

Supporting information

**Differentially cross-linkable core-shell nanofibers for tunable
delivery of anticancer drugs: Synthesis, characterization and its
anticancer efficacy**

S. Uday Kumar^a, I. Matai^a, P. Dubey^a, B. Bhushan^a, A. Sachdev^a and P.Gopinath^{*a,b}

^aNanobiotechnology Laboratory, Centre for Nanotechnology,

^bDepartment of Biotechnology,

Indian Institute of Technology Roorkee, Roorkee, Uttarakhand-247667, India.

Fax: +91-1332-273560;

Tel: 91-1332-285650;

**E-mail: pgopifnt@iitr.ernet.in, genegopi@gmail.com*

Experimental Section

1.1 Materials

Poly(ethyleneoxide) (PEO) (M_v -900,000) and branched poly(ethyleneimine) (bPEI) (M_w -25 kDa) was purchased from Sigma-Aldrich and were used without any modification. Anticancer drugs, 5-FU and curcumin (purity > 99%, from *Curcuma longa*) were procured from Sigma-Aldrich and stored at appropriate storage conditions until used. All polymeric blend was prepared in ultrapure water at $\sim 37^\circ\text{C}$. Gluteraldehyde (25 vol %) was obtained from SRL Pvt. Ltd., India. The cell staining dyes Hoechst 33342 and Rhodamine B were purchased from life technologies and diluted to working concentrations. A549 cells (Non-small lung cancer cells) were received from National Centre for Cell Science, Pune, India. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and 1% Penicillin-streptomycin in the 37°C incubator with 5% CO_2 and 95% air.

1.2 Co-axial electrospinning

Two variations of PEO-PEI core-shell nanofibers were fabricated with different core composition. In type I core-shell nanofibers, the core solution is blend of PEO and PEI (3.5 wt% PEO and 1.16 wt% bPEI) whereas in type II core-shell nanofiber, the core solution comprised of PEO (3.5 wt %) alone. The shell solution in both the cases was PEO-PEI blend of higher weight percentage (4.5 wt% PEO and 1.5 wt % bPEI). Apart from the polymer, core and shell polymer solutions were supplemented with anticancer drugs 5-FU (2.5wt% of PEO) and curcumin (3wt% of PEO), respectively. All polymer-drug blends were prepared at room temperature and homogenized by magnetically stirring for overnight before being used for electrospinning. The co-axial spinneret of electrospinning unit comprised of a 10-gauge inner needle concentrically positioned within an outer needle of 20-gauge diameter. The solutions were fed into vertical co-axial electrospinning apparatus with two independent programmable peristaltic microsyringe pump. The spinneret tip was connected to a variable high voltage (DC) power supply to dissipate charge into the polymer solution. The instrument was operated at 11kV for type I nanofiber synthesis and 8kV for type II nanofiber synthesis. The flow rate for core and shell drug-polymer blend was maintained at 0.27mL/h and 0.3mL/h, respectively. The nanofibers produced in the process were collected over a grounded stationary metal collector positioned at a distance of 25 cm from the spinneret. The nanofibers deposition was carried out for stipulated time under a controlled cabinet temperature of 32°C and constant humidity. After deposition the fibers were removed from metallic collector and kept in a desiccator for overnight in order to remove the remnant solvent.

1.3 Crosslinking nanofibers

The presence of bPEI moieties in as-synthesized core-shell nanofibers extends a possibility of introducing inter-chain polymer crosslinks by gluteraldehyde treatment. The extent of crosslinking will directly influence the rate of drug (i.e.5-FU) release from the nanofibers. Thus the effect of crosslinking time over the release profile of 5-FU from two different core-shell nanofibers (type I and type II) was carried out as part of crosslinking time optimization process. About 5 mg of drug loaded core-shell nanofibers of type I and type II were subjected to gluteraldehyde vapors for different time span (crosslinking time)

at 35 °C. After gluteraldehyde treatment, the fibers were again vacuum dried in a desiccator to remove excess unreacted gluteraldehyde from the fibers. These fibers were further given a mild wash with ultrapure water and then UV sterilized for 45 minutes prior to cell culture study.

