4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

Table 4.1 Chemicals used in the study

S. No.	Chemicals	Source
1	TNZ (TNZ)	USV Pharmaceuticals, Baddi, India.
2	Poly (ε- caprolactone) (PCL) (Mw = 80000 g/mol)	Sigma-Aldrich, India
3	GE	SD Fine-Chem Ltd., Mumbai, India
4	Chitosan (CH)	Hi Media, Mumbai, India
5	Hexafluoro-2-propanol (HFP)	Merck Ltd., Mumbai, India
6	Acetic acid	SD Fine-Chem Ltd., Mumbai, India
7	Formic acid	SD Fine-Chem Ltd., Mumbai, India
8	Methanol	Merck Ltd., Mumbai, India
9	Chloroform	Merck Ltd., Mumbai, India
10	Acetone	Merck Ltd., Mumbai, India
11	Citric acid	Merck Ltd., Mumbai, India
12	Sodium hydroxide	Qualigens Chemicals, Mumbai, India
13	Potassium dihydrogen orthophosphate	Qualigens Chemicals, Mumbai, India
14	Disodium hydrogen orthophosphate	Merck Ltd., Mumbai, India
15	Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific (USA).
16	Penicillin-streptomycin (10000 U/ml)	Thermo Fisher Scientific USA
17	Fetal bovine serum (FBS)	Thermo Fisher Scientific USA
18	Ethilon Suture	Johnson & Johnson Ltd.
19	Fluorescein diacetate dye	Thermo Fisher Scientific USA
20	Ethidium bromide	Thermo Fisher Scientific USA

4.1.2 Equipments

Table 4.2 List of major instruments used in the study

S. No.	Instruments	Source
1	High Voltage power supply	Goldstar, New Delhi, India
2	Syringe pump	AYRA N801 New era pumps, USA
3	Magnetic stirrer	Decibel Instruments, Chandigarh, India
4	Digital electronic balance	Shimadzu, Japan
5	Digital pH meter	IKON Instruments, New Delhi, India
6	Dissection box	Camlin Ltd., Mumbai, India
7	Cooling centrifuge	REMI C20, Mumbai, India
8	Aluminium foil (fresh wrap)	Hindalco Industries Ltd., Mumbai, India
9	Hot air oven	IKA, Germany
10	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
11	Sonicator (bath type)	IKA, Germany
12	Stability chamber	NSW-175, New Delhi, India
13	UV-visible spectrophotometer	Shimadzu 1800, Japan
14	Foam tape	Camlin Ltd., Mumbai, India
15	Differential scanning calorimeter	Mettler Toledo, Switzerland
16	Powder X-Ray diffractometer	Rigaku, Japan
17	FT-IR spectrophotometer	Shimadzu FT-IR-8400, Japan
18	Scanning electron microscope	ZEISS EVO 18 Research, USA
19	HR-SEM	FEI, Quanta 200F, Japan
20	Sputter coater	Q150RES QUORUM, Germany
21	Atomic force microscopy	NT-MDT, Russia
22	Camera assisted light microscope	Dewinter Optical, Inc., New Delhi, India
23	Membrane filters (0.22 μm and 0.45 μm)	Hi Media, Mumbai, India

4.1.3 Software

Table 4.3 List of software used in the study

S. No.	Name of Software	Developer
1	Microsoft Office 2010	Microsoft Corp., USA
2	Adobe Acrobat Reader 11.0	Adobe Systems Inc. USA
3	Design Expert	Version 7.0.6.1, Stat-Ease Inc., USA
4	OriginPro	Version 8.1, Origin Lab Corp., USA
5	GraphPad Prism	Version 5.03, Graph Pad Software Inc., USA
6	EndNote 6.0	EndNote, Wintertree Software Inc., USA
7	Grammarly	Grammarly Inc., San Francisco, USA

4.2 Experimental

4.2.1 Preformulation studies

Preformulation studies include

- ➢ Analytical method development by UV-visible spectroscopy

- ➢ Solubility studies

4.2.1.1 Analytical method development and validation for estimation of TNZ by UV-Visible spectroscopy

4.2.1.1.1 Standard stock solution preparation

4.2.1.1.1.1.1 Standard stock solution preparation in water

10 mg of TNZ was accurately weighed and transferred to a 100 ml of volumetric flask and then dissolved completely in 70 ml of water. The resultant solution was sonicated, and the volume was made up to the mark (100 ml) with distilled water to get resultant stock solution containing 100 μg/ml of TNZ.

4.2.1.1.1.1.2 Standard stock solution preparation in McIlvaine buffer pH 6.6

10 mg of TNZ was accurately weighed followed by transferring to a 100 ml of volumetric flask and then was dissolved completely in 70 ml of McIlvaine buffer (pH 6.6). The resultant solution was sonicated, and volume was made up to the mark (100

ml) with McIlvaine buffer (pH 6.6) to get final stock solution containing 100 μg/ml of TNZ.

4.2.1.1.1.1.3 Identification of λ_{max}

The stock solutions were then successively diluted with a respective solvent, to obtain a final concentration of 8 μg/ml. The resultant solutions were subjected to scan between 200-400 nm using a UV-Visible spectrophotometer, and wavelength maximum (λ_{max}) was determined.

4.2.1.1.1.1.4 Sample Preparation of known drug concentration

From the standard stock solution of TNZ (100 μg/ml), different volumes of aliquots were pipetted out and transferred into a series of volumetric flasks of 10 ml capacity. The volume was then adjusted up to the volumetric mark with respective solvents to obtain final drug concentrations of 4, 8, 12, 16, 20 and 24 μg/ml.

