounds.^{4,5} However, most of these treatment strategies do not address all the prerequisites necessary of diabetic wound care because of the multifactorial pathophysiology of the condition and the high cost associated with these treatments.^{6,7} Although the pathogenesis of the diabetic wound is multifactorial, the continuous inflammation with improper tissue management is reported to be the major cause of impaired healing.^{8,9}

Increased levels of inflammatory and proinflammatory cytokines in diabetic wounds cause a chronic inflammatory condition that leads to decreased levels of growth factors.¹⁰ Improper matrix formation is because of the rapid degradation of formed matrix because of the increased levels of matrix metalloproteinases (MMPs). In the family of MMP, MMP-2 and MMP-9 are the major metalloproteinases responsible for the chronic inflammation.¹¹ Literature also suggest a rapid increase in the levels of MMP-9 compared with those of MMP-1, 2, and 8 in the chronic wound fluid.¹² Inhibition of the MMP-9 is reported to lead to efficient healing of wound in diabetic condition.^{12,13} Local treatment with inflammatory mediators block MMP-9, and thus restores cutaneous homeostasis and matrix formation and leads to better wound healing.

Pioglitazone (Pio), an MMP-9 inhibitor was selected to suppress the prolonged inflammatory phase that is caused by the upregulation of major inflammatory mediator MMP-9 in diabetic condition.^{14,15} There is no report on the topical delivery of Pio in treating diabetic wounds. For formation of scaffolds containing Pio, collagen (COL) and chitosan (CS) were selected. Selection is based on the fact that COL helps in providing the structural matrix which, in turn, is responsible for significant wound healing.¹⁶ CS, on the contrary, is structurally similar to glycosaminoglycan. Glycosaminoglycan is involved in several stages of wound healing such as regulation and secretion of inflammatory mediators including interleukin-1 β , prostaglandin-E, and so on. CS is also reported to possess significant antibacterial activity.¹⁶ COL/CS scaffolds of Pio are, therefore, formulated with the aim to decrease the inflammation in diabetic wounds and support cell proliferation, differentiation, and matrix formation.

MATERIALS AND METHODS

Reagents and cell line

- Pio hydrochloride (Cat. no. E6910; Sigma Chemicals Co. Ltd, Mumbai, India).
- Chitosan medium molecular weight (Cat. no. 448877; Sigma Chemicals Co. Ltd).
- Type-1 collagen from rat tail (Cat. no. C7661; Sigma Chemicals Co. Ltd.).
- Polysorbate 80 (Cat. no. 59924; Sigma Chemicals Co. Ltd.).
- Glacial acetic acid (Cat. no. 100066; Merck Millipore, Mumbai, India).
- Ethanol (Cat. no. 100980; Merck Millipore).
- Polyethylene glycol (PEG) 400 (Cat. no. 807485; Merck Millipore).
- 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Cat. no. 800907; Merck Millipore).
- *N*-hydroxysuccinimide (NHS) (Cat. no. 804518; Merck Millipore).
- 2-(*N*-morpholino) ethane sulfonic acid (MES) (Cat. no. 137074; Merck Millipore).
- Sodium dihydrogen orthophosphate (NaH₂PO₄) (Cat. no. Q20335; Qualigen Fine Chemicals, Mumbai, India).
- Sodium hydroxide (NaOH) pellets (Cat. no. Q27815; Qualigen Fine Chemicals) glyceryl monostearate (Cat. no. K06119; Kemphasol, Mumbai, India).
- Poloxamer 188 (Cat. no. 24040032; Thermo Fisher Scientific, Mumbai, India).
- Mouse embryonic fibroblast cells (3T3-L1) from National Centre for Cell Sciences (Pune, India).