1.4 Characterization of core-shell morphology of nanofibers

The core-shell morphology of the nanofibers was visualized by field emission-scanning electron microscope (FE-SEM) operating at an accelerating voltage of 20 keV. The fibers collected were gold coated for a duration of 30 seconds and then mounted for observation. The images obtained were processed by ImageJ software to obtain the mean fiber diameter and fiber diameter distribution. Transmission electron microscope (TEM) analysis was performed using a JEOL 2100 UHR-TEM operating at 200 keV. To observe the core-shell morphology of nanofibers, the fibers were directly deposited onto carbon-coated copper TEM grids (200 mesh $\times$ 125 μ m). In order to obtain clear contrast between core and shell layer of the nanofibers, the fibers deposited over copper mesh were immersed in water for 24 hours, then air dried at 37 °C and observed under TEM.

1.5 Functional characterization of core-shell nanofibers

Drug entrapment efficiency

Entrapment efficiency is defined as the weight ratio of amount of drug loaded in the nanofiber to the total amount of drug added in the feed solution. To determine the amount of drug loaded in nanofibers (type I and type II), the un-crosslinked fibers of predetermined mass are sonicated in 5 mL of phosphate buffered saline (PBS) for 15 minutes, with pulse duration of 2 seconds-“On” and 3 seconds “Off”. The release medium was then centrifuged for 5 minutes at 2000g to bring down the nanofiber fragments. The total amount of drug released in PBS was estimated by UV-Vis analysis of the resultant release medium and then correlating the absorbance values with standard plot of 5-FU and curcumin respectively. The entrapment efficiency for both curcumin and 5-FU was calculated by the following equation;

Entrapment efficiency = (Total mass of drug released from nanofiber/Mass of total drug added) $\times$ 100

Degree of swelling and weight loss

Controlled release of drugs loaded in polymeric nanofibers is determined to a large extent by solvent penetration into the polymer matrix and polymer dissolution rate. Estimation of degree of swelling and weight loss provides a clear perspective of occurrence of such phenomenon during the release studies. The time dependent variation in swelling and weight loss of drug loaded nanofibers (type I and type II) can be directly correlated with different phases of drug release kinetics. Both studies were carried out in PBS (pH = 7.2) at 37°C at 24 hours of incubation. The degree of swelling and weight loss was calculated according to the following equations:

Degree of swelling % (W_s) = ($W_1 - W_2$) / $W_2 \times 100$

Weight loss % (W_L) = ($W_2 - W_3$) / $W_2 \times 100$

Where,

W_1 - weight of swollen nanofiber (after removing excess water)

W_2 – initial weight of the sample before incubation.

W_3 – weight of PBS incubated sample after drying at 40°C (until constant weight is attained).

Contact angle

Static contact angles of water on type I and type II nanofiber surface were measured using the sessile drop method with Drop Shape Analysis System-DSA30 (Krüss, Hamburg, Germany). 30 μ L of ultrapure water was dropped onto dry nanofiber at 37°C and the contact angle was calculated after 60 s of incubation time in order to avoid discrepancy in contact angle values due to location and time. Three variation of type I and type II nanofibers were used in this study, (i.e. uncrosslinked bare PEO-PEI nanofibers, uncrosslinked drug loaded PEO-PEI nanofibers and crosslinked drug loaded PEO-PEI nanofibers) to study the effect of drug loading and crosslinking on contact angle.

X-ray diffraction (XRD) analysis

XRD analysis was carried out to study the drug distribution and crystallinity in the electrospun nanofibers. XRD analysis of drug loaded nanofibers provided a perspective on effect of drug loading on nanofiber polymer matrix. XRD patterns for drug loaded and bare polymer fibers were obtained by Bruker AXS D8 Advance powder X-ray diffractometer (Cu-K α radiation, λ = 1.5406Å) in the range of 10°–90° at a scan speed of 0.050/min.

