6. Summary and Conclusions

6.1 Summary

Recent advances in electrospinning technology have led to emergence of topical products that do more than just cover and conceal, but address specific issues in open full thickness and highly infected wounds. The electrospun scaffolds facilitate dermis layer regeneration by mimicking the native extracellular matrix and releases the drugs in sustained manner at the wound site which causes complete wound closure in anticipated time. Taking into consideration the advantages of nanofibers, we had developed three novel combinations of nanofibers by electrospinning technique for the healing of a full thickness wound: (1) PCL based nanofibers loaded with ciprofloxacin hydrochloride and quercetin (PCL-CH-Que nanofibers); (2) PCL-GE based nanofibers loaded with ciprofloxacin hydrochloride and quercetin (PCL-GE-CH-Que nanofibers); (3) PLGA-GE based nanofibers loaded with ciprofloxacin hydrochloride and quercetin (PLGA-GE-CH-Que nanofibers). The fabricated nanofibers were extensively evaluated for different *in-vitro* as well as *in-vivo* characterizations and results are summarized as follows:

1. PCL-CH-Que Nanofibers

PCL based nanofibers of different composition were successfully electrospun using acetic acid and formic acid solvent system, which is one of the most commonly used solvents composition for electrospinning of PCL solution. This combination (acetic acid : formic acid; 7:3 ratio) produced a solvent system of optimum conductivity, which resulted into stable Taylor cone, continuous and homogenous nanofibers formation. The fabricated scaffold exhibited bead-free, continuous nanofibers with fewer secondary fibrils and thin

diameter (101.59±29.18 nm). The porosity of scaffold was within optimum range (69.36%) which was able to support cellular infiltration and proliferation, and exchange of gases and nutrients across the scaffold. Solid-state characterization by FT-IR and XRD confirms the absence of drugs and polymer interaction, and encapsulation of drugs in amorphous form, respectively. The non-volatile nature of drugs and complete mixing with PCL resulted into very high encapsulation efficiency (CH=92.04% and Que=94.32%). Although high encapsulation of drugs had significantly lowered the contact angle of drugs loaded nanofiber membrane (80.73±2.66°) in comparison to PCL membrane (100.1±2.28°), still contact angle was quite high for sufficient tissue attachment. The sustained release of encapsulated drugs (CH=98.98% and Que=85.09% in 6 days) with initial burst release was reasonably effective to inhibit bacterial load and excess free radicals activity in wound area. Further, the inhibition of *S. aureus* growth on an agar plate for consecutive 7 days and attenuation of DPPH in methanol solution to 40.13% extent established its antimicrobial and antioxidant activity, respectively. Quercetin loaded nanofiber was able to preserve the functionality of RBCs by protecting it against lipid peroxidation and it lysed 0.98% RBCs. Similarly, it maintained the fibroblast viability by shielding it against oxidative damage. Thus, the developed nanofiber proved its hemocompatibility and cytocompatibility activity. Further, *in-vivo* healing of full thickness wound demonstrated that PCL-CH-Que-nanofiber has promising wound healing property. The H&E stained granulation tissues exhibited developed epidermis and dermis layer. This result was further corroborated by biochemical analysis of granulation tissue, which showed that scaffold had effectively restored the SOD, catalase and hydroxyproline levels.

Although, PCL is one of the extensively examined, biodegradable**,** biocompatible with good mechanical properties, it suffers from hydrophobic nature, low biodegradation rate and absence of cell recognition site in PCL chain which results into inefficient attachment of scaffold at wound site, partial degradation and assimilation of nanofibers during the course of healing, and incomplete closure of wound, as observed in above finding. Therefore, the blending of PCL with gelatin was proposed to obtain a scaffold with optimum hydrophilicity, biodegradation rate, and optimum mechanical strength.