4.2.1.1.1.1.5 Calibration curve preparation

The absorbance of each of the prepared sample solution was measured at 319 nm by using UV-Visible spectrophotometer against respective solvent as a blank. The calibration curve of absorbance vs. concentration (μg/ml) was plotted, and regression analysis was performed to get the straight-line equation. The calibration curves were generated on three consecutive days using freshly prepared samples each time.

4.2.1.1.1.1.6 Method Validation

The suitability of the developed method for quantitative estimation of TNZ was determined by evaluating the different validation parameters as per the guidelines of ICH Q2B. The developed method was validated for the typical validation characteristics, i.e., accuracy, precision, linearity range, specificity, selectivity, limit of detection (LOD) and limit of quantification (LOQ) [Albalasmeh et al. 2013].

4.2.1.1.1.1.7 Accuracy

The accuracy of the analytical method can be defined as the closeness of test results obtained by that method to the true value. The accuracy of the analytical method was determined by performing recovery study using standard addition method. Recovery is determined by taking the ratio of the observed result to that of the predicted result and usually expressed as a percentage. Three different concentrations designated as low (4 μg/ml), medium (8 μg/ml) and high (12 μg/ml) of TNZ were selected. Each of the samples was scanned in UV-Visible spectrophotometer on the same day (n = 3) and over three consecutive days (n = 3) to assess intra-day and inter-day variation, respectively. The accuracy was expressed in the form of % bias, and the acceptable values as per the requirements should be within±15% at all concentrations levels [Bressolle et al. 1996]. The % bias was calculated by the following equation:

$$\% \, \text{Bias} = \frac{Observed \; concentration - Predicted \; concentration}{Predicted \; concentration} \times 100 \qquad \text{Eqn. (4.1)}$$

4.2.1.1.1.1.8 Precision

Precision is known as the degree of agreement amongst individual test results, obtained by the series of measurements performed from multiple sampling of the same homogeneous sample using the developed analytical method. The precision of the analytical method was determined by comparing the relative standard deviations (RSD) of a series of measurement. Three different drug concentrations (4, 8 and 12 μg/ml) of TNZ were scanned in UV-Visible spectrophotometer on the same day (n = 3) as well as three consecutive days (n = 3), to measure intra-day and inter-day precision, respectively. The acceptable values for % RSD should be within 15% at all concentrations levels [Shah et al. 1992]. The % RSD was calculated by the following equation:

$$\% \, RSD \; = \; \frac{Standard \; deviation}{Mean \; observed \; concentration} \times 100 \qquad \qquad \text{Eqn. (4.2)}$$

4.2.1.1.1.1.9 Linearity and Range

The linearity of an analytical method is the ability of the method to produce test results which are directly proportional to the concentration of an analyte in samples. The range is an expression of the lowest and highest levels of analyte concentration and can be easily determined, either directly or indirectly with acceptable precision, accuracy and linearity. Linearity and range was determined by measuring in triplicate the absorbance of a series of samples over the concentration range of 4-20µg/ml over three consecutive days.

4.2.1.1.1.1.10 Specificity and Selectivity

Specificity is a procedure used to detect the analyte quantitatively in the presence of other components, which may be expected to be present in the solvent media. Selectivity is the procedure used for qualitative analysis of the analyte, in the presence of other components in the solvent media. Specificity and selectivity of the analytical method towards TNZ was determined by the scanning of known concentration (8 µg/ml) of TNZ at 319 nm in UV-Visible spectrophotometer.

4.2.1.1.1.1.11 Limits of detection (LOD)

The limit of detection (LOD) can be defined as the lowest concentration of an analyte in a sample, which can be detected, but not necessarily quantifiable as exact value, under the stated experimental conditions. The LOD of TNZ in the different medium was calculated by using the following equation:

$$LOD \; = \; \frac{3.3 \times \sigma}{S} \qquad \qquad \text{Eqn. (4.3)}$$

Where σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

4.2.1.1.1.1.12 Limits of quantification (LOQ)

The limit of quantitation (LOQ) is the lowest concentration of analyte which can be determined with acceptable precision and accuracy under the stated experimental conditions. The LOQ of TNZ in the different medium was calculated by using following equation:

$$\text{LOQ} = \frac{10 \times \sigma}{S} \qquad \text{Eqn. (4.4)}$$

Where σ is the standard deviation of y-intercept of the regression equation, and S is the slope of the calibration curve.

4.2.1.2 Solubility of TNZ in distilled water and McIlvaine buffer pH 6.6

Saturation solubility of TNZ in distilled water and McIlvaine buffer pH 6.6 was determined by adding an excess amount of TNZ into 10 ml of each of the medium separately. The solution was agitated at 25 °C for 72 hrs on a magnetic stirrer. The clear solution was then obtained through 0.45-micron filter which was diluted to 250 ml. After that, drug content of samples was measured spectrophotometrically at λ_{max} 319 nm. This study was conducted in triplicate, and the average value of the three determinations was taken as the final result.

4.2.2 Formulation development

4.2.2.1 Preparation of TNZ-PCLNF membrane

Electrospinning process was carried out using 8-12% w/v of the polymer solution. A homogeneous polymeric solution of PCL was obtained by dissolving it in the mixed solvent system of formic acid and acetic acid under magnetic stirring for 3 hr at room