Fourier transform infrared spectroscopic (FTIR) analysis

FTIR analysis was performed for all combination of drugs (5-FU and curcumin) and polymer (PEO and bPEI) so as to confirm absence of covalent interaction between various components of polymer matrix and the drug loaded within them. Presence of such covalent interaction can possibly introduce unfavorable alterations in drugs and might compromise their therapeutic efficacy. Apart from this, FTIR analysis of crosslinked and un-crosslinked core-shell nanofibers was also carried out to verify completion of glutaraldehyde mediated crosslinking reaction. The FTIR spectra were obtained by Thermo Nicolet spectrometer using KBr pellets in the range 4000–400 cm^{-1} .

Atomic force microscope (AFM) analysis

The core shell nanofibers deposited over aluminum foil were flushed with distilled water to completely extract PEO core (in type I fiber) and to a small extent PEO- PEI shell. The samples were then air dried for overnight before observation under AFM. The nanofiber surface topology was analyzed by AFM (NTEGRA PNL) operating in semi-contact mode. The images were further processed using NOVA software.

Thermal gravimetric (TG) analysis

The as-synthesized core-shell nanofibers bulk compositional analysis was carried out by TG analysis. The nanofibers susceptibility to higher temperatures and effect of crosslinking and drug loading on the stability of nanofibers was interpreted from the thermograms obtained by TG analysis. About 10 mg of respective polymeric nanofiber mats were heated from 32°C to 600°C at a constant rate of 10°C/ min in EXSTAR TG/DTA 6300. A constant nitrogen atmosphere was maintained throughout the TG

analysis of all samples. Various phases of weight loss in the thermogram were corroborated with degradation of specific components of the drug loaded nanofibers.

1.6 Drug release study

Drug release from crosslinked and un-crosslinked core-shell nanofibers

The drug loaded nanofibers (type I and type II) were cut into small pieces of ~4 mg and then subjected to crosslinking in glutaraldehyde vapor for different time duration i.e. 2 min, 5 min, 10 min and 15 min at 37° C in a controlled environment. After drying each of crosslinked nanofibers were placed in PBS. The amount of drug released at the end of 24 hours was estimated by measuring the absorbance of the release medium (i.e PBS) at 265 nm and 427 nm for 5-FU and curcumin, respectively. Equivalent amount of samples from same batch of nanofibers (type I and type II) were used in this study in order to interpret the effect of crosslinking time on drug release profile. The linear equation obtained from the standard plot for curcumin and 5-FU in PBS was used for estimating the amount of drug released from the fibers. The release study was carried out at 37°C in static sink conditions. To avoid interference of polymeric fibers in the UV-Vis observation the release media was centrifuged at low speed (2000 rpm for 2 minutes) and the supernatant was taken for UV-Vis analysis.

Drug release profile of type I and type II core-shell nanofibers

About 10 mg of drug (5-FU and curcumin) loaded core-sheath nanofibers (15 mins crosslinked) were immersed in 4 mL PBS (pH 7.0) for stipulated (48 hours and 96 hours) time period under static conditions. At given time point, 500 µL of release medium was withdrawn for UV-Vis absorbance studies. An equivalent amount of release medium was added in order to maintain constant (volume) sink conditions during the release study. The absorbance value of the release medium at 265 nm and 427 nm were corroborated with standard curve of 5-FU and curcumin to calculate the amount of drug released with respect to incubation time. Curcumin release with each time point could not be quantified owing to use of lower concentration of drug and limits of detection by UV-Vis spectrophotometer (at lower concentrations) for initial phase of release study. Moreover the only difference between type I and type II fibers is extent of crosslinking in 5-FU loaded nanofiber core, thus throughout this article only 5-FU release profile is considered for evaluating the PEO-PEI based core-shell nanofibrous drug delivery system. All the samples in the experiment were in triplicates. The results were represented in terms of cumulative percentage drug release with respect to time;

Cumulative percentage of drug released = $M_t/M_{\infty} \times 100$

Where,

M_t – Mass of drug released at time t

M_{∞} - Total mass of drug in the nanofiber.