Preparation of nanostructured lipid carriers of Pio

Pio is a practically water-insoluble drug having log *p*-value of 3.46. Nanostructured lipid carriers (NLCs) were prepared by solvent evaporation technique. First, oil-in-water (o/w) microemulsions were prepared by using 10% of glyceryl monostearate as lipid phase, 0.1% of PEG 400 as solubilizer, and 1% of polysorbate 80 as hydrophilic surfactant (in double distilled water). The lipid phase was heated to 60°C and Pio was added. Aqueous solution (50 mL) containing polysorbate 80 was heated to the same temperature and the lipid phase was added using microneedle syringe to the aqueous phase with constant stirring at 100 rpm. Stirring was continued for 15 min after

addition of lipid phase. Pio-NLCs were obtained by dispersing the warm o/w microemulsion dropwise into ice cold water in a beaker under continuous stirring for 15 min. The formed NLC dispersions were subjected to ultrasonication for 15 min for further size reduction.¹⁷

Evaluation of Pio-NLC

Particle size determination and zeta potential. Size and zeta potential of the prepared particles were measured by a particle size analyzer with a zeta potential measurement facility (Malvern Instruments). The polydispersity index was also measured to find the range of size distribution.^{18,19}

Entrapment efficiency. Entrapment efficiency of the Pio-NLCs was assessed using the centrifugation technique.^{20,21} The developed formulation was centrifuged at 15,000 rpm at 4°C using cold centrifuge (Remi Equipments Ltd, Mumbai, India) for 15 min. The supernatant was collected, diluted suitably, and absorbance was recorded at 282 nm. Drug entrapment was calculated using the formula:

$$\text{Entrapment efficiency} = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug}} \times 100.$$

Morphological characterization. Morphology of Pio-NLCs was observed by scanning electron microscopy (SEM). Formulations were diluted with water, spread over a glass sample holder, and dried under vacuum. The dried samples were coated with gold and examined with SEM at 30 s run time.²²

Preparation of composite scaffold

COL solution (2% w/v) was prepared in deionized water at room temperature. CS solution (2% w/v) was prepared in 1.5% v/v acetic acid solution. The pH of the COL solution was adjusted to 7.0 by adding 2 M NaOH at 4°C. The composite scaffolds were prepared by incorporating Pio-NLCs into CS solution. The Pio-NLCs were added to achieve 1% w/w concentration of Pio in COL-CS. This dispersion was then added to cold COL solution. The dispersion was stirred overnight at 4°C to achieve uniform homogeneity. The entrapped bubbles in the dispersion were removed by sonication on a bath sonicator (RK 100 H; Bandelin, Germany) for 5 min. Then this dispersion was poured in Petri dishes and kept in the deep freezer at -60°C (Lab-line) for 24 h, followed by lyophilization (Christ Alpha 1-2) for 72 h to obtain the Pio composite scaffolds.²³

Cross-linking of scaffold

MES (0.488 g) was dissolved in 50 mL of double distilled water to get a pH of 5.6. Pio-NLC-loaded scaffolds (50 mg) were added to 20 mL of MES buffer and stirred for 30 min. In another beaker, 19.5 mL of MES buffer was mixed well with 0.1264 g of EDC and 0.014 g of NHS. Soaked scaffold was kept dipped in the buffer mixture for 4 h for cross-linking. Cross-linked scaffold was filtered out and washed twice after keeping in contact with 0.1 M sodium dihydrogen orthophosphate for 1 h, 1 M NaCl for 2 h, and 2 M NaCl for 1 day successively.^{23,24} Both the prepared Pio-NLCs, cross-linked and noncross-linked scaffolds were subjected to further evaluation.

Characterization of scaffold

Morphological characterization using SEM. Field emission SEM (FE-SEM; Hitachi S-4800) was used for determination of the cross-sectional morphology of scaffolds. Samples (5×5 mm) of scaffolds were coated with thin layer of gold, and placed on adhesive stub. The operational voltage for FE-SEM was kept 10 kV. Fifty pores were selected randomly for the determination of pore size. From each group, three scaffolds were viewed for three different cross-sections and pore size was determined.²⁵

Porosity. The porosity of the scaffolds was determined using liquid displacement method. Ethanol was chosen as displacement liquid as it can effortlessly penetrate the pores of scaffolds, and does not stimulate contraction or swelling of scaffolds. Porosity of scaffolds is calculated according to the following equation.

$$\text{Porosity (\%)} = \frac{W_w - W_d}{SV} \times 100,$$

where W_w and W_d are the wet and initial dry weights of the scaffolds, respectively, whereas SV is the scaffold volume.^{26,27}

Water absorption test. Weight of dry scaffold was measured. The weighed scaffold was incubated in phosphate-buffered saline (PBS) at pH 7.4 at 37°C for 72 h. The swollen scaffold was weighed after removing the excess water by blotting with filter paper. This was subjected to lyophilization and the freeze-dried scaffolds were weighed.²³ The water content of scaffold was calculated by the following formula.

$$Ead = \frac{[W_e - W_o]}{W_o} \times 100,$$

where E_{ad} is the percentage water adsorption of the scaffold at equilibrium. W_o and W_e represent the dry weight and wet weight of the scaffold, respectively.