temperature. TNZ in the range of 10-30% (w/w) of the polymer was then added to the polymeric solution of PCL and stirred for 2 hr followed by bath sonication (WUC-1.8L, Fisher Scientific, India) for 15 min to remove air bubbles from the solution. The resulting clear solution was then transferred to 10 ml syringe, fitted with a 23 Gauge needle. The solution was pumped at a flow rate of 0.5-1.5 ml/hr while maintaining the applied positive voltage in the range of range of 20-24 kV. Electrospinning was carried out at room temperature (22±2 ℃) and a relative humidity of 65±5%. The resulting fibers were collected on a grounded aluminum foil covered screen. The distance between the tip and the collector was fixed at 11.5 cm [Khan et al. 2016a]. The steps involved in the fabrication of nanofiber membrane are represented in Figure 4.1
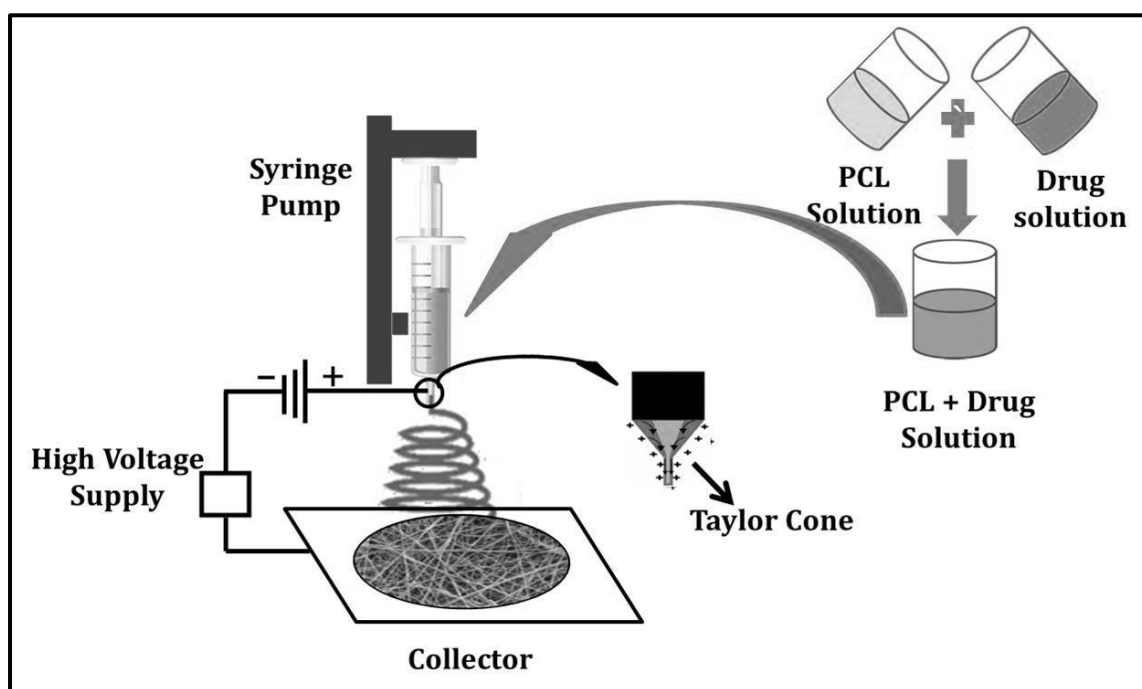

Figure 4.1 Schematic presentation of steps involved in the fabrication of electrospun nanofiber membrane (TNZ-PCLNF).

4.2.2.2 Preparation of TNZ-PGHNF membrane

TNZ-PGHNF membrane was fabricated by electrospinning technique. A solution strategy was adopted in GE/PCL blend. PCL (6% w/v) and GE (6% w/v) were dissolved separately in hexafluoro-2-propanol (HFP). GE/PCL blend was prepared by mixing these

solutions in an appropriate ratio where the GE:PCL weight ratio was varied between10:90, 30:70 and 50:50. After that TNZ in the range of 10-30% (w/w) was added to the above prepared polymeric blend and stirred for 24 hr followed by bath sonication. The final solution was then transferred to a 10 ml syringe, and subjected to electrospinning (within a voltage range of 10-14 kV) using grounded aluminium foil covered screen as a collector which was placed at an optimum distance from the needle. The solution was fed at 0.8 - 1.0 ml/hr by a syringe pump, and electrospinning was carried out at room temperature (22±2 ℃) and a relative humidity of 65±5%. The steps involved in the fabrication of nanofiber membrane are represented in Figure 4.2.
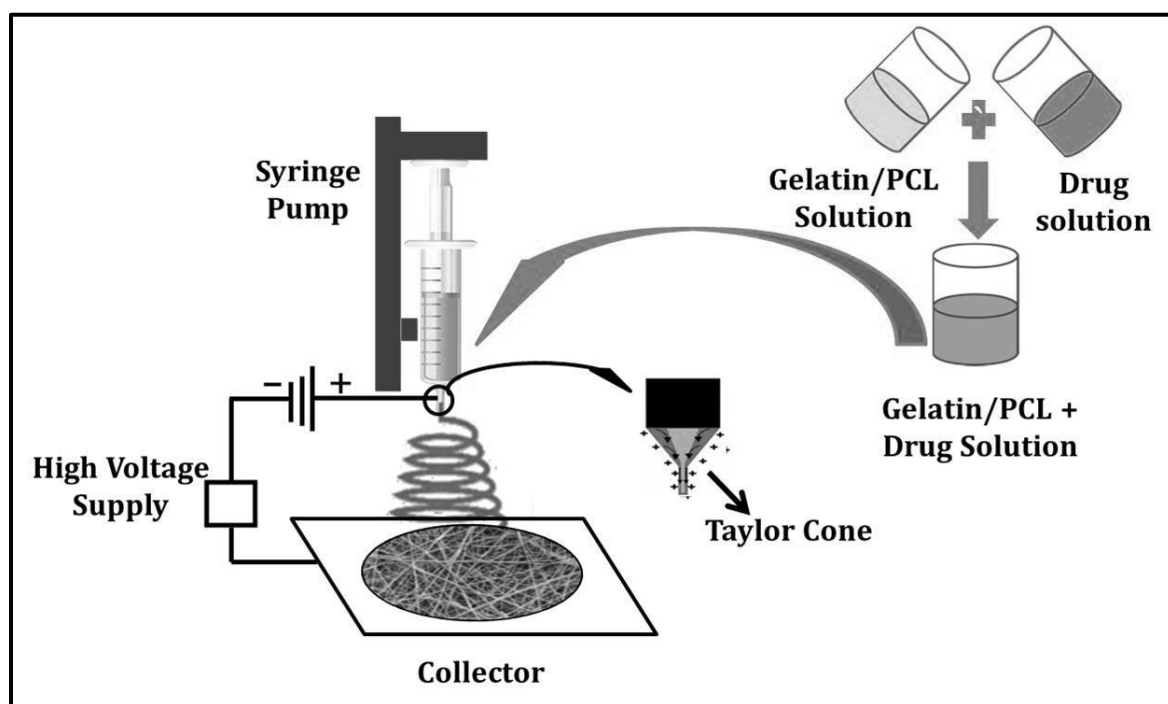

