4. Materials and Methods

4.1. Materials

Following chemicals, equipment and software were used in my research work.

Table 4.1: List of chemicals utilized during study

S. No.	Chemicals	Source
1	Acetic acid	SD Fine-Chem Ltd., Mumbai
2	Acetone	Himedia, Mumbai.
3	Antibiotic Solution 100X Liquid	Himedia, Mumbai.
4	Chloramine-T	SRL Pvt Ltd, Maharashtra
5	Ciprofloxacin Hydrochloride (Gift Sample)	Cipla, Mumbai
6	Disodium hydrogen orthophosphate	Merck Ltd., Mumbai
7	Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific (USA).
8	Ehrlich's solution	Sigma-Aldrich, India
9	Ethanol	SD Fine-Chem Ltd., Mumbai
10	Fetal Calf Serum (FCS)	Thermo Fischer Scientific, UK
11	Formic acid	SD Fine-Chem Ltd., Mumbai
12	Gelatin from porcine skin, Type A	Himedia, Mumbai
13	Hydrochloric Acid (HCl)	Thermo Fischer Scientific, Mumbai
14	Hydrogen Peroxide (H_2O_2)	Merck Life Sciences Pvt Ltd
15	Hydroxyproline amino acid	Sigma-Aldrich, India
16	Methanol	Merck Ltd., Mumbai, India
17	Methionine	SRL Pvt Ltd, Maharashtra
18	MTT	SRL Pvt Ltd, Maharashtra
19	Nitro blue tetrazolium	SRL Pvt Ltd, Maharashtra
20	Phenolphthalein Solution	SRL Pvt Ltd, Maharashtra
21	Poly (D,L-lactide-co-glycolide) (Mw = 66000-10700)	Sigma-Aldrich, India
22	Poly (ε- caprolactone) (Mw =80000)	Merck, India
23	Potassium dihydrogen orthophosphate	Qualigens Chemicals, Mumbai
24	Quercetin	Cayman Chemical, USA
25	Riboflavin	SRL Pvt Ltd, Maharashtra
26	Sodium hydroxide	Qualigens Chemicals, Mumbai
27	Trypsin-EDTA Solution 1X	Himedia, Mumbai
28	1,1,1,3,3,3-hexafluoro-iso-propanol (HFIP)	Spectrochem Pvt. Ltd, Mumbai
29	3T6-Swiss albino fibroblast	National Centre for Cell Science, Pune

Table 4.2: List of major equipment utilized during study

S. No.	Instruments	Source
1	Aluminium foil (fresh wrap)	Hindalco Industries Ltd., Mumbai, India
2	Camera assisted Optical microscope	Dewinter Optical, Inc., New Delhi, India
3	Cooling centrifuge	REMI C20, Mumbai, India
4	Digital electronic balance	Shimadzu, Japan
5	Digital pH meter	Perfit India
6	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
7	Dissection box	Camlin Ltd., Mumbai, India
8	Fourier Transform Infrared (FT-IR) Spectrophotometer	Shimadzu FTIR-8400S, Japan
9	High voltage power supply	Goldstar, New Delhi, India
10	High-resolution Scanning Electron Microscopy HR-SEM	FEI, Quanta 200F, Japan
11	Hot air oven	IKA, Germany
12	Magnetic stirrer	Decibel Instruments, Chandigarh, India
13	Microplate reader	Synergy HTX multi-mode reader (Bio Tek, USA)
14	Powder X-Ray Diffractometer	Mini Flex 600, Rigaku, Japan
15	Scanning Electron Microscope	ZEISS EVO 18 Research, USA
16	Sonicator (bath type)	IKA, Germany
17	Syringe pump	AYRA N801 New era pumps, USA
18	UV-visible Spectrophotometer	UV-1800, Shimadzu, Japan

Table 4.3: List of software utilized during study

S. No.	Name of Software	Developer
1	Adobe Acrobat Reader 11.0	Adobe Systems Inc. USA
2	EndNote 6.0	Wintertree Software Inc., USA
3	Grammarly	Grammarly Inc., San Francisco, USA
4	GraphPad Prism 5.03	Graph Pad Software Inc., USA
5	Microsoft Office 2010	Microsoft Corp., USA
6	OriginPro 8.1	Origin Lab Corp., USA

4.2. Experimental Sections

4.2.1. Pre-formulation studies

Pre-formulation studies include;

1. Development of analytical method for the estimation of ciprofloxacin hydrochloride and quercetin by UV-Vis spectroscopy
2. Validation of developed method as per ICH guidelines for the estimation of ciprofloxacin hydrochloride and quercetin by UV-Vis spectroscopy
3. Solubility studies of both drugs in phosphate buffer (pH 7.4)

4.2.1.1. Development of analytical method for the estimation of ciprofloxacin hydrochloride and quercetin by UV-Vis spectroscopy

4.2.1.1.1. Preparation of phosphate buffer (pH 7.4)

A phosphate buffer solution (pH 7.4) was prepared according to standard method given in Indian Pharmacopoeia, 2014. Briefly, 250 mL of 0.2M KH_2PO_4 solution and 195.5 mL of 0.2M NaOH solution were mixed together and made up the volume upto 1000 mL. The pH of the solution was adjusted to 7.4 using above NaOH solution. Digital pH-meter was used for adjusting pH of the buffer solution.

4.2.1.1.2. Preparation of stock solution (1000 µg/ml)

Accurately weighed amount of ciprofloxacin hydrochloride (25 mg) and quercetin (25 mg) were transferred in 25 ml volumetric flask separately and dissolved in methanol. The obtained solutions were sonicated for 5 min and volume was made up to 25 ml by methanol for each drug.

4.2.1.1.3. Preparation of working standard solution 100 µg/ml)

10 mL of each stock solution was transferred in 100 mL volumetric flask separately and volume was made up to 100 mL with phosphate buffer (pH 7.4) for each.