Drug release kinetics for type I and type II nanofibers

The therapeutic efficacy of drugs loaded in such delivery system is dictated by the drug release profile and the release mechanism driving it. Correlation of experimental drug release profile with existing mathematical models can aid in deducing the mechanism of

5-FU release. Four such mathematical models have been considered for the analysis (Table S1)

Higuchi model of drug release derives its basis from Fick's law of diffusion as this model considers diffusion as the prime mechanism of mass transfer. Such models do not account for certain non-Fickian mass transfer diffusion phenomenon (which involves polymer swelling and dissolution) and thus in order to investigate the existence of such events in the system under study, another well-established model, Korsmeyer-Peppas is also sought for the fitting equation. Korsmeyer-Peppas model clearly categorizes the drug release profile based on the release exponent (n) value obtained for the fitting curve. If the n value is less than or equal to 0.5 the release mechanism follows Fickian diffusion, and higher values between the range of 0.5 - 1 is obtained when mass transfer follows anomalous non-Fickian model. In accordance with prerequisite of Korsmeyer-Peppas model only the initial phase of drug release was considered for the study. Such information of the drug delivery system is of vital importance as it provides hindsight of critical phenomena governing drug release which can further be fine-tuned to deliver the right amount of drug at the right time.

The regression (R^2) values were calculated independently for the fitting curve of each model with respect to the observed 5-FU release profile from type I and type II nanofibers. These regression values signify how closely each model represents the system and thereby undermine the basic mechanism driving the drug release kinetics.

1.7 *In-vitro* studies

Cell viability assay

(a) Drug IC₅₀ determination

The cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, A549 cells in the log phase were seeded in 96 well plate at a seeding concentration of 5,000 cells/ well. Once the cells were attached, they were treated with different concentration of curcumin (10 μ M - 80 μ M) and incubated at 37 °C in incubator with 5% CO₂ for 48 hours. After 48 hours, the spent media was removed and cells were given a brief PBS wash. Fresh media (DMEM) (~ 90 μ L) supplemented with 10 μ L of MTT (stock concentration-500 μ g/mL) was added to each well. The cells were incubated at 37 °C for 3-4 hours during the course of which mitochondrial dehydrogenases enzyme converts MTT to insoluble formazan crystals. The dark-blue intracellular formazan crystals thus formed are then solubilized in dimethyl sulfoxide (DMSO) after carefully removing the supernatant spent media. In order to completely dissolve the crystals, the 96 well plate was placed over gyratory shaker for 15 minutes and then readings of each well were subsequently taken in microplate reader at 574 nm and 690 nm. In the above mentioned assay DMSO is used as solvent for curcumin and thus a control with equivalent amount of DMSO alone is taken as reference. The absorbance values obtained were represented as percentage viable cells relative to the control. All experiments were carried out in triplicates in order to establish consistency of results. Similar assay was performed independently for determination of 5-FU IC₅₀ against A549 cells for 48 hours and 96 hours.

(b) MTT assay for drug loaded nanofibers

The core-shell nanofiberous mats (type I and type II) were punched into small circular disks of 7 mm in diameter, weighing 1.45 mg, each of them were loaded with the predefined stipulated amount of drug. The nanofiber disks were placed in wells of 96 well plate and then UV-sterilized for 45 minutes. As controls for nanofibers and drugs, bare PEO-PEI nanofibers, curcumin loaded PEO-PEI nanofibers and 5-FU loaded PEO-PEI nanofibers were also included in the assay. A549 cells were seeded onto these fibers at a seeding concentration of 5000 cells/ well for cell viability assay. MTT assay was carried out for two different concentration of drug loaded in type I and type II core-shell fibers in order to establish concentration dependent viability. The experiments were performed for two different time periods i.e. 48 hours and 96 hours independently in triplicates. The control adapted for cell viability includes A549 cells seeded as such in 96-well plate and A549 seeded over bare core-shell PEO-PEI nanofibers at same cell seeding concentration as that of other wells. In order to establish the synergistic effects of drugs, 5-FU and curcumin loaded PEO-PEI core-shell nanofibers were also included in the experiment. The same protocol as described above for determining drug IC₅₀ concentration was adapted with slight modification. In brief, before taking 96-well plate for microplate readings, the solubilized formazan crystals were transferred to free wells in the following lane for UV-Vis observation in order to avoid interference of nanofibers. The obtained absorbance was normalized with respective reference values and correlated with positive controls to arrive at percentage cell viability. Similarly, an independent MTT assay was carried out for estimating the efficacy of drug loaded nanofibers against U-87 MG at two time points i.e. 48 hours and 96 hours.