Figure 4.2 Schematic presentation of steps involved in the fabrication of electrospun nanofiber membrane (TNZ-PGHNF).

4.2.2.3 Preparation of TNZ-PCHNF membrane

TNZ-PCHNF membrane was fabricated by using the technique of electrospinning. The solution strategy was adopted in CH/PCL blending. PCL (8% w/v) and CH (2% w/v) were initially dissolved separately in a mixed solvent system consisting of formic acid and acetic acid. CH/PCL blends were prepared by mixing these solutions in appropriate

ratio wherein the weight ratio of CH:PCL was varied between10:90, 20:80 and 30:70. After that TNZ 10-30% (w/w) was added to the above prescribed polymeric blend and stirred for 2 hr followed by bath sonication. After that, the final solution was transferred to a 10 ml syringe and subjected to electrospinning (within a voltage range of 18-22 kV) using grounded aluminium foil covered screen as a collector placed at an optimum distance from the needle. The solution was fed at the rate of 0.8 ml/hr by a syringe pump, and electrospinning was carried out at room temperature (22 ± 2 ℃) and a relative humidity of $65\pm5\%$ [Khan et al. 2017]. The steps involved in the fabrication of nanofiber membrane are represented in Figure 4.3.
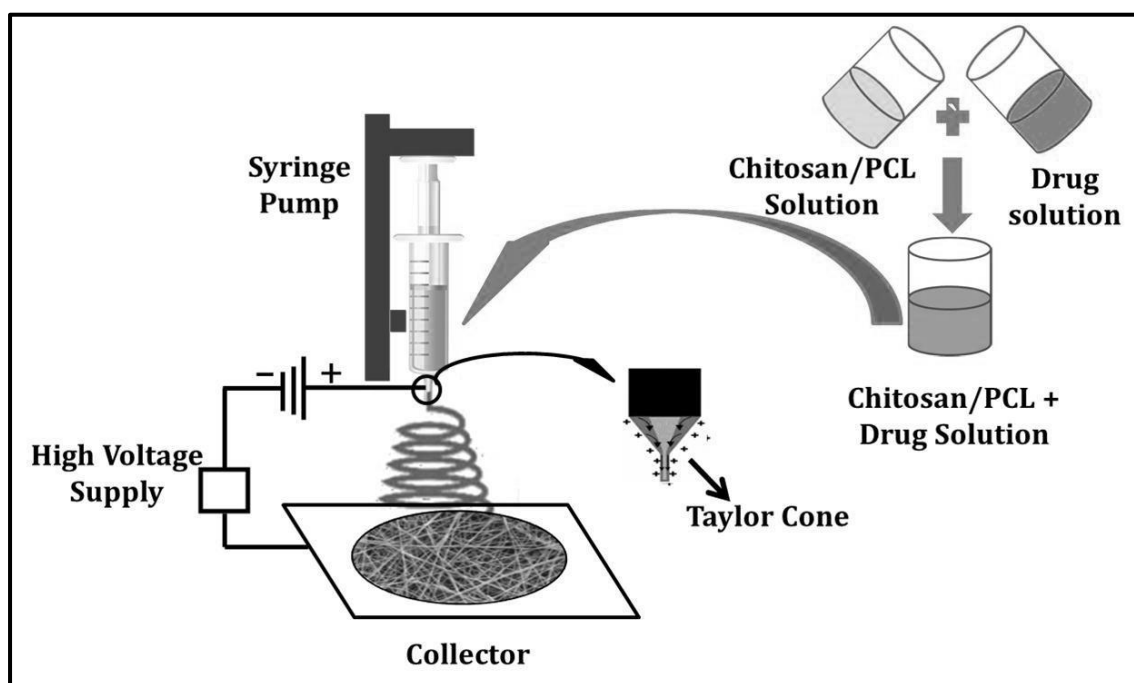

Figure 4.3 Schematic presentation of steps involved in the fabrication of electrospun nanofiber membrane (TNZ-PCHNF).