4.2.1.1.4. Determination of λ_{max}

The working standard of both drugs was diluted separately with phosphate buffer to obtain a final concentration of 8 µg/mL. The resultant solution was scanned against blank in the entire UV-Visible range (200-800 nm) to determine the absorbance maxima. Clear peaks for ciprofloxacin hydrochloride and quercetin were observed at 271 nm and 370 nm, respectively. Therefore, these absorbance maxima were selected as the λ_{max} value for these drugs. The zero-order overlay spectra of ciprofloxacin hydrochloride and quercetin with their absorbance maxima are shown in Figure 4.1.

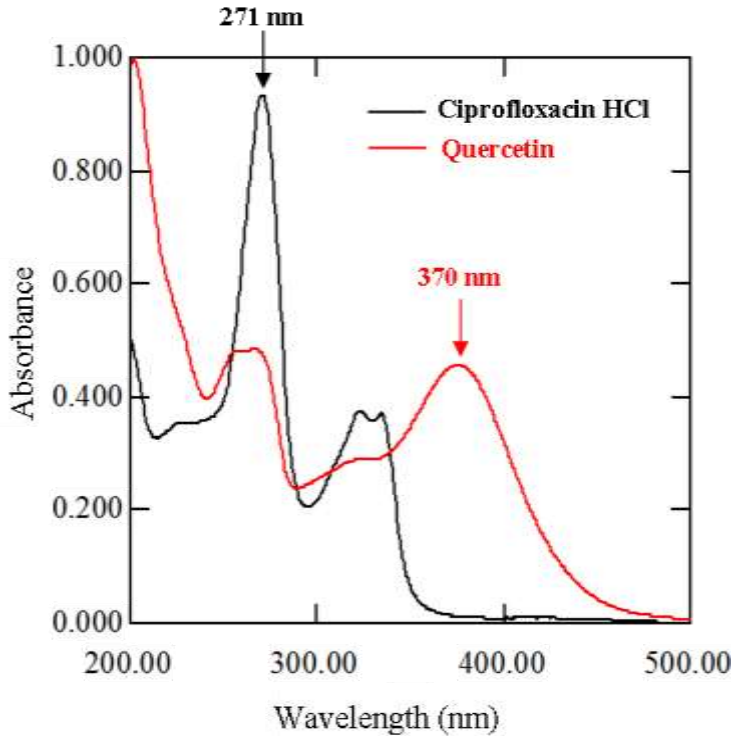

Figure 4.1: Overlay spectra of ciprofloxacin hydrochloride and quercetin

4.2.1.1.5. Calibration curve preparation

Standard solutions of ciprofloxacin hydrochloride and quercetin were prepared in the concentration range of 1-8 µg/ml and 1-16 µg/ml, respectively, by serial dilution from the corresponding working standard solutions in the phosphate buffer (pH 7.4). The absorbance values of these solutions were measured at 271 nm and 370 nm, respectively. The calibration curve was plotted between absorbance and corresponding drug concentrations to verify the Beer's & Lambert's law, and to calculate molar absorptivity value for both drugs at both wavelength values.

Absorptivity at respective wavelengths was calculated using following Beer's Lambert Law.

$$A = log\left(I_0/I\right) = \varepsilon cl \quad (1)$$

Where, I_0 = intensity of light incident upon sample cell, I = Intensity of light leaving sample cell, c = molar concentration of solute (mole/litre), l = length of sample cell (cm), ϵ = molar absorptivity (Liter/mole/cm).

4.2.1.1.6. Simultaneous equation method for drugs estimation

Simultaneous equation method also known as Vierordt's method is a typically used method for the estimation of two or more than two drugs in combination in a single dosage form. It is based on measuring the absorbance at two selected absorption maxima wavelengths i.e. 271 nm (λ_{max} of Ciprofloxacin HCl) and 370 nm (λ_{max} of Quercetin) (Fig. 4.1). This method can be applied only when ciprofloxacin hydrochloride and quercetin have well-separated absorption maxima. The concentrations of ciprofloxacin hydrochloride and quercetin was quantified using the following equations [Beckett and Stenlake 1988].

$$Quercetin\ concentration = \frac{A_{271} \times a_{370} - A_{370} \times a_{271}}{b_{271} \times a_{370} - b_{370} \times a_{271}} \quad (2)$$

$$Ciprofloxacin\ Hydrochloride\ concentration = \frac{A_{370} \times b_{271} - A_{271} \times b_{370}}{b_{271} \times a_{370} - b_{370} \times a_{271}} \quad (3)$$

Where, A_{271} and A_{370} denotes the absorbance of supernatant at 271 and 370nm, respectively. a_{271} and a_{370} is the absorptivity of ciprofloxacin hydrochloride at 271 and 370nm, respectively. b_{271} and b_{370} is the absorptivity of quercetin at 271 and 370nm, respectively.

4.2.1.2. Validation of developed method as per ICH guidelines for the estimation of ciprofloxacin hydrochloride and quercetin by UV-Vis spectroscopy

Validation is an important part of quality assurance program and aims to demonstrate that the developed analytical method is suitable for the simultaneous estimation of CH and Que and it is safe to run. Method validation was performed following ICHQ2(R1) specifications for linearity, accuracy, precision, limit of detection, limit of quantitation and mixture analysis [ICHQ2(R1) 2005].

4.2.1.2.1. Linearity and range

As per ICHQ2(R1), "the linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample". Mathematically, the concentration range over which Beer's Lambert law is obeyed is linearity. "The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity". [ICHQ2(R1) 2005]. Linearity and range

of analyte at different wavelengths was determined from corresponding calibration curve of drugs.