1.8 Study of cell morphology

(a) Acridine Orange-Ethidium Bromide (AO-EB) staining

AO-EB staining was carried out to monitor time dependent apoptosis/necrosis induction in cells seeded over drug loaded type I and type II core-shell nanofibers. After growing the cells for specified time period over type I and type II nanofibers (i.e. 4, 12, 48, 96 hours) cell were stained with 2 μ L of AO-EB mixture (10 μ g/mL working concentration) to monitor the apoptotic cells. The cells were incubated at 37°C along with dyes for 10-15 minutes and then given PBS wash to remove the excess dyes (to avoid background fluorescence of free dye). After staining, the cells were fixed with 3% paraformaldehyde for 10 minutes. The cellular morphology was then observed under EVOS cell imaging system (life technologies, USA) and images were captured under blue filter, green filter and transmitted mode.

(b) RhoB and Hoechst 33342 staining

The cytoplasm and nucleus of A549 cells seeded over drug loaded nanofibers undergoes drastic morphological changes while undergoing apoptosis and necrosis. Such cellular changes could be tracked by combination of fluorescent stains RhoB and Hoechst 33342. Rho B stains the mitochondria and cytoplasmic vesicles and can be tracked under green filter whereas Hoechst 33342 is a cell permeable DNA stain which binds to the AT rich regions in nucleus which can be visualized under DAPI filter. After specific time spans, the cells treated with drug loaded nanofibers were given a brief PBS wash and then

stained with 1 μ L Rhodamine B (working stock concentration - 1mg/mL) for 10 mins. After incubation time, the excess RhoB was removed and replaced with PBS supplemented with 2 μ L of Hoechst 33342 (working concentration – 10 mg/mL). The stained cells were fixed with 3.7vol % glutaraldehyde and visualized by EVOS cell imaging system under DAPI filter, blue filter and green filter. An overlay of images obtained in two filters represented the location and morphology of nucleus and cytoplasm of A549 cells along with the auto-fluorescent nanofibers.

(c) FE-SEM analysis of cell morphology

The A549 cells were seeded over glass coverslips coated with 5-FU and curcumin loaded nanofibers. On completion of desired time span of therapeutic study, the treated cells were washed twice with PBS and then fixed with paraformaldehyde as mentioned previously. The fixed cells were then sputter coated with gold for observation under FE-SEM.

S.No.	Model	Governing Equation	Parameters
1	Zero order	$f_t = K_0 t$	t - time f_t - fraction of drug dissolved in time t , K_0 - apparent dissolution rate constant.
2	First order	$Q_t = Q_0 e^{-K_1 t}$	Q_t - amount of drug released in time t Q_0 - initial amount of drug in the solution K_1 - first order release constant
3	Higuchi	$f_t = K_H t^{1/2}$	K_H - Higuchi dissolution constant,
4	Korsmeyer-Peppas	$M_t/M_\infty = a t^n$	M_t - mass of drug released at time t M_∞ - total mass of drug loaded in the fiber a, n - constant