4.2.3 Experimental design

The "Quality by Design" approach was used for statistical optimization of formulation variables precisely, using response surface methodology (RSM). On the basis of extensive literature survey and preliminary formulation study, four factors (X_1) polymer content, (X_2) drug concentration, (X_3) volume of Formic acid/Acetic acid and (X_4)

applied voltage was selected as formulation/process variables. In this study, a 3-factor, 3-level BBD experimental design was used to examine the quadratic response surfaces by evaluating the influence of independent variables on the dependent response variables, i.e., diameter (Y_1) and encapsulation efficiency (EE) (Y_2). All the independent variables with their actual and coded values are shown in Table 4.4, Table 4.5, and Table 4.6. All the experiments were performed in a randomized order to circumvent any probable source of experimental bias and to increase the predictability of the model [Patel et al. 2014b, Solanki et al. 2007]. The non-linear polynomial equation generated by the BBD is given as

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_2X_3 + A_6X_1X_3 + A_7X_{21} + A_8X_{22} + A_9X_{23}$$
$$+ E \hspace{5cm} \text{Eqn. (4.5)}$$

where, Y is the measured response of the dependent variable associated with each factor level combination; A_0 is an intercept; A_1−A_9 are the coefficients of X_1, X_2 and X_3; A_1 − A_3 are the main outcome of X_1−X_3; A_4−A_6 are the interaction of the main factors; A_7−A_9 are the quadratic effects of the independent variables, and E is the random error [Gannu et al. 2009].

The fitness of the model was evaluated by analyzing the significance of independent formulation variables on the basis of quality indicator correlation coefficient R^2 (maximum) and analysis of variance ($p < 0.05$).

Table 4.4 Independent formulation variables with their coded levels and dependent variables with their constraints in Box–Behnken experimental design for TNZ-PCLNF membrane.

	Coded levels of variables		
	Low	**Medium**	**High**
	-1	**0**	**+1**
Independent variables			
X_1 = Polymer concentration (w/v)	8	10	12
X_2 = Drug concentration (w/w)	10	20	30
X_3 = FA/AA ratio (v/v)	0.1	0.25	0.4
Dependent variables		**Constraints**	
Y_1 = Diameter		Minimize	
Y_2 = Entrapment Efficiency		Maximize	

Table 4.5 Independent formulation variables with their coded levels and dependent response variables with their constraints in Box–Behnken experimental design for TNZ-PGHNF.

	Coded levels of variables		
	Low	**Medium**	**High**
	-1	**0**	**+1**
Independent variables			
X_1 = GE/PCL ratio (v/v)	0.11	0.42	1.0
X_2 = Drug concentration (w/w)	10	20	30
X_3 = Applied voltage (KV)	8	10	12
Dependent variables		**Constraints**	
Y_1 = Diameter		Minimize	
Y_2 = Entrapment Efficiency		Maximize	

Table 4.6 Independent formulation variables with their coded levels and dependent response variables with their constraints in Box–Behnken experimental design for TNZ-PCHNF

	Coded levels of variables		
	Low	**Medium**	**High**
	-1	**0**	**+1**
Independent variables			
X_1 = CH/PCL ratio (v/v)	0.1	0.25	0.4
X_2 = Drug concentration (w/w)	10	20	30
X_3 = FA/AA ratio (v/v)	2.33	5.66	9
Dependent variables		**Constraints**	
Y_1 = Diameter		Minimize	
Y_2 = Entrapment Efficiency		Maximize	

4.2.4 Characterization

4.2.4.1 Solid-state characterizations by FTIR, DSC and PXRD studies

Fourier Transform Infrared (FTIR) spectra of the prepared samples were recorded using FTIR spectrophotometer (SHIMADZU 8400S, Tokyo, Japan) with an objective to investigate the possible interaction between the drug and the excipients. Briefly, the solid samples were crushed and blended with potassium bromide (IR grade), finally compressed using pressed pallet technique and, then mounted in FTIR in transmittance mode. Samples were scanned in the region of 4,000–400 cm^{-1} while keeping the number of reference scan 20 [Patel et al. 2016]. All the optimized formulation samples, their components as well as the physical mixture (PM) were analyzed by FTIR, and the spectra was compared to study any interaction occurred between drug and any components of the formulation.

The nature of distribution and thermal stability of the drug in the nanofiber membrane was determined by performing differential scanning calorimetric (DSC) for all the optimized formulation samples and their components as well as PM. Thermograms were measured with the differential scanning calorimeter (Mettler, Toledo, 822). Before

performing the experiment, the DSC instrument was calibrated by using 5 mg of indium by maintaining the heating rate of 10 °C/min. The thermal behavior of the compound was analyzed by heating the weighed amount of the samples at a constant rate of 10 ºC/min from 25 ºC to 350 ºC in a pan which was hermetically sealed, and containing a pinhole in the lid under a nitrogen purge of 20 ml/min.

All the optimized formulation samples, their components, and PM were characterized for Powder X-ray diffraction (PXRD) conducted on a Rigaku Mini Flex 600 X-ray diffractometer equipped with the D-Tex Ultra detector for analyzing their crystallographic structure and the change in the physical state of the drug TNZ during the electrospinning. The sample was packed into the sample holder, and the respective diffraction patterns were recorded over 2θ scanning range from 5º to 50º at a scan rate of 2º/min.

4.2.4.2 Morphological study using SEM and AFM

The atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to characterize the surface morphology of the prepared electrospun nanofiber membranes. The samples to be observed were coated with gold, before analysis under the scanning electron microscope (ZEISS EVO 18 Research, USA) using a sputter coater (Q150RES QUORUM, Germany). The diameter of the nanofiber was measured by using Image J software at different 10 random locations of obtained SEM micrograph. The thickness of the nanofiber membrane was measured with the help of a micrometer. The apparent density and porosity of the nanofiber membrane were calculated using the following equations [Xue et al. 2014c]:

$$Porosity\ (\%) \ = \ \left(1 - \frac{Apparent\ density\ (g/cm^3)}{Bulk\ density\ (g/cm^3)}\right) \times 100 \qquad \text{Eqn. (4.6)}$$

$$Apparent\ density\ (g/cm^3) \ = \ \frac{Mass\ of\ membrane\ (g)}{Membrane\ thickness\ (cm) \times Membrane\ area\ (cm^2)} \text{Eqn. (4.7)}$$

To perform AFM study, the electrospun nanofibers were initially collected on mica substrates (freshly cleaved) for about 90 seconds and then allowed to air dry so that it forms a thin film. After that, the surface morphology of the nanofiber membrane was picturized by mounting the thin film on the AFM scanner (NT-MDT, Moscow, Russia) and by imaging in semi-contact mode at the spring constant of 1–5 N/m and 152 kHz resonance frequency. The diameters of the nanofiber were determined from the respective height of the fibers.