4.2.1.2.2. Accuracy

As per ICHQ2(R1), "the accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found"[ICHQ2(R1) 2005]. The accuracy was determined by standard addition method. In this method, a known amount of standard stock solution of ciprofloxacin hydrochloride and quercetin was added at three different levels, i.e. 80%, 100% and 120% in the pre-analyzed solution of both the drugs. The solution was reanalyzed by the proposed method. The concentration of added analyte was calculated from the respective simultaneous equation and accuracy was reported in terms of recovery (%) by using following equation:

$$Recovery\ (\%) = \frac{Calculated\ concentration}{Theoretical\ Concentration} \times 100 \quad (4)$$

4.2.1.2.3. Precision

According to the ICH guideline, "the precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions" [ICHQ2(R1) 2005]. It was assessed for both intra-day and inter-day. The accuracy was displayed in terms of percentage relative standard deviation (%RSD) and calculated using the following equation:

$$\%RSD = \frac{Standard\ deviation\ of\ values}{Mean\ value} \times 100 \quad (5)$$

4.2.1.2.4. Limit of detection and limit of quantitation

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily exactly quantified. The limit of quantitation (LOQ) is the lowest amount of analyte in a sample which can be quantitated with precision and accuracy. For each determination, y-intercept was calculated and the standard deviation (SD) of the y-intercept was computed. From these values, LOD and LOQ were calculated on the basis of response and slope (S) of the regression equation obtained from the linearity studies as follows.

$$DL = 3.3\left(\frac{\sigma}{S}\right) \quad (6)$$

$$QL = 10\left(\frac{\sigma}{S}\right) \quad (7)$$

Where σ = the standard deviation of the intercept
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out from the standard deviation of y-intercepts of regression lines.

4.2.1.3. Solubility studies

When a deep wound take place, the skins acidic milieu and pH (4 to 6) is disturbed, revealing the more neutral pH (7.4) of the underlying tissue [Jones et al. 2015]. Therefore, pH 7.4 was chosen for solubility determination to simulate pH of underlying tissue. Solubility of ciprofloxacin hydrochloride and quercetin were determined in phosphate buffer (pH 7.4) by shake-flask method. In this method, excess amounts of both drugs were

separately added to 10 ml of phosphate buffer (pH 7.4) contained in air tight glass vials and samples were stirred on magnetic stirrer for 24 h for complete dissolution. The formed suspensions were filtered to remove excess of undissolved drug. The solutions were diluted with phosphate buffer (pH 7.4) and assayed for drug content using developed UV method.

4.2.2. Formulation development

In the present study three nanofibers of different combinations were developed by electrospinning technique: (1) PCL based nanofibers loaded with ciprofloxacin hydrochloride and quercetin (PCL-CH-Que nanofibers); (2) PCL-Gelatin based nanofibers loaded with ciprofloxacin hydrochloride and quercetin (PCL-GE-CH-Que nanofibers); (3) PLGA-Gelatin 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.

4.2.2.1. Fabrication of PCL based nanofiber loaded with ciprofloxacin hydrochloride and quercetin

Electrospinning solutions of different compositions were prepared in acetic acid : formic acid (AA:FA = 7:3 v/v) solvent mixture as per the scheme given in Table 4.4. Although acetic acid is an independent solvent for PCL and ciprofloxacin hydrochloride dissolution, owing to the medium dielectric constant, 6.2 at 25 °C and poor electric conductivity, it is not suitable for electrospinning. Mixing it with formic acid (high dielectric constant solvent, 57.9 at 25 °C) facilitates the electrospinning process. Dimethyl sulfoxide was used as co-solvent for solubilization of quercetin. The electrospinning was carried out with an in-house assembled electrospinning equipment fitted with a 5cc syringe and a blunt-end 24G metallic needle, a high voltage DC power supply to generate sufficient electric field

for charge repulsion in the solution, a syringe pump to regulate the flow rate of the electrospinning solution and a flat metallic collector covered with aluminum foil to collect the nanofibers. The electrospinning parameters for PCL-Gelatin based nanofiber are shown in Table 4.5. All the experiments were performed at ambient room condition (temperature– 24°C; relative humidity– 65±5%). Steps involved in electrospinning are represented in Figure 4.2.

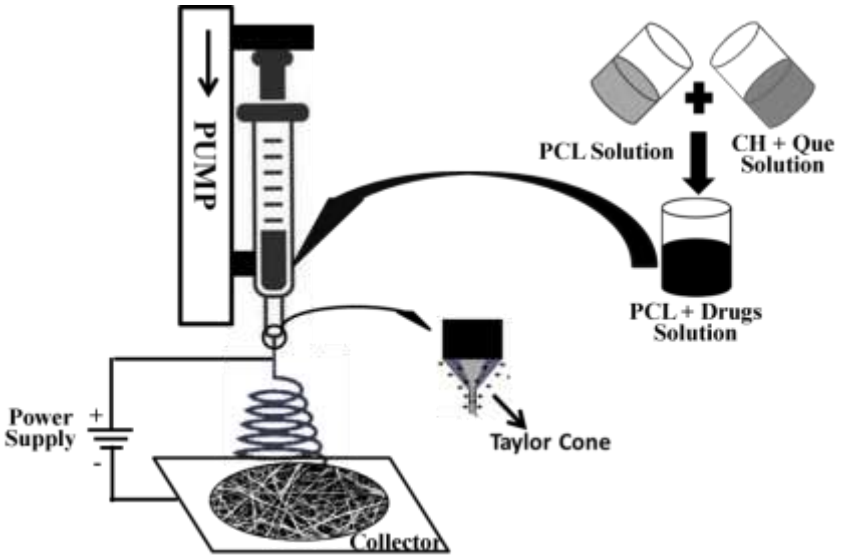

Figure 4.2: Graphic demonstration of steps involved in the PCL-based electrospun nanofiber preparation.