2. PCL-GE-CH-Que Nanofibers

PCL-GE based nanofibers functionalized with quercetin and ciprofloxacin hydrochloride was successfully developed by using hexafluoro-iso-propanol solvent, which is most commonly used solvent for solubilizing the gelatin. The mixing of gelatin had significantly increased the nanofiber diameter (725.94±201.97nm) with smooth shape and uniform size distribution. The porosity of scaffold was found 81.79%, which was sufficient for nutrient and gases exchange, and fibroblast and proliferation in-growth. Solid-state characterization by FT-IR and XRD confirms the absence of drugs and polymers interaction, and encapsulation of drugs in amorphous form, respectively. The high encapsulation efficiency (CH=87.31% and Que=90.10%) of PCL-GE based nanofibers was attributed to non-volatile nature of drugs and complete mixing with polymers. The addition of gelatin had enhanced the hydrophilicity (contact angle=48.8±2.95°) and biodegradation rate. The resultant high rate of biodegradation had reduced the drugs release time, and 57.98% ciprofloxacin hydrochloride and 44.78% quercetin were released within 8h, which was desirable for reducing bacterial infection and ROS attenuation during the early healing phase. The developed nanofibers had effectively reduced the *S. aureus* growth on the agar

plate for one week, and scavenged 55.14% DPPH in methanol, which confirms the potential anti-microbial and anti-oxidant property of nanofibers, respectively. Enhanced hydrophilicity and cell recognition site provided by gelatin improved the fibroblast viability on the nanofibers and scaffold exhibited >100% cell growth on its surface. Further, the protective action of quercetin against RBCs lysis by inhibiting lipid peroxidation confirmed the biocompatible nature of the scaffold and proved its application for open full thickness wound healing. The application PCL-GE-CH-Que nanofiber membrane on full thickness wound displayed excellent healing efficiencies and scaffold was able to close the wound completely by the end of 16 days. The H&E stained granulation tissues exhibited well developed epidermis and dermis layer along with dense collagen deposition in all treatment group. The biochemical analysis of SOD, catalase and hydroxyproline in the granulation tissue confirmed *in-vivo* antioxidant activity and fibroblast protective action of scaffold.

Although blending of gelatin with PCL resulted in significantly fast *in-vitro* degradation rate, hydrophilicity with comparatively higher cost and optimum mechanical strength than PCL based scaffold, however, PCL-GE solution separated into two phases during long-duration electrospinning and requires continuous mixing. Further, due to PCL content, the resulting nanofibers membrane did not biodegrade and assimilates completely during the course of healing. To avoid mentioned problems, blending of PLGA with gelatin was proposed to obtain a solution which should not separate into two layers during electrospinning, and resulting scaffold should completely biodegrade and assimilate during healing.

3. PLGA-GE-CH-Que Nanofibers

PLGA-GE based nanofibers loaded with ciprofloxacin hydrochloride and quercetin was successfully fabricated by using hexafluoro-iso-propanol, most commonly used solvent for solubilization of PLGA and gelatin. Mixing of PLGA and gelatin resulted into smooth and thick nanofibers (642.49±301.04 nm) with fewer fibrils and uniform size distribution. The scaffold had sufficient porosity (72.53%) for cell in-growth, exchange of nutrient, gases and exudates across the scaffold. Solid-state characterization by FT-IR and XRD confirms the absence of drugs and polymer interaction, and encapsulation of drugs in amorphous form, respectively. The high encapsulation efficiency (CH=92.04% and Que=94.32%) of PLGA-GE based nanofibers was due to and complete mixing of drugs with polymers and non-volatile nature of drugs. Owing to combination of PLGA and gelatin, the surface hydrophilicity of scaffold was very high and water drop absorbed on the surface immediately leaving no time to take an image by Drop Shape Analyzer. Mixing of PLGA and gelatin also increased the biodegradation rate of scaffold which resulted into shortening of drugs release duration (CH = 97.43%, Que = 80.21% in 49 h). This much of short duration release was required for reducing bacterial infection and attenuation of ROS during the early healing phase, however not effective for preventing long term infection. Inhibition of *S. aureus* growth on the agar plate for one week, and attenuation of DPPH (61.89%) in methanol, confirmed the anti-microbial and anti-oxidant property of ciprofloxacin hydrochloride and quercetin loaded nanofibers. Further, biocompatible nature of scaffold was examined by hemocompatibility and cell viability on scaffold surface. Flavonoid loaded nanofiber was able to protect the functionality of RBCs by reducing the lipid peroxidation in RBCs membrane. Similarly, it upheld the fibroblast growth on its surface by protecting it against oxidative damage. Finally, drugs loaded nanofiber

membrane was applied on full thickness wound and it had shown good healing efficiencies. The scaffold was able to close the wound completely by the end of third weeks. The histological examination confirmed the development of epidermis and dermis layer. The analysis of SOD, catalase and hydroxyproline in the granulation tissue confirmed fibroblast protective action and *in-vivo* antioxidant activity of scaffold.