4.2.4.3 Surface pH and Uniformity of Drug Content

Surface pH was determined to evaluate any possible damage which may be caused to mucosa by the nanofiber membrane. Samples were immersed in GCF simulated McIlvaine buffer (pH 6.6) for 2 hr. Samples were taken out, and the pH of each nanofiber membrane was measured by placing the probe of pH meter in contact with the wet sample [Avachat et al. 2013]. A mean of the three readings was recorded. After that, the total drug content, between batches and within batches, was determined by dissolving individually weighed nanofiber membrane (n = 3) in a volumetric flask containing 5 ml solvent mixture of FA/AA in case of PCL and CH/PCL nanofiber whereas HFP solvent was used for GE/PCL nanofiber membrane. After gentle stirring for an hour, the volume was made up to 100 ml with McIlvaine buffer pH 6.6. The resultant solution was then filtered through a G-2 glass filter (40 μm). Following adequate dilutions, the filtrate was analyzed by UV-spectrophotometer at λ_{max} of 319 nm, and amount of TNZ was obtained from the standard calibration curve of TNZ.

4.2.4.4 Contact angle of nanofiber membrane

Measurement of contact angle was used to determine the surface wettability of the prepared nanofiber membranes. A video contact angle instrument (KRUSS GmbH

Germany, DSA 10) was used to measure the contact angle of nanofiber membrane by using sessile drop method. In the method mentioned above, a small drop of water (4 µl) was placed on the nanofiber membrane surface. The changes occurring in the shape of the placed water bead was recorded, and the measurement of surface contact angles was performed. The water contact angle of each of the membrane was taken to be the average value obtained from three different locations [Jing et al. 2015].

4.2.4.5 *In vitro* Mucoadhesion studies

The mucoadhesive strength of the electrospun nanofiber membrane, as well as the placebo nanofiber membrane, was determined by using texture analyzer (TA.XT Plus, Stable Micro System, UK). In this study, Nanofiber sample (1×1 cm^2) was attached to the mobile upper arm of the machine and excised buccal mucosal membrane of the goat was (obtained from local slaughterhouse) mounted securely on the lower platform with cyanoacrylate adhesive. Both of these arms were kept in close contact by applying a weight of 1000 gm for 15 s. After that, the force needed for the fibers to detach from the mucosal membrane was calculated [Sharma et al. 2016].

4.2.4.6 Entrapment efficiency

The drug entrapment efficiency of prepared nanofiber membrane was assessed by employing the direct method. A known weight of a previously rinsed sample of the nanofiber membrane was dissolved in 2 ml solution of FA: AA (in the ratio of 80:20 v/v). The solution was then added dropwise to 20 ml methanol, following which the drug (TNZ) was found to be solubilized in methanol, and the polymer was found to be precipitated. After centrifuging the obtained methanol solution, the liquid supernatant was decanted, and analysis using UV (SHIMADZU UV-1800, Tokyo, Japan) was performed at λ_{max} of 319 nm [Xue et al. 2014c]. The amount of TNZ was obtained from

the standard calibration curve, and the percentage entrapment efficiency was calculated using following equation:

$$\% \, EE \; = \; \frac{weight \; of \; drug \; in \; the \; nanofiber \; membrane \times 100}{theoritical \; weight \; of \; drug \; loading \; in \; the \; nanofiber \; membrane} \qquad \text{Eqn. (4.8)}$$

4.2.4.7 *In vitro* drug release studies

The nanofiber membrane was cut into $1 \times 1 \; cm^2$ pieces, weighed accurately, and then placed in a vial containing 10 ml McIlvaine buffer (pH 6.6), previously warmed at 37 ºC, without shaking to simulate the stagnant intrapocket condition [Sridevi et al. 1995]. 1 ml of the buffer solution was removed at predefined time intervals, followed by spectrophotometric analysis at λ_{max} of 319 nm to measure the amount of the drug released by the calibration curve of TNZ in the McIlvaine buffer (pH 6.6). In order to sustain the sink conditions, the leftover media was removed while replacing it with 10 ml of fresh buffer. The results were recorded as an average of the three distinct determinations. Similarly, the release study was also carried out for the drug (TNZ) suspension by using dialysis bag in McIlvaine buffer (pH 6.6). To comprehend the kinetics and the mechanism of the drug release, correlation coefficients (R^2), as well as the release rate constants (K) for different release kinetic models, i.e., zero order, Higuchi, first-order, and Korsmeyer–Peppas models, were calculated for all the batches by using the Microsoft Excel 2010. The release model which presented the R^2 value close to 1 was concluded to be the best fit model [Costa and Lobo 2001].

4.2.4.8 *In vitro* antibacterial activity

The fabricated nanofiber membrane (at different TNZ concentration 10%, 20% and 30%) and placebo were tested for their antibacterial activity against *S. aureus* (MTCC1303) using well diffusion method [Khan et al. 2016b, Sharma et al. 2012]. Muller Hinton agar (MHA) plates were developed by pouring 15 ml of the molten media into previously

sterilized Petri plates. These plates were then used for the antibacterial screening. The 18 hr cultured bacteria were adjusted to 0.5 McFarland standards in sterilized saline to obtain the desired concentration of 107 CFU/ml. After that 1.0 ml of the above solution was spread on the surface of prepared agar plates with the aid of sterile swab sticks. Then, 5×4 mm^2 nanofiber membranes were placed in the well of agar medium followed by incubation at the temperature of 37 °C for a period of 24 hr. At the end of the incubation period, the inhibition zones developed were examined around the membrane and measured with the help of a transparent ruler. This nanofiber membrane was further transferred into another swabbed sterile MHA plate, and the respective zone of inhibition was calculated on different days 1, 3, 7, 14 and 21.