Table 4.4: Composition of different PCL-based electrospinning solution

Sample	PCL (% w/v)	Ciprofloxacin Hydrochloride (% w/w of PCL)	Quercetin (% w/w of PCL)
PCL (8%)	8	0	0
PCL (12%)	12	0	0
PCL-CH	12	10	0
PCL-CH-Que	12	10	5

Table 4.5: Electrospinning parameters for PCL-based nanofibers

Parameters	Description
Solvent system	Acetic Acid : Formic Acid (7:3 v/v)
Co-solvent for Quercetin	Dimethyl Sulphoxide
Syringe	A 5mL syringe fitted with a 24G blunt-end needle
Distance between needle and collector	8-10cm
Solution Flow rate	0.6 mL/h
Applied DC Voltage	16 kV

4.2.2.2. Fabrication of PCL-Gelatin based nanofiber loaded with ciprofloxacin hydrochloride and quercetin

A polymeric blend of PCL and Gelatin was prepared by mixing 12% w/v of PCL in hexafluoro-iso-propanol (HFIP) and 12% w/v of Gelatin in HFIP in the equal volume ratio (1:1). A small quantity (0.2% v/v of HFIP) of glacial acetic acid was added to the mixture for complete solubilization of gelatin and PCL and also for avoiding the phase separation during electrospinning [Anjum et al. 2017]. DMSO was used as a co-solvent for quercetin solubilization. Electrospinning solutions of various compositions had been developed in accordance with the scheme in Table 4.6. The final solution was filled in a 5mL syringe which was kept at 8-10cm distance from the collector. The electrospinning solution was pumped from the syringe and electrospun at a voltage of 14kV. The electrospinning parameters for PCL-Gelatin based nanofiber are shown in Table 4.7. Steps involved in electrospinning are shown in Figure 4.3. The nanofiber membrane was carefully removed from the collector and stored in a vacuum desiccator for further use.

Table 4.6: Composition of different PCL-Gelatin-based electrospinning solution

Sample	PCL (% w/v)	Gelatin (% w/v)	Ciprofloxacin Hydrochloride (% w/w of Polymers)	Quercetin (% w/w of Polymers)
PCL-GE	12	12	0	0
PCL-GE-CH	12	12	10	0
PCL-GE-CH-Que	12	12	10	5

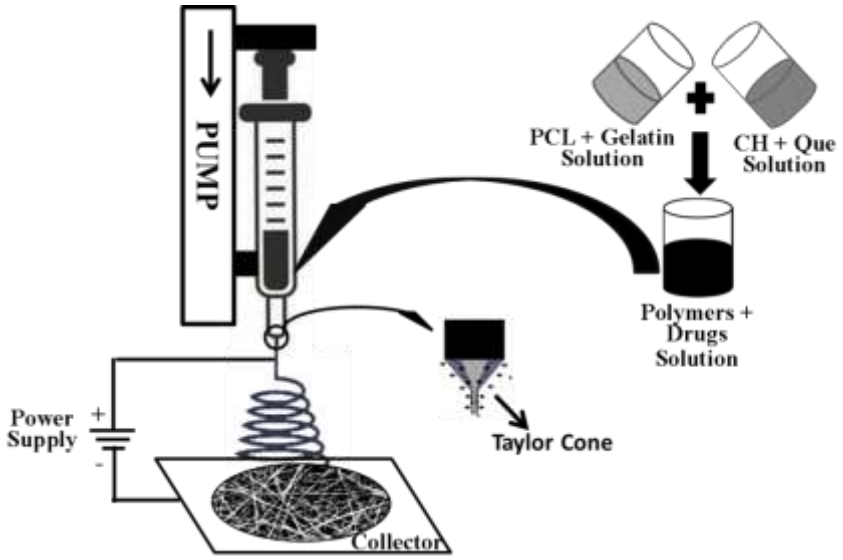

Figure 4.3: Graphic demonstration of steps involved in the PCL-GE based electrospun nanofiber preparation.

Table 4.7: Electrospinning parameters for PCL-Gelatin-based nanofibers

Parameters	Description
Solvent system	Hexafluoro-2-propanol (HFIP)
Co-solvent for Quercetin	Dimethyl Sulphoxide
Syringe	A 5 mL syringe fitted with a 24G blunt-end needle
Distance between needle and collector	8-10 cm
Solution Flow rate	0.5 mL/h
Applied DC Voltage	14 kV

4.2.2.3. Fabrication of PLGA-Gelatin based nanofiber loaded with ciprofloxacin hydrochloride and quercetin

A polymeric blend of PLGA and Gelatin was prepared by mixing 15% w/v of PLGA in HFIP and 15% w/v of Gelatin in HFIP in the equal volume ratio (1:1). DMSO was used as a co-solvent for quercetin solubilization. Electrospinning solutions of various compositions had been developed in accordance with the scheme in Table 4.8. The electrospinning was carried out with an in-house assembled electrospinning equipment fitted with a 5cc syringe and a blunt-end 24G metallic needle, a high voltage DC power supply to generate sufficient charge in the solution, a syringe pump to regulate the flow rate of the electrospinning solution and a flat metallic collector covered with aluminum foil to collect the nanofibers. Various electrospinning parameters are shown in Table 4.9. The nanofiber membrane was carefully removed from the collector and stored in a vacuum desiccator for further use. Steps involved in electrospinning are represented in Figure 4.4.

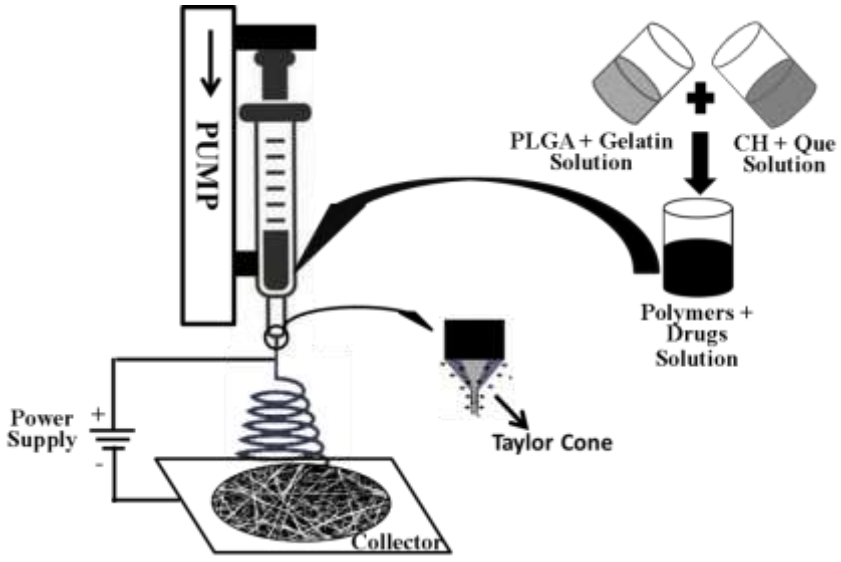

Figure 4.4: Graphic demonstration of steps involved in the PLGA-GE based electrospun nanofiber preparation.