Matrix degradation. Scaffold (1×1 cm) was immersed in PBS of pH 7.4 with 10,000 U/mL of lysozymes and incubated at 37°C for 7 days. The scaffold was then washed with distilled water to remove any ions adsorbed on its surface and then lyophilized. Initial weight and weight after lyophilization of the scaffold were noted as W_o and W_t , respectively.²³ The rate of degradation was calculated using the formulae:

$$\text{Degradation \%} = \frac{[W_o - W_t]}{W_o} \times 100.$$

In vitro drug release studies. The composite scaffold (3×3 cm) was immersed in 20 mL PBS of pH 7.4 at 37°C. The supernatant was taken out periodically and replaced by equivalent volume of PBS. The collected samples were filtered through

Diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg) prepared in citrate 0.1 M buffer of pH 4.5. Monitoring of blood glucose levels (Glucometer; AccuChek) was started 2 days after induction. Animals with ≥300 mg/dL of blood glucose were separated and monitored for seven more days. Only the animals that demonstrated constantly increased levels of blood glucose were considered for the study. An open wound of an area measuring 2×2 cm² was created on dorsal region under anesthetic conditions (Ketamine 100 mg/kg and xylazine mg/kg). The control wounds were dressed with sterile gauze, whereas the remaining groups were covered with the respective scaffolds throughout the duration of study. Animals were housed individually after the anesthetic recovery and monitored closely. The animals were divided into three groups. Wounds in group 1 were covered with sterile gauze (control), group 2 animals were treated with NLC-COL-CS scaffold (cross-linked scaffold without Pio), and group 3 animals were treated with Pio-NLC-COL-CS cross-linked scaffold (test group).^{23,28} Percentage wound contraction was calculated using the formula:

$$\% \text{ wound contraction} = \frac{\text{wound area on day 0} - \text{wound area on a particular day}}{\text{wound area on day 0}} \times 100.$$

0.2 μm membrane filter for further analysis. The drug release was determined using ultraviolet-visible spectrometer at 282 nm using Pio standard curve. The percentage of Pio released was determined.²³

In vitro biocompatibility studies

MTT [(3-(4,5 dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide)] assay was used to assess the biocompatibility of the prepared scaffolds. Scaffolds of standard dimensions (15 mm diameter) were sterilized for 30 min in 75% ethanol, and then washed with sterile water for 5 min. One scaffold was then placed in each of the well of the 24 well plates with 2 mL of Dulbecco's modified Eagle's medium. To this 24 well plate, 3T3-L1 cells were added. Test was performed for 72 h, wherein both control and sample media were maintained for proper comparison.

In vivo wound healing study

Healthy male Wistar albino rats of 6–12 weeks of age weighing 180–200 g were used for the study. All the animals were housed in optimized laboratory condition with free access to the standard chow diet and water for 10 days for acclimatization.

The protocol of animal experiments was approved by the Institutional Animal Ethical Committee of JSS College of Pharmacy, Ooty (protocol number: JSSCP/IAEC/M.Pharm/PH-CEUTICS/02/2016–2017).