4.2.4.9 Cytocompatibility

1. Cell cultures

Mouse fibroblasts (L-929 cell lines) were used for cytocompatibility study. L-929 cell lines have been proven to exhibit the same order of toxicity as exhibited by human gingival fibroblasts (HGF). Furthermore, L-929 cell lines are found to be more sensitive than HGF [Schweikl and Schmalz 1996, Thonemann et al. 2002]. Mouse fibroblasts cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM,) with 10% fetal calf serum, penicillin-streptomycin (10000 U/ml) at 37 °C in an air atmosphere with 5% CO_2.

2. Generation of DMEM aliquots

To perform viability studies of the nanofiber membranes, aliquots from nanofiber membranes consisting of distinct TNZ concentrations (10–30.0%, w/w) were developed. Briefly, the nanofiber membranes were accurately weighed to get equivalent 2 mg of TNZ. The membranes were then placed into 24- well plates followed by exposing them to 2 ml of DMEM. The aliquots required for the study were collected on successive days

(1–7), followed by weeks (day 14 and 21). After the removal of each of the aliquot, the nanofiber membranes were rinsed with 1 ml of DMEM, followed by wetting with the fresh liquid medium. DMEM containing 10% of fetal calf serum has been used as the new aliquot medium. The plates were then stored at 37 ºC with 5% CO_2. All the aliquots were kept in Eppendorf tubes at the temperature of -20 ºC until they had to be used for further tests.

3. MTT-Assay

To evaluate the effect of test aliquots on the viability of L-929 cell lines, cells were treated with water-soluble dye 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT). The viability of cells can be analyzed by measuring the optical density obtained due to glycolysis equivalent transformation of the colourant MTT. Briefly, the cells were added at a density of 1×10^5 cells/ml to each well of tissue culture plate (96-well) and incubated for 24 hr. Post incubation, the medium was changed with 100 µl of negative control (DMEM), positive control (10% dimethyl sulfoxide with DMEM) and test aliquots, respectively and again incubated in humidified surroundings with 5% CO2 at 37 °C. At chosen time points 10µl MTT (10 mg/ml) was added and incubated for 4 hr. Afterwards, 100 µl SDS (10% in 0.01M HCl) was added to dissolve purple formazan crystals then measured absorbance at 570 nm with a reference wavelength of 630 nm.

4. Direct exposure of cells to TNZ-PCLNF membrane

Nanofiber membranes containing different proportions of TNZ (0%, 10.0%, 20.0% and 30.0%, w/w) were sterilized by exposing to UV-light for 30 min on each side and clamped into cell-crown (Scaffdex Oy, Finland) cell culture inserts. The nanofiber membranes were rinsed with 2 ml PBS, and then cell suspensions (10000 cells/cm^2) was applied to each of the nanofiber membranes after that covered with 2 ml of DMEM

medium and incubated for 48 hr. Post incubation, membranes were removed from cell culture inserts, rinsed with PBS and stained with live/dead dye combination, fluorescein diacetate (FDA) and ethidium bromide (EtBr) and then evaluated microscopically by using a confocal microscope LSM 510 META (Carl Zeiss Inc., USA) at different magnifications with excitation wavelength of 540 nm [Sigusch et al. 2009].

4.2.4.10 *In vivo* study

A ligature-induced rat model for periodontitis was used to evaluate the *in vivo* performance of the developed nanofiber membranes. Earlier ligature-induced periodontitis in rats has been reported for the evaluation of some antimicrobial agents against periodontitis [Muià et al. 2006, Napimoga et al. 2009, Oz and Puleo 2011, Xu and Wei 2006]. The animal study protocol was approved by the Central Animal Ethical Committee (CAEC) of Banaras Hindu University, Varanasi (no. Dean/2015/CAEC/984). All the animal studies were carried out in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal), Ministry of Social Justice and Empowerment, Government of India, New Delhi. The animals were treated as per the plan is given in Table 4.7. Each nanofiber membrane ($6\,mm^2$) applied to group C had sufficient amount of drug (~300 µg) to maintain the drug concentration locally in the periodontal pocket above MIC during the treatment period (14 days). Two parameters viz. cementoenamel junction (CEJ)-bone distance and septal bone resorption were examined for the study of bone loss. Further continuity of the epithelium and the transseptal fibers of the interdental papilla were evaluated separately using a semi-quantitative grading given in Table 4.8 (Xie et al., 2011)

Table 4.7 Treatment plan for the ligature-induced periodontitis study in rats

Group code	No. of rats	Treatment
A	6	No treatment (control)
B	6	TNZ gel
C	6	Drug loaded nanofiber

Table 4.8 Grading scale for semi-quantitative examination of the Interdental papilla

Observation	Grade
Continuity of epithelium	
Intact (continuity of the epithelium along the whole interdental papilla)	0
Partly absent (small or large disruptions of the epithelium)	1
Absent (complete absence of epithelium on the interdental papilla, but no exudates of inflammatory cells)	2
Absent + exudate (same as grade 2, but presence of an exudate)	3
Continuity of transseptal fibers	
Intact (no disruption of transseptal fibers)	0
Partly absent (some transseptal fibers still present)	1
Absent (complete disruption of transseptal fiber system)	2