Table 4.8: Composition of different PLGA-Gelatin-based electrospinning solution

Sample	PCL (% w/v)	Gelatin (% w/v)	Ciprofloxacin Hydrochloride (% w/w of Polymers)	Quercetin (% w/w of Polymers)
Gelatin	0	15	0	0
PLGA-GE	15	15	0	0
PLGA-GE-CH	15	15	10	0
PLGA-GE-CH-Que	15	15	10	5

Table 4.9: Electrospinning parameters for PLGA-Gelatin-based nanofibers

Parameters	Description
Solvent system for Polymer (PCL)	Hexafluoro-2-propanol
Co-solvent for Quercetin	Dimethyl Sulphoxide
Syringe	A 5 mL syringe fitted with a 24G blunt-end needle
Distance between needle and collector	6-8 cm
Solution Flow rate	0.7-0.8 mL/h
Applied DC Voltage	16 kV

4.2.3. Characterization of nanofiber membrane

4.2.3.1. Morphological Study

Morphology of the fabricated electrospun nanofibers were observed by high-resolution scanning electron microscopy (HR-SEM) (FEI, Quanta 200F, Japan) operated at an acceleration voltage of 10 kV. Before SEM scanning, samples were sputtered with gold for 2 min to increase their conductivity. ImageJ software was used to measure fibers diameter and porosity, as reported previously [Liu et al. 2014]. At least 100 filaments of each sample from random locations were analyzed to obtain the average fiber diameter. The data was further processed by GraphPad Prism 5 to get the histogram of fiber diameter with respect to its distribution frequency.

4.2.3.2. Solid-state characterizations

FT-IR spectroscopy was done to explore the possible chemical interaction between drugs and polymer(s), stability of drugs as well as effect of electrospinning on the functional groups of drugs loaded in the formulation [Gaonkar et al. 2017, Khan et al. 2017]. SHIMADZU 8400S spectrophotometer was utilized for scanning the samples. The FTIR spectra of individual drug, polymer and nanofiber film were obtained by scanning the KBr pellet in the range of 400 to 4000 cm^{-1} with a resolution of $2\,cm^{-1}$.

Powder X-ray diffraction was employed to determine the change in crystalline property of the drugs encapsulated in nanofiber film. The diffraction pattern of individual drug, polymer(s) and nanofiber film were examined by an X-ray diffractometer equipped with a CuKα X-ray source ($\lambda = 1.54\,°A$), a detector (D/tex Ultra) and under 40 kV, 15 mA operating condition. The samples were scanned in a rotating holder over a 2θ range from 5° to 60° at a scan rate of $4°\,min^{-1}$.

4.2.3.3. Contact angle of nanofiber

The hydrophilicity of the nanofiber surface was evaluated by measuring the contact angle between a sessile drop of water and membrane surface. For contact angle measurement, a 2µL drop of water was dropped on the nanofiber surface with the help of a microsyringe and static image was taken by Drop Shape Analyzer (DSA25S, KRUSS, Germany). An average value of three measurements at different position on the scaffold surface represented as the contact angle value of that scaffold.

4.2.3.4. Entrapment efficiency and in-vitro cumulative drug release profile

The amount of drugs entrapped in nanofiber was quantified by dissolving known weight of nanofiber film in 1 mL HFIP using 0.2% v/v acetic acid. Obtained clear solution was mixed into 10 mL methanol which was an anti-solvent to the polymer(s). After centrifugation of mixture, polymer(s) gets precipitated, and drugs remain solubilized into supernatant which was assayed by UV-Vis Spectrophotometer, and concentration was calculated by simultaneous equation methods- equation (2) & (3). Entrapment efficiency was determined by the equation (8):

$$Quercetin\ concentration = \frac{A_{271} \times a_{370} - A_{370} \times a_{271}}{b_{271} \times a_{370} - b_{370} \times a_{271}} \quad (2)$$

$$Ciprofloxacin\ Hydrochloride\ concentration = \frac{A_{370} \times b_{271} - A_{271} \times b_{370}}{b_{271} \times a_{370} - b_{370} \times a_{271}} \quad (3)$$

$$Entrapment\ Efficiency(\%) = \left(\frac{Amount\ of\ drug\ in\ the\ sample\ (mg)}{Theoretical\ amount\ of\ drug\ loading\ in\ the\ sample\ (mg)}\right) \times 100 \quad (8)$$

Where, A_{271} and A_{370} denotes the absorbance of supernatant at 271 and 370 nm, respectively. a_{271} and a_{370} is the absorptivity of ciprofloxacin hydrochloride at 271 and 370 nm, respectively. b_{271} and b_{370} is the absorptivity of quercetin at 271 and 370 nm, respectively.

In-vitro drug release characteristics of developed nanofiber membrane were determined in phosphate buffer (pH 7.4). A known weight and approximate dimension ($1 \times 1\ cm^2$) of membrane was soaked into 2 mL phosphate buffer in a sealed vial. The vials were immersed in a water bath shaker running at 50 strokes per min at 37 °C. At specified time point, the whole portion of buffer was withdrawn and replaced with an equivalent amount of fresh buffer to maintain sink condition. Experiments were run in triplicate per sample.