Hydroxyproline estimation

Skin including the wound was excised carefully on days 7, 14, and 21 for hydroxyproline estimation. Skin samples were hydrolyzed by autoclaving at 120°C for 20 min. To the hydroxylate 450 μL of Chloramine-T was added, mixed gently, and allowed to stay for 25 min at room temperature to undergo oxidation. To the above mixture, 500 μL of Ehrlich's reagent (*p*-dimethyl amino benzaldehyde in perchloric acid/*n*-propanol) was added, and the mixture was incubated for 20 min at 65°C. The absorbance of complex with reddish purple color was measured spectrophotometrically at 550 nm. Concentration of hydroxyproline in the tissue was expressed as micrograms per milligrams.²⁹

Histopathological studies

Wound tissues were isolated on the days 7, 14, and 21, and fixed in 10% formalin solution (10%) prepared in neutral buffer. Tissues were cut into

Figure 1. (a) NLCs particle size analysis by using dynamic laser light scattering revealing a particle size of 185.7 ± 5.2 nm. (b) Pio-NLC particle size analysis by using dynamic laser light scattering revealing a particle size of 192.2 nm. All data analysis was performed in automatic mode and particle size results are represented as the average value of 20 runs. (c) Positive zeta potential (22.2) distribution of NLCs. (d) Positive zeta potential (32.5) distribution of Pio-NLCs. NLC, nanostructured lipid carrier; Pio, Pioglitazone.

6 μm thickness using a microtome (Model No: RM2135; Leica, United Kingdom). Sections were mounted on a glass slide and hematoxylin and eosin were used for staining.²³ All the images were taken by using motic microscope (Model No: MLX-i Plus; Magnus, India) under 40 $\times$ magnification.

Enzyme-linked immunosorbent assay test

Estimation of MMP-9 using enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) was carried out in accordance with the instructions provided by the manufacturer. Tissue samples isolated from the wound area were minced using tissue homogenizer and the homogenate was subjected to centrifugation. The collected supernatant was diluted 100-folds using assay buffer. MMP-9 concentrations of the samples were estimated for all the groups (control, NLC-COL-CS, and Pio-NLC-COL-CS scaffold) on day 21.³⁰

Statistical analysis

The results obtained were expressed as mean $\pm$ standard deviation. Statistical significance was obtained by one-way analysis of variance followed by Dunnett's *post hoc* test. Statistical analysis was performed using GraphPad Prism v5.01 (San Diego, CA). Values with $p < 0.05$ or lower were considered significant.

RESULTS

Characterization of NLCs and Pio-NLCs

The prepared NLCs and Pio-NLCs were evaluated for particle size and zeta potential. Particles were found to lie in the size range of 185.7 ± 5.2 nm for NLCs, whereas Pio-NLCs has shown particle size range of 192.2 ± 5.6 nm (Fig. 1a, c). Pio-NLCs have a zeta potential of 32.5 ± 1.2 mV compared with NLCs alone (22.2 ± 1.5 mV) (Fig. 1b, d). The SEM results (Fig. 2) of the PIO-NLC revealed that the particles were spherical in shape. Size of the particles observed by SEM correlated well with the particle size measured by the zeta sizer.

Characterization of the composite scaffolds

Pio-NLC-COL-CS composite scaffolds prepared by lyophilization were found to be porous with interconnected pores. The macroscopic characteristics and the three-dimensional structure of scaffolds were observed to be like a sponge, consisting of a homogenous porous arrangement. On visual examination, the composite scaffolds were found to be off-white in color, stiff, and inelastic. Scanning electron micrographs of the NLC-COL-CS and Pio-NLC-COL-CS scaffolds are given in Figure 3a, b. These confirmed the incorporation of Pio-NLC with uniform distribution all over the scaffold.

Figure 2. Morphology of Pio-derived NLC determined by SEM at a scale range of 1 μm . SEM, scanning electron microscopy.

The data related to porosity and water absorption of noncross-linked and cross-linked scaffolds are given in the Table 1. The porosity of noncross-linked Pio-NLC-COL-CS scaffold was found to be more compared with that of cross-linked Pio-NLC-COL-CS scaffold. The percentage water absorption was found to be more in noncross-linked Pio-NLC-COL-CS scaffold compared with that in cross-linked Pio-NLC-COL-CS scaffold because of the increased mechanical strength. The biodegradation studies were performed for both noncross-linked and cross-linked Pio-NLC-COL-CS scaffolds. The noncross-linked scaffold degraded progressively after incubation with phosphate buffer pH 7.4 solution in the presence of lysozymes for 7 days. Initial degradation rate in the first 3 days was found to be high after which the rate decreased slightly in the following 4 days. Contrary to this, the cross-linked scaffold showed lower degradation rate indicating enhanced resistance to the enzymatic degradation (Fig. 4). From the data given in Figure 5, a slow release of drug in all formulations was observed, which was followed by a sustained release. In the initial 1 h, $11.79\% \pm 2.9\%$ drug was released from Pio-NLC-COL-CS noncross-linked scaffold, whereas only $5.62\% \pm 3.2\%$ drug was released from Pio-NLC-COL-CS cross-linked scaffold. After 6 h, the release of the drug from the scaffolds increased by $31.52\% \pm 2.99\%$ in noncross-linked scaffold and by $13.02\% \pm 3\%$ in cross-linked scaffolds. After 24 h, there was a release of $>50\%$ drug from the noncross-linked scaffold,