Procedure of the study

The animals (Adult Charles Foster albino rats of either sex with weight ranging between 150-200 g) were kept under standard laboratory conditions, wherein the temperature of 25±2 ℃ and the relative humidity of 55±5% was maintained. The animals were housed in cages made up of polypropylene, wherein six animals were kept in each cage. The animals had free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal gm^{-1} energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) with water ad libitum. The animals were anesthetized, after which a non-absorbable suture (ETHILON, Johnson & Johnson Ltd.) was applied on the upper incisor teeth to allow accumulation of plaque aimed at inducing several changes in the animal periodontal tissues which would be similar to that present in human periodontitis. The

administration of each of the treatment was started on the second day after placing the ligature. The rats were sacrificed at the end of 2 weeks, which was the end of the experimental period. 10% formalin was prepared freshly in phosphate buffered saline (PBS). The teeth of the animals were dissected and fixed in above-prepared formalin for 24 hr, followed by decalcification with 10% EDTA and embedding in paraffin. Serial parasagittal sections of 7 μm thicknesses were cut from paraffin-embedded tissue blocks. These sections were then mounted on the microscope slides followed by staining with haematoxylin and eosin for general tissue survey by using Dewinter classic microscope (Dewinter Technology, Italy) [Botelho et al. 2010, Xie et al. 2011, Xu and Wei 2006].

Preparation of TNZ Mucoadhesive Gel

Carbopol 934P was dissolved in 50 ml McIlvaine buffer pH6.6 and TNZ (0.5% w/v) in about 25 ml of the same solvent separately. The solution of TNZ was added to the solution of Carbopol 934P slowly with continuous stirring at 100 rpm. It was then cooled by placing it in an ice bath. A weighed amount of Poloxamer 407 was then added slowly with continuous stirring (100 rpm) at 5 °C temperature. The volume was made up to 100 ml with McIlvaine buffer pH 6.6. The prepared gel was kept for 24 hr at room temperature for complete polymer dissolution [Bansal et al. 2009].

4.2.4.11 Clinical study

Clinical performance of best-developed formulation, i.e., TNZ-PCHNF membrane was carried out by using single-blind, split-mouth study design for periodontitis. This model was earlier reported for the evaluation of antimicrobial agents against periodontitis [Bansal et al. 2016, Khan et al. 2016b]. In the present study, total 10 patients of either gender (20-50 years) suffering from chronic periodontitis having pocket depth ≥ 5 mm

in at least three permanent teeth were selected for clinical evaluation. The study was conducted at the Faculty of Dental Sciences, Institute of Medical Sciences, Banaras Hindu University, India, in compliance with the relevant laws and institutional guidelines after obtaining approval from the ethical committee of the Institute [Dean/2015-16/EC/1569]. Further, informed consent was also obtained from human subjects for the application of developed nanofiber membrane for local treatment of periodontitis. Patients were initially examined for a sign of chronic periodontitis and screened by following criteria. The inclusion criteria were as follows: (1) patients with good general health; (2) demonstrate bleeding on probing from the base of pocket and having pocket depth more or equal to 5 mm; (3) no history of periodontal therapy in the last 6 months; (4) no history of usage of antibiotics for 1 month prior to the study; (5) no pregnant or lactating women. The exclusion criteria were as follows: (1) poor oral hygiene maintenance during the treatment; (2) acute oral disease (3) allergic history to imidazole group of drugs (4) habits like smoking and tobacco chewing etc.

Treatment procedure

In the present study, three periodontal sites in every patient were taken in the study so as a sum of 30 periodontal sites from 10 patients were incorporated for evaluation. These three periodontal sites of every patient were randomly and blindly divided into the following three groups: i) Group 1-SRP; ii) Group 2-SRP + placebo nanofiber; iii) Group 3 - SRP with medicated nanofiber. Before insertion of the nanofiber membrane into the periodontal pocket, it was sterilized, and periodontal dressing (Coe-pack) was used to secure the nanofiber membrane at the periodontal pockets. After one week, the periodontal dressing was removed. All the recruited patients were recalled at scheduled follow up i.e. 1st week, 2nd week, 4th week, and 8th week for examination and recording

of clinical parameters such as probing pocket depth (PPD), gingival index (GI), clinical attachment level (CAL) and bleeding on probing (BOP) by using a semiquantitative grading given in Table 4.9. The patients were instructed to avoid the use of brush and to keep hard or sticky food away from the injury to the gingival tissue, and supragingival scaling was also provided if needed at the follow-up visits.

Table 4.9 Grading scale for gingival index and bleeding on probing

Observation	Score
Gingival index [Löe and Silness 1963]	
A periodontal probe is drawn horizontally along the soft tissue wall of the entrance to the gingival sulcus.	
Normal gingiva	0
Mild inflammation, slight change in colour, slight edema, and no bleeding on probing	1
Moderate inflammation, redness, edema and glazing, and bleeding on probing	2
Severe inflammation, marked redness and edema, ulcerations, and tendency for spontaneous bleeding	3
Bleeding on Probing	
The periodontal probe was carefully introduced parallel to the longitudinal axis of the tooth to the bottom of the pocket and gently moved along the pocket wall. BoP was recorded immediately or after 30 to 60 sec of insertion of the probe.	
Absence of bleeding	0
Presence of bleeding	1

4.2.4.12 Statistical Analysis

All the statistical comparison among experimental groups from the clinical study was performed by two-way analysis of variance (ANOVA) followed by Bonferroni post-tests whereas one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was used for *in vivo* study. All the results were presented as mean ± standard deviation (SD), except in clinical results which were expressed as mean ± standard error of mean (SEM). The significance level was set at $p < 0.05$ (95% confidence interval). Graph Pad Prism 5.03 (Graph Pad Software Inc., USA) software was used for statistical analysis and graph preparation.