The results shown that PLGA-GE based nanofibers exhibited highly hydrophilic surface, high DPPH scavenging efficacies and complete biodegradation and assimilation at wound site than PCL-GE based nanofiber membrane, however, it suffered from poor tensile strength which resulted into fast release of drugs, and it also required soft handling. Moreover, the cost of PLGA-GE based nanofibers was comparatively quite high (PLGA P1941-1G: Rs 8,919 + Gelatin MB169-500G: Rs 4200) than PCL based and PCL-GE based nanofiber membrane.

The comparative analysis of different *in-vitro* and *in-vivo* observations of PCL-CH-Que nanofibers, PCL-GE-CH-Que nanofibers and PLGA-GE-CH-Que nanofibers are displayed in Table 6.1.

Table 6.1: Comparative analysis of *in-vitro* and *in-vivo* observations for three different types of nanofiber membranes

Parameters	PCL-CH-Que Nanofibers	PCL-GE-CH-Que Nanofibers	PLGA-GE-CH-Que Nanofibers
Fiber Morphology	Bead-free, continuous nanofibers with lateral perturbations	Smooth nanofibers with fewer fibrils	Smooth nanofibers with fewer fibrils
Diameter distribution & porosity	101.592±29.184 nm; 69.36%	725.943±201.965 nm; 81.79%	642.489±301.039 nm; 72.53%
FT-IR & P-XRD	No physico-chemical interaction was reported between drugs and polymer(s)		
Contact angle	80.73±2.65º	48.76±2.95º	Highly hydrophilic
Entrapment efficiency	CH = 92.04% Que = 94.32%	CH = 87.31% Que = 90.10%	CH = 85.45% Que = 92.03%
***In-vitro* release profile**	CH = 98.98% Que = 85.09% in 6 days	CH = 99.18% Que = 88.09% in 4 days	CH = 97.43% Que = 80.21% in 49 h (around 2 days)
***In-vitro* antioxidant activity**	40.31% DPPH scavenging in 0.5h	55.14% DPPH scavenging in 0.5h	61.89% DPPH scavenging in 0.5h
***In-vitro* antibacterial activity**	Yes	Yes	Yes
Hemocompatibility	Yes	Yes	Yes
Cell Viability	All three nanofiber membranes allowed the Swiss albino 3T6 fibroblast growth on its surface		
Wound Healing	92% wound contraction in 16 days	~100% wound closure in 16 days with sealing effect was observed since day 12	96% wound contraction in 16 days
Collagen deposition	A lot white spaces (or lack of collagen) were observed in H&E stained tissues.	Very few white spaces were observed.	Moderate amount of white spaces was observed.
Comparative Tensile Strength	Good	Optimum	Poor
Comparative Cost	Low	Medium	High

6.2. Conclusions

The current research focused on the development of a scaffold for prompt healing of full-thickness wounds. Considering the advantages of nanofibers as wound healing materials, and importance of an antimicrobial and antioxidant, we proposed to fabricate a nanofiber loaded with ciprofloxacin hydrochloride and quercetin by electrospinning technology. Three different combinations of electrospun nanofibers (PCL-CH-Que based, PCL-GE-CH-Que based, and PLGA-GE-CH-Que based) were fabricated one by one to alleviate the shortcomings of the previous one. On the basis of research findings, it was found that although all three fabricated nanofiber membrane were able to completely close the full thickness wound within three weeks. However on the basis of various parameters evaluated such as surface hydrophilicity (contact angle), *in-vitro* drugs release profile, free radicals scavenging efficacies, wound healing capability, collagen deposition efficiencies, tensile strength and cost of formulation, we found PCL-GE-CH-Que nanofiber membrane better than the other two, i.e., PCL-CH-Que and PLGA-GE-CH-Que nanofiber membranes. Conclusively, this study endorses the use of ciprofloxacin hydrochloride and quercetin loaded nanofiber membrane as a potential wound healing dressing material.

Future perspective: Although current research supports the application of PCL-GE-CH-Que nanofiber membrane for healing of a full-thickness wound, it further requires safety study (such as toxicity study, skin irritation, cutaneous toxicity, contact sensitization, contact photodermatitis, effect of percutaneous absorption), efficacy study by well-controlled clinical study, and manufacturing controls since in this research in-house assembled equipment was used for fabrication of nanofibers. After obtaining appropriate results, the PCL-GE-CH-Que nanofiber membrane can be endorsed for human application.