Figure 3. (a) SEM images of NLC-COL-CS scaffold. (b) SEM images of Pio-NLC-COL-CS scaffold with an average of 200 nm particle size. COL, collagen; CS, chitosan.

Table 1. Percentage porosity and water absorption properties of noncross-linked and cross-linked nanostructured lipid carrier–collagen–chitosan and Pio-nanostructured lipid carrier–collagen–chitosan scaffold

Scaffold Type	% of Porosity	% Water Absorption
Noncross-linked NLC-COL-CS	94.12 ± 1.41	925
Noncross-linked Pio-NLC-COL-CS	93.30 ± 1.34	920
Cross-linked NLC-COL-CS	80.37 ± 1.61	812
Cross-linked Pio-NLC-COL-CS	78.23 ± 1.10	810

COL, collagen; CS, chitosan; Pio-NLC, pioglitazone-nanostructured lipid carrier.

whereas cross-linked scaffold took 72 h to release >50% of the drug. Based on the above results, cross-linked Pio-NLC-COL-CS was selected for further evaluation.

In vitro cell viability studies

The results have shown that inclusion of the Pio-NLC-COL-CS scaffold into the growth media did not stimulate any cytotoxicity toward the 3T3-L1 cells. As given in Figure 6, cell viability was higher than that of control indicating better growth of fibroblasts in the presence of scaffolds.

In vivo wound healing

Contraction of the wound area in each group was determined using grid technique on days 0, 7, 14, and 21 (Fig. 7a, b). Significantly ($p < 0.001$) faster contraction of wound was noticed in cross-linked scaffold-treated group in comparison with that in control and NLC-COL-CS-treated group. The mean percentage of wound contraction in Pio-NLC-COL-CS-treated group was found to be significantly ($98.94\% \pm 3.2\%$ on day 21, $p < 0.001$) faster in com-

parison with that in the control ($67.77\% \pm 5.3\%$ on day 21, $p < 0.01$) and NLC-COL-CS ($81.88\% \pm 2.9\%$ on day 21, $p < 0.01$) groups.

Hydroxyproline estimation

COL is available in wound granulation tissue and is created by new fibroblasts. The estimation of hydroxyproline levels indirectly indicates the COL amalgamation of tissue. Hydroxyproline content was found to be higher in the Pio-NLC-COL-CS-treated group compared with that in the control group. Despite the fact that the hydroxyproline content of NLC-COL-CS-treated group was marginally higher than in Pio-NLC-COL-CS-treated group, the level was not very significant as given in Figure 8.

Histopathological studies

The histopathological studies revealed that all the wounds were free from oozing after seventh day postinjury. Results are given in Figure 9. Observation of cell infiltration on the seventh day after injury showed that in the control group, neutrophils prevailed at a time point with intermittent lymphocyte or macrophage. In the NLC-COL-CS-treated animals, smaller number of neutrophils and macrophages were observed than in the control group. The Pio-NLC-COL-CS-treated group showed a low number of neutrophils and macrophages in comparison with those in the control.

On the 14th day after injury, in the control group, neutrophils still prevailed with few multinucleated massive cells and histiocytes. In the NLC-COL-CS treatment group, small number of histiocytes and multinucleated massive cells were

Figure 4. Matrix degradation study of NLC-COL-CS, Pio-NLC-COL-CS noncross-linked and cross-linked scaffolds from day 1 to 7 in phosphate buffer pH 7.4 at 37°C showing noncross-linked scaffold degraded progressively for 7 days. Contrary to this, the cross-linked scaffold degradation rate decreased greatly indicates enhanced resistance to enzymatic degradation.