Table S1: Different kinetic models considered for 5-FU release studies

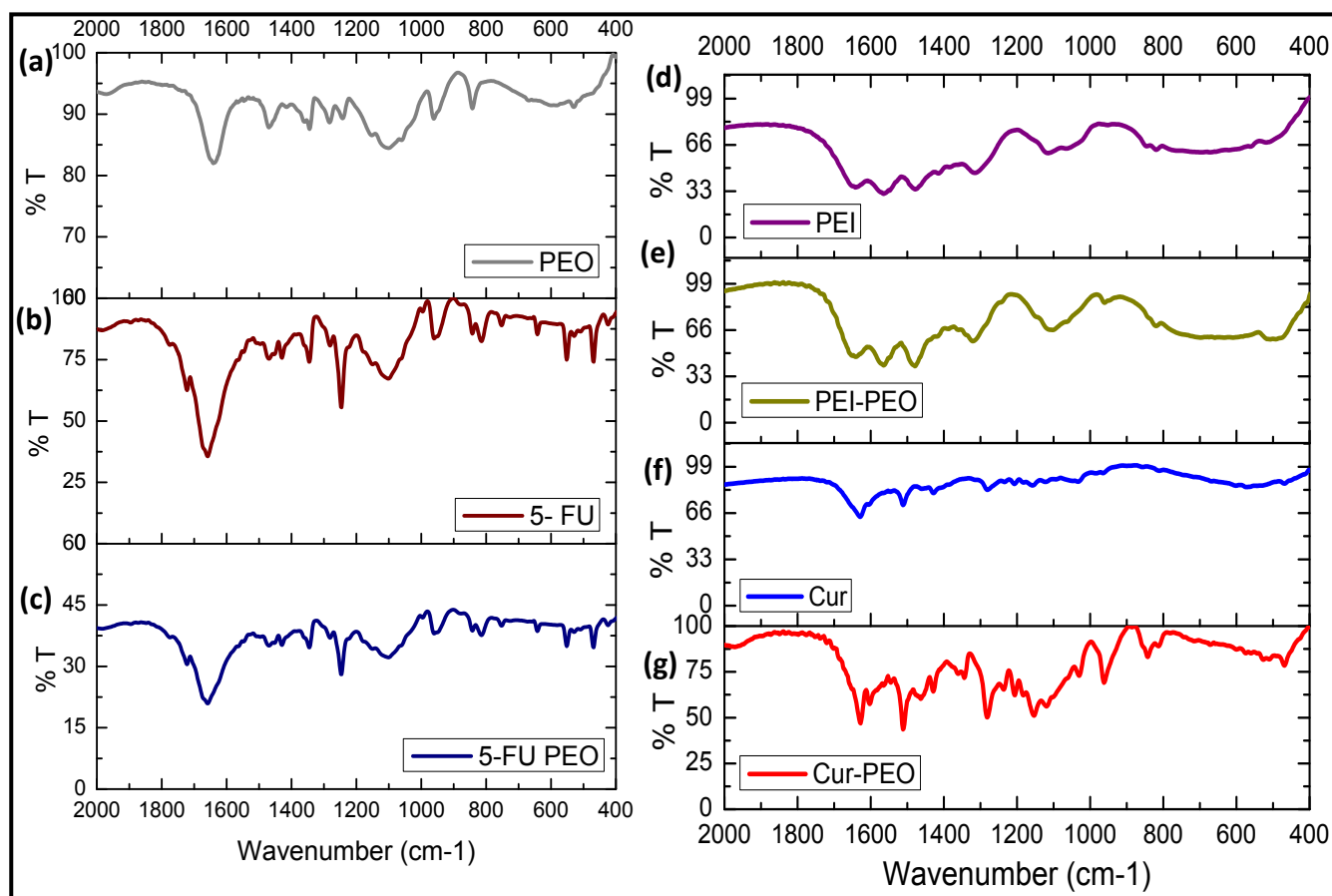


Fig. S2. FTIR spectra of drugs ((b) 5-fluorouracil and (f) curcumin) in polymer ((a) PEO and (d) PEI) at (c)core and (e)&(g)shell composition.