The cumulative amount and percentage of the drug released was calculated based on the initial amount of entrapped drug in the nanofiber film.

4.2.3.5. Free-radical scavenging efficiency of nanofibers

Antioxidant activity of the nanofiber membrane was studied by estimating 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability of fabricated scaffold, according to the earlier reported method [Selvaraj and Fathima 2017]. The fundamental principle behind this assay is that DPPH is a stable free radical which gives purple color solution in methanol with maximum absorbance at 517 nm. It changes its color from purple to yellow on accepting a hydrogen (H) or an electron from the antioxidant (like quercetin) and is reduced itself to $DPPH_2$. UV-spectrophotometer measures the reduction of purple color intensity at 517 nm and utilized for quantification of antioxidant concentration in the solution [Mishra et al. 2012]. Briefly, 5 mg nanofiber membrane was immersed in 3 mL methanolic solution of DPPH (100 µM) and the reaction mixture was incubated in the dark condition at room temperature. After incubating for 0.5 h, the absorbance of the reaction mixture was measured at 517 nm by UV-Vis Spectrophotometer. DPPH scavenging efficacies was determined by following equation:

$$DPPH\ scavenging\ efficacies(\%) = \left(\frac{A_{DPPH\ pure} - A_{sample}}{A_{DPPH\ pure}}\right) \times 100 \quad (9)$$

where, $A_{DPPH\ pure}$ and A_{sample} represents the absorbance of pure DPPH solution and nanofiber-incubated DPPH solution in methnol, respectively.

4.2.3.6. In-vitro antibacterial assessment

Antibacterial activity of fabricated nanofiber membrane against *Staphylococcus aureus* (*S. aureus*) (MTCC1303), which is commonly found Gram-positive bacteria in open wounds

infection, was determined by film diffusion method [Almeida et al. 2014, Unnithan et al. 2012]. The microorganism was pre-cultured overnight in Luria–Bertani medium in a rotary shaker at 37±1 °C. Subsequently, broth was centrifuged at 12000 rpm for 3 min, resulting pellet was re-suspended and diluted to obtain a standard working suspension (10^8 cells/mL). The 100 µL of standard working bacterial suspension was spread on nutrient agar plate surface using sterile swab sticks. Thereafter, nanofiber films of 5 mm diameter were placed on an agar plate surface and incubated at 37±1 °C for 24 h. After 24 h, films were shifted to another fresh agar plate seeded with *S. aureus.* At each time point, the microbial growth on the petri-plate was observed directly and inhibition diameter was measured.

4.2.3.7. Biocompatibility studies of nanofiber membrane

4.2.3.7.1. Hemocompatibility assessment of nanofiber membrane

As the scaffolds were intended for open wound application, the assessment of hemolytic property became essential to check whether the formulation was able to preserve the integrity of RBCs in newly developed blood vessels. Hemocompatibility of the nanofibers was done as described by Vijayakumar et al. [Vijayakumar et al. 2016]. RBCs were collected from 2 mL human blood by centrifugation at 2200 rpm for 15 min and rinsed thrice with normal saline (0.9%) to separate out traces of plasma. Finally, erythrocytes pellet were re-suspended gently up to 10 mL in normal saline to obtain standard erythrocytes suspension. 5 mg of each sample were mixed with 2 mL of standard suspension of RBCs and incubated at room temperature for 0.5 h. Equal volume of standard erythrocytes suspension was mixed with 1% Triton X-100 and normal saline, separately and utilized as positive (completely lysed RBCs) and negative control, respectively. After incubation, the samples were centrifuged and supernatant was separated out and further

incubated for 0.5h for oxidation of hemoglobin in to oxy-hemoglobin. The absorbance of oxy-hemoglobin was measured at 540 nm by UV-Vis spectrophotometer. The percentage of RBCs lysed was calculated by following equation:

$$Hemolysis\ (\%) = \left(\frac{A_{test} - A_{negative\ control}}{A_{positive\ control} - A_{negative\ control}}\right) \times 100 \qquad (10)$$

Whereas A_{test}, $A_{negative\ control}$ and $A_{positive\ control}$ represent the absorbance of supernatant incubated with nanofibers, normal saline and Triton X-100, respectively.

4.2.3.7.2. *In-vitro* cell viability assessment

The Viability of 3T6-Swiss albino fibroblast on nanofiber was examined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as described by Selvaraj and Fathima, 2017. The basic principle of this assay is that yellow color MTT is reduced by mitochondrial NAD(P)H-dependent oxidoreductase enzymes to a intracellular insoluble purple color formazan. The cells are then solubilised with an organic solvent and release the solubilized purple color formazan reagent, which is measured spectrophotometrically. Since only metabolically active cells contain-NAD(P)H-dependent oxidoreductase enzymes, therefore this assay directly measures of viable cells. Briefly, the fibroblast were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal calf serum, 10% antibiotics in a humified CO_2 incubator (95% air/5% CO_2) at 37°C. Sterilized nanofiber membrane was attached on a coverslip and put into 6-well microplate. Subsequently the cultured fibroblasts were detached by trypsin-EDTA and seeded onto fixed-scaffold at a density of 2×10^4 cells/well, and further incubated for 24h, 48h, 72h in triplicate. A similar cell suspension seeded into a well without any scaffold was considered as control. At the end of incubation period, 10µL MTT (10mg/mL in PBS solution) was added in each well and incubated for another 4h. Afterward, MTT solution

with culture medium was pipette put completely and 150 µL DMSO was added in each well to solubilize the internalized insoluble formazan that were proportional to viable cell number. After agitating the plate for 10 min, formazan turn to purple color solution which was measured at 570 nm. Cell viability was calculated by following equation:

$$Cell\ Viability\ (\%) = \left(\frac{A_{test} - A_{DMSO}}{A_{control} - A_{DMSO}}\right) \times 100 \qquad (11)$$

where A_{test}, $A_{control}$, and A_{DMSO} represent the optical density of culture medium incubated with a nanofiber, without a nanofiber, and DMSO only, respectively.