Figure 5. *In vitro* drug release profile of Pio from Pio-NLC-COL-CS noncross-linked and cross-linked scaffolds in phosphate buffer pH 7.4 at 37°C showing a slow release of drug in all formulation followed by a sustained release and then a plateau. Data are expressed as mean $\pm$ SD ($n=3$). SD, standard deviation.

seen, whereas in the Pio-NLC-COL-CS-treated group abundant histiocytes, multinucleated giant cells, and single lymphocytes were observed.

On the 21st day postinjury, the dominant response of inflammation by neutrophils in the control group was decreased. However, the control group showed recurrence of macrophages and histiocytes. The NLC-COL-CS group showed moderate number of histiocytes and lymphocytes,

Figure 6. *In vitro* biocompatibility studies in Fibroblast 3T3-L1 cells depicting higher percentage of cell growth in Pio-NLC-COL-CS followed by NLC-COL-CS scaffold, Pio, and control, respectively.

whereas those treated with Pio-NLC-COL-CS were found to be rich in histiocytes, multinucleated massive cells, and lymphocytes.

Observation on course of healing on seventh day postinjury showed disruption of all skin layers at the edges of the wound in all groups. In the control group, intermittent visibility of epidermis was noticed. In NLC-COL-CS group, moderate visibility of epidermis was noticed, whereas in the Pio-NLC-COL-CS group, wound was found to be in the phase of reepithelization and also covered with slim layer of epidermis. Granulation tissue was also seen more often in cross-linked scaffold-treated groups.

On the 14th day after injury, in all the groups, wounds were found to be covered with epidermis. In the control group, the formed epidermis was observed to be a slim layer. In the NLC-COL-CS group, the epidermis layer formed was thicker than that in the control, whereas in the Pio-NLC-COL-CS-treated group, the epidermis was clearly thicker than that in the remaining groups. The zones of dense fibroblasts were also observed in the Pio-NLC-COL-CS group.

On the 21st day postsurgery, the epidermis was observed to be considerable thicker in the Pio-NLC-COL-CS-treated group than that in the control group. Of interest, recovery of the hair follicles was also observed in the Pio-NLC-COL-CS-treated group, followed by NLC-COL-CS group. The histopathological observation was correlated well with the macroscopic observation during the study tenure.

ELISA test

MMP-9 content in the Pio-NLC-COL-CS and NLC-COL-CS-treated groups was found to be less in comparison with those in the control group. However, MMP-9 content of NLC-COL-CS was marginally decreased compared with that in the Pio-NLC-COL-CS group as given in Figure 10.

DISCUSSION

Because of the poor water solubility of Pio, it was first converted into NLCs and then incorporated into scaffold. The particle size of the Pio-NLCs was slightly greater than that of NLCs with more positive zeta potential of 32.5 ± 1.2 mV that may be because of the entrapment of the drug in Pio-NLCs that helps in the better adhesion to the negatively charged cell membrane. The results obtained were in compliance with Karri *et al.*²³

Composite scaffold was prepared with a combination of COL and CS keeping in mind the contribution of these two polymers in wound healing. COL is the most abundant protein of extracellular

Figure 7. (a) Images representing the wound contraction in the control, NLC-COL-CS, and Pio-NLC-COL-CS-treated groups from days 0 to 21 postwounding. **(b)** Graphical representation of *percentage* wound reduction in the control, NLC-COL-CS, and Pio-NLC-COL-CS-treated groups from days 0 to 21 postwounding. Data are expressed as mean $\pm$ SD ($n=6$ wounds/group). ** $p \leq 0.005$; *** $p \leq 0.001$.