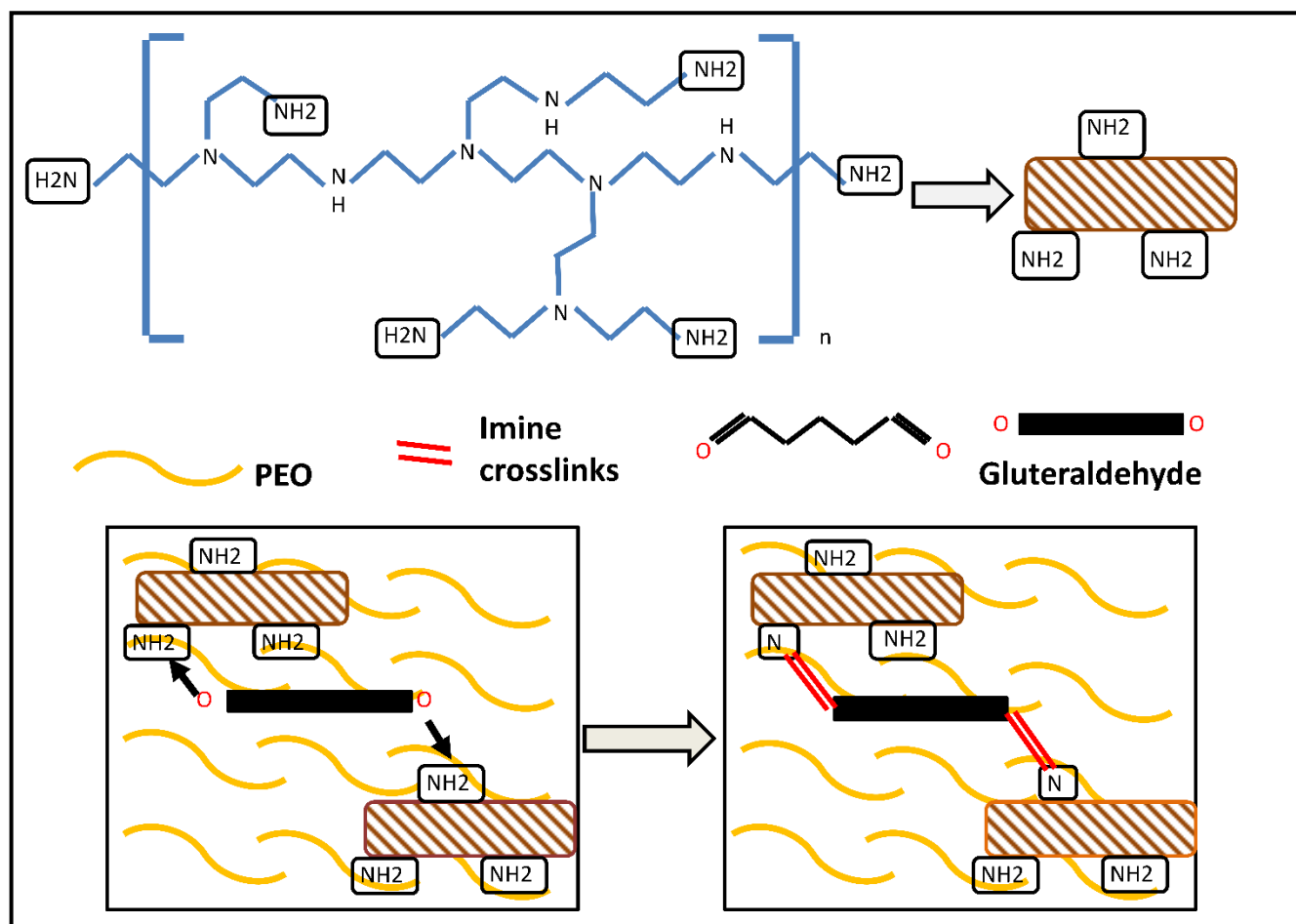


Fig. S3. Schematic representation of glutaraldehyde mediated crosslinking reaction.