4.2.3.8. Pilot study- wound healing efficiency of PCL-CH nanofiber membrane in comparison to CIPLOX cream

Since quercetin is not available in any marketed formulation for topical application, therefore a comparative pilot study was performed to check wound healing efficiency of PCL-CH nanofiber only with respect to a ciprofloxacin marketed formulation [CIPLOX cream (0.5% w/w)- manufactured by Cipla]. All the experimental protocol was pre-approved by the Central Animal Ethical Committee (CAEC) of Banaras Hindu University, Varanasi (No. Dean/2018/CAEC/638). The healthy adult Wistar rats, both female and male, weighing 220-250 mg were anesthetized separately by an intraperitoneal injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). The dorsal surface of animals was shaved carefully and sterilized with ethanol (70% v/v in water) and skin was excised to create a full thickness circular wound with an approximate diameter of 2.5 cm. The excised animals was randomly distributed into three groups (n=6), namely gauze treated, CIPLOX treated and PCL-CH nanofibers treated. The gauze treated animals' wound was covered with gauze to reduce contamination from dirt, bedding husk etc. A weight amount of CIPLOX cream

having equivalent quantity of drug with respect to PCL-CH nanofibers was applied in 2nd group. Third group was covered with PCL-CH nanofibers membrane. On regular interval of post-surgery, the wounds were snapped and the size of the wound was outlined using a tracing paper, obtained wound boundary was traced back on a graph paper (2 mm^2) and wound area was calculated. The following equation was utilized to determine the progression of wound healing area:

$$Wound\ closure\ (\%) = \left(\frac{A_0 - A_t}{A_0}\right) \times 100 \qquad (12)$$

A_0 is wound area at day 0 and A_t is wound area on day 4, 8, 12 and 16 post surgery.

The result of comparative pilot study was applied further to explore the wound healing efficiency of quercetin and ciprofloxacin hydrochloride loaded scaffolds.

4.2.3.9. In-vivo wound healing study

Developed nanofiber was evaluated for wound healing in animal by excising circular wound. All the experimental protocol was same as described in Subsection 4.2.3.8. Here, the excised animals was randomly distributed into four groups (n=6), namely gauze treated, placebo scaffold treated, CH loaded scaffold treated, and CH and Que loaded scaffold treated. On regular interval of post-surgery, the wound area was measured as described in Subsection 4.2.3.8. Then, on days 8 and 16, granulation tissue from healed area was harvested and divided into three portions. The first portion was preserved in formalin for further evaluation of histological changes. The second portion was rinsed with ice-cold saline, homogenized in ice-cold phosphate buffer saline (pH 7.4, 50 mM). The tissue homogenates were centrifuged for 25 min at 5000 rpm at 5 °C, and supernatants were collected for endogenous antioxidant (SOD and catalase) analysis in granulation tissue [El-

Ferjani et al. 2016]. The third portion was acid hydrolyzed for hydroxyproline content analysis.

4.2.3.9.1. Histological examination

In order to evaluate the histological changes, formalin-preserved granulation tissues were fixed in paraffin, dissected into 4 µm thin slice and stained using a routine hematoxylin−eosin (H&E) procedure. Microscopic changes at histological level were visualized under the optical microscope.

4.2.3.9.2. Antioxidant enzyme assay

1. Assay of superoxide dismutase (SOD)

Superoxide free radical scavenging activity of SOD was determined in the tissue supernatant as per the method described by Beauchamp and Fridovich [Beauchamp and Fridovich 1971] and modified by Ak and Gülçin [Ak and Gülçin 2008]. Superoxide free radicals ($O_2^{-\cdot}$) were produced in riboflavin/methionine/fluorescent illuminated system and quantitatively evaluated by the reduction of nitro blue tetrazolium (NBT) to form color product (blue formazan). The total volume of the reaction mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT were 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reaction mixture was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated $O_2^{-\cdot}$ which reduced NBT to form blue formazan, which was measured spectrophotometrically at 560 nm. When SOD containing supernatant was added in the above solution, SOD started scavenging the superoxide, thereby inhibiting the reduction of NBT. The reduced absorbance of the reaction mixture indicates improved superoxide scavenging activity. A reaction mixture devoid of SOD

served as control. The superoxide radical scavenging activity was calculated by using the following equation:

$$Superoxide\ radical\ scavenging\ activity\ (\%) = \left(\frac{A_C - A_S}{A_C}\right) \times 100 \qquad (13)$$

where, A_C denotes the absorbance of the reaction mixture without supernatant, and A_S is the absorbance of the reaction mixture with supernatant.

The activity of SOD was expressed as Unit/mg protein, with the unit being the amount of SOD required to inhibit 50% NBT reduction by superoxide radicals. The possible reactions during SOD assay are shown below:

$$Riboflavin + Methioine \xrightarrow{O_2/fluorescent\ illumination} O_2^{-\cdot}$$

$$Nitro\ blue\ tetrazolium + O_2^{-\cdot} \xrightarrow{reduction} Blue\ formazan\ (abs.560nm)$$

If SOD is present in solution, then it scavenges the $O_2^{-\cdot}$, thereby inhibiting the NBT reduction and hence reduction in formazan concentration:

$$SOD + O_2^{-\cdot} \xrightarrow{reduction} H_2O_2$$

2. Assay of catalase

The Hydrogen peroxide scavenging activity of catalase in the supernatant was determined by method described by Aebi [Aebi 1984]. The assay is based on the direct measurement of decrease in absorbance of H_2O_2 (at 240 nm) due to its decomposition by catalase into water and molecular oxygen. 0.17M ethanol (0.01 mL/mL of supernatants) was added in the supernatants to prevent the formation of inactive complex-II (catalase-H_2O_2 complex-II). The reaction mixture was prepared by adding 1 mL H_2O_2 (30 mM in potassium phosphate buffer, pH 7.4) in 2 mL of above supernatants. The reaction was started immediately after addition of H_2O_2. Diminishing UV-absorbance of the sample was recorded at 240 nm for 3 min at intervals of 30 sec. The catalase activity was calculated by the following equation:

$$Catalase\ activity = \left(\frac{2.3}{\Delta t}\right)\left(\frac{a}{b}\right)\left(\log\frac{A_1}{A_2}\right) \quad (14)$$

where, A_1 and A_2 are absorbances of solution at two consecutive time, a is dilution factor, b is protein content (mg/mL), and Δt is time interval i.e. 0.5 min.