Figure 8. Result representing the estimation of hydroxyproline content of wound on days 7, 14, and 21 as an indicator of indirect collagen level estimation. The results are represented in microgram hydroxyproline/milligram of dry wound tissue. The data represent mean $\pm$ SD ($n=6$ wounds/group). Statistical significance was obtained by one-way ANOVA followed by Dunnett's *post hoc* test. * $p \leq 0.027$; ** $p \leq 0.009$; *** $p \leq 0.002$. ANOVA, analysis of variance.

matrix that is present in living tissues and provides the tissue matrix strength and integrity. Along with this, it also interacts with the cells to generate the required signals essential for proliferation, migration, and survival of the cells.¹⁶ CS, on the

contrary, is a nontoxic, biocompatible material known for its antimicrobial property and usually exhibits positive charge that helps in better adherence of the formulation to the negatively charged cell membrane.^{31,32} As the scaffolds were observed to be like a sponge, they consist of homogenous pores which, in turn, provide favorable biological environment for cell growth and also allow oxygen supply to the wound site.^{23,25} The porosity of the scaffold is expected to assist in absorption of wound exudates, cell binding, proliferation, and migration that are essential aspects of tissue formation. High porosity, however, leads to decreased mechanical strength of the scaffold. The decrease in porosity of the scaffold after cross-linking may be attributed to the formation of strong molecular interaction by EDC/NHS with CS and COL.^{33,34} Thus, cross-linking helped in maintaining the mechanical strength while providing favorable environment for cell growth.³⁵

Water absorption property of the scaffold influences not only in maintenance of its shape and form, but also affects the cell growth. Percentage water absorption was found to be more in noncross-linked scaffold than in cross-linked scaffold. Similar results were reported by Karri *et al.*²³ Con-

Figure 9. Histological changes during the wound healing process in STZ-induced diabetes Wistar albino rat skin on days 7, 14, and 21 after without (control) and with treatment (NLC-COL-CS, Pio-NLC-COL-CS) in a full thickness excision wound model. Arrows denote the following: N, neutrophils; M, macrophage; H, histiocytes; MM, multinucleated massive cell; MNGC, multinucleated giant cell, F, fibroblast. STZ, streptozotocin.

Figure 10. Graph representing the MMP-9 levels in fluids obtained from healing open dermal wounds in STZ-induced diabetic mouse model on day 21. Levels of MMP-9 were determined in 100-fold aliquots of wound fluids using ELISA analysis. The data represent mean $\pm$ SD ($n=3$). Statistical significance was obtained by one-way ANOVA followed by Dunnett's *post hoc* test. ** $p \leq 0.05$; *** $p \leq 0.005$. ELISA, enzyme-linked immunosorbent assay; MMPs, matrix metalloproteinases.

centration of the CS and cross-linking plays a major role in water absorption. The increase in the CS concentration decreases the water absorption capacity. Cross-linking of the scaffold attributed to increased mechanical strength that helped in maintaining the shape without affecting the cell growth.³⁶ The biodegradation studies showed that noncross-linked scaffold degraded more quickly compared with the cross-linked scaffold. Cross-linking leads to increased network strength and mechanical properties that, in turn, result in decreased degradation rate because of resistance to the enzymatic deterioration.³⁷

In vitro drug release studies showed a delay in the initial release of the drug, probably because of the time taken for complete wetting of scaffold. The cross-linked scaffold showed significantly slower release in comparison with noncross-linked scaffold. The cross-linking of scaffold increases the chemical and mechanical bonding compared with that of noncross-linked scaffold that, in turn, results in slower water absorption and swelling, thus sustaining the release of drug as stated by Pillay and Fassihi.³⁸ This results in prolonged release of Pio to combat chronic inflammatory condition in diabetic wounds.

The *in vitro* cell viability assay was performed on fibroblast cells (3T3-L1). Fibroblasts are the key cells that mediate the production of extracellular

matrix and protein (COL). They play a vital role in the formation of structural framework required for normal wound healing. The test scaffold did not induce any cytotoxicity, and cell growth was found to be more in Pio-NLC-COL-CS followed by NLC-COL-CS scaffold and Pio-treated group in comparison with that of the control group. These results indicate that uniform growth of fibroblast took place in the pores of the scaffold indicating that the scaffolds developed were biocompatible. The increase in cell growth in both the groups was because of the presence of COL and CS as matrix in scaffold form. Furthermore, comparatively higher growth in Pio-treated scaffolds might be because of stimulation of PPARs by Pio (PPARs agonist) that can stimulate fibroblast differentiation. It is pertinent to add here that similar results were observed by Mao-Qiang *et al.*³⁹

Diabetes causes multifactorial symptoms that include biochemical, vascular, neuropathic, and immune function abnormalities that delay the healing process in the wounds.^{26,40} To mimic the condition in rats, high dose of streptozotocin (STZ) was used intraperitoneally resulting in selective damage of the pancreatic β -cells. This leads to inhibition of synthesis and release of insulin leading to type 1 diabetes after 2–3 days. Various complications are typically accompanied with STZ-induced diabetic animal model.^{41–45} Open wounds were created in the diabetic rats and the treatment was carried out for 21 days. Reduction in the mean percentage of wound area was calculated for all the groups on days 0, 7, 14, and 21. Rats treated with the Pio-NLC-COL-CS scaffold showed a varying degree of wound contraction compared with NLC-COL-CS and untreated groups. The initial inflammatory responses to injury provided the necessary framework to the subsequent production of a new functional barrier, and next stage of wound healing is proliferative phase that lasted for 3–14 days. More than 60% of the reduction in the wound area was observed in the Pio-NLC-COL-CS group, whereas only 52% and 39% reduction of wound was seen in the NLC-COL-CS and control group on day 7, respectively. Contraction of the wound starts once the inflammatory and proliferative phase completes. This clearly depicts that Pio helped in suppressing the inflammation phase, whereas COL and CS helped in the proliferation and migration of cells resulting in 99% of wound healing in 21 days.

Control wounds on day 7 postinjury predominantly showed neutrophils along with some monocytes and lymphocytes. As stated in literature, neutrophils are responsible for the wound debridement in the early stage of wound healing.

However, excess number of neutrophils is known to negatively influence the wound repair and healing process by destroying the normal tissue. As reported by Dovi *et al.* reduction in the number of neutrophils accelerates reepithelization of the tissue.⁴⁶

In the Pio-NLC-COL-CS-treated group, the collection of macrophages and their morphological variants such as multinucleated massive cells and histiocytes were observed. The presence of these cells favors the atmosphere for wound healing. Macrophages have a more important role in repair than that in defense, and promote the COL fiber synthesis that helps in the wound closure and healing.

The ELISA test results showed a significant decrease of MMP-9 levels in the Pio-NLC-COL-CS-treated group in comparison with those in the control group. As stated earlier, excess levels of MMPs are responsible for the breakdown of formed COL during wound healing. Pio, an anti-inflammatory agent inhibited the MMP-9 levels that may be considered as the reason to prevent the breakdown of COL leading to accelerated healing. Similar type of results was obtained by Zhang *et al.*¹⁵

CONCLUSION

The prepared novel composite frameworks fulfilled the requisites of an optimum diabetic injury dressing in terms of mechanical resilience, swelling, porosity, biodegradation, biocompatibility, controlled discharge, cell bond, and multiplication with anti-inflammatory properties that are considered to be indispensable for tissue recovery in diabetic injuries. Therefore, this study indicates that the synergistic combination of Pio as an anti-inflammatory agent, COL as a structural matrix stabilizer, and CS as a controlled drug carrier is a potential approach in treating diabetic wounds.

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AUTHOR DISCLOSURE AND GHOSTWRITING

The authors declare that there are no conflicts of interest in this study. The authors alone are responsible for the content and writing of the article.

AUTHORS' CONTRIBUTIONS

Natarajan Jawahar was the author and synthesized the literature. Bharat Kumar Reddy Sanapalli was involved in complete *in vitro* and *in vivo* studies along with drafting the article and critical revision of the article. Veera Venkata Satyanarayana Reddy Karri provided conceptual inputs. Mehajabeen Bano Sachin Kumar Singh and Monica Gulati was involved in drafting the article. All authors read and approved the final version of the article.

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Abbreviations and Acronyms

ANOVA	= analysis of variance
COL	= collagen
CS	= chitosan
DM	= diabetes mellitus
EDC	= 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	= enzyme-linked immunosorbent assay
FE	= field emission
MES	= 2-(<i>N</i> -morpholino) ethane sulfonic acid
MMPs	= matrix metalloproteinases
NHS	= <i>N</i> -hydroxysuccinimide
NLC	= nanostructured lipid carrier
PBS	= phosphate-buffered saline
PEG	= polyethylene glycol
Pio	= pioglitazone
SD	= standard deviation
SEM	= scanning electron microscopy
STZ	= streptozotocin