The catalase activity was expressed as Unit/mg protein, with the unit expressed as mM of H_2O_2 consumed per min.

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

4.2.3.9.3. Hydroxyproline content in granulation tissues

Collagen is the most abundant protein, accounting for 30% of the total protein in the human body. Hydroxyproline forms a major constituent of collagen (comprising 13-14% of the total amino acid content) and its distribution is highly restricted to collagen [Pang et al. 2017].Therefore, its quantitative estimation can be utilized as an indicator for collagen synthesis. For Hydroxyproline assay, the procedure was adapted from Reddy and Enwemeka, with some modification [Reddy and Enwemeka 1996]. Granulated tissues were treated thrice with acetone for 6 h to remove any possible fat. Fat-free samples were acid-hydrolyzed (6 N HCl, 50 mg sample/mL) for 6 h at 120 °C to yield individual amino acid. Hydrolyzed samples were allowed to cool and neutralized with 2 N NaOH solution (using phenolphthalein solution). 50 µL of each hydrolyzate was added to 96-well plate and mixed with chloramine-T (450 µL) and incubated for 25 min to form oxidation product, pyrrole-2-carboxylate. In each reaction mixture 500 µL of Ehrlich's solution was added and incubated for 25 min at 65 °C, which resulted in the conversion of pyrrole-2-carboxylate to a chromophore (reddish purple complex). The absorbance of chromophore was measured at 550 nm with a microplate reader. The possible reaction during hydroxyproline assay is

shown in Figure 4.5. Hydroxyproline content was determined from the standard curve of pure amino acid (10-100 µg/mL).

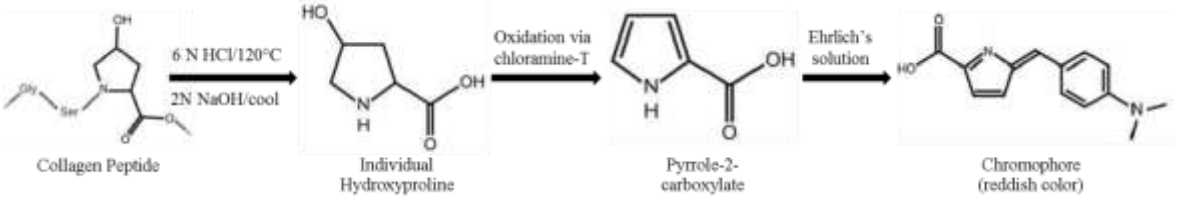

Figure 4.5: Overview of the hydroxyproline assay [Cissell et al. 2017].

Preparation of standard curve of hydroxyproline

Standard solutions of hydroxyproline were prepared in the concentration range of 10-100 µg/ml, by serial dilution from the corresponding working standard solutions (1mg/mL) in the distilled water. 450 µL of the Chloramine-T reagent was added to each standard and oxidation was allowed to proceed at room temperature. 500 µL of Ehrlich's solution was added and incubated for 25 min at 65 °C to form a reddish-purple color chromophore. The absorbance of chromophore was measured at 550 nm with a microplate reader and standard curve was plotted between concentration and absorbance and curve is shown in Figure 4.6.

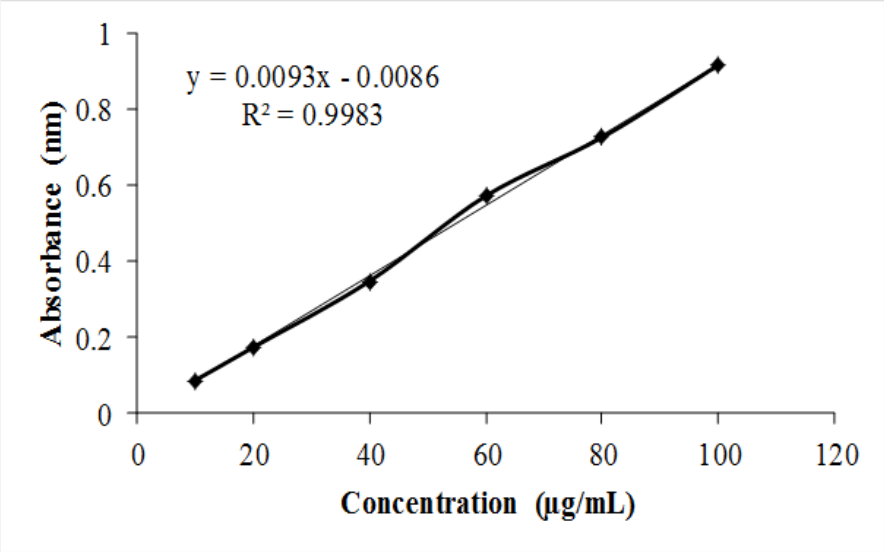

Figure 4.6: Calibration curve of hydroxyproline.

4.2.4. Statistical analysis

All the results were presented as mean±standard deviation (SD) and statistical comparisons were carried out by GraphPad Prizm software, version 5.01. The obtained data were either subjected to one-way ANOVA followed by Tukey's post-test or two-way ANOVA followed by Bonferroni post-tests. The experimental result with 95% confidence level ($p < 0.05$) was considered as statistically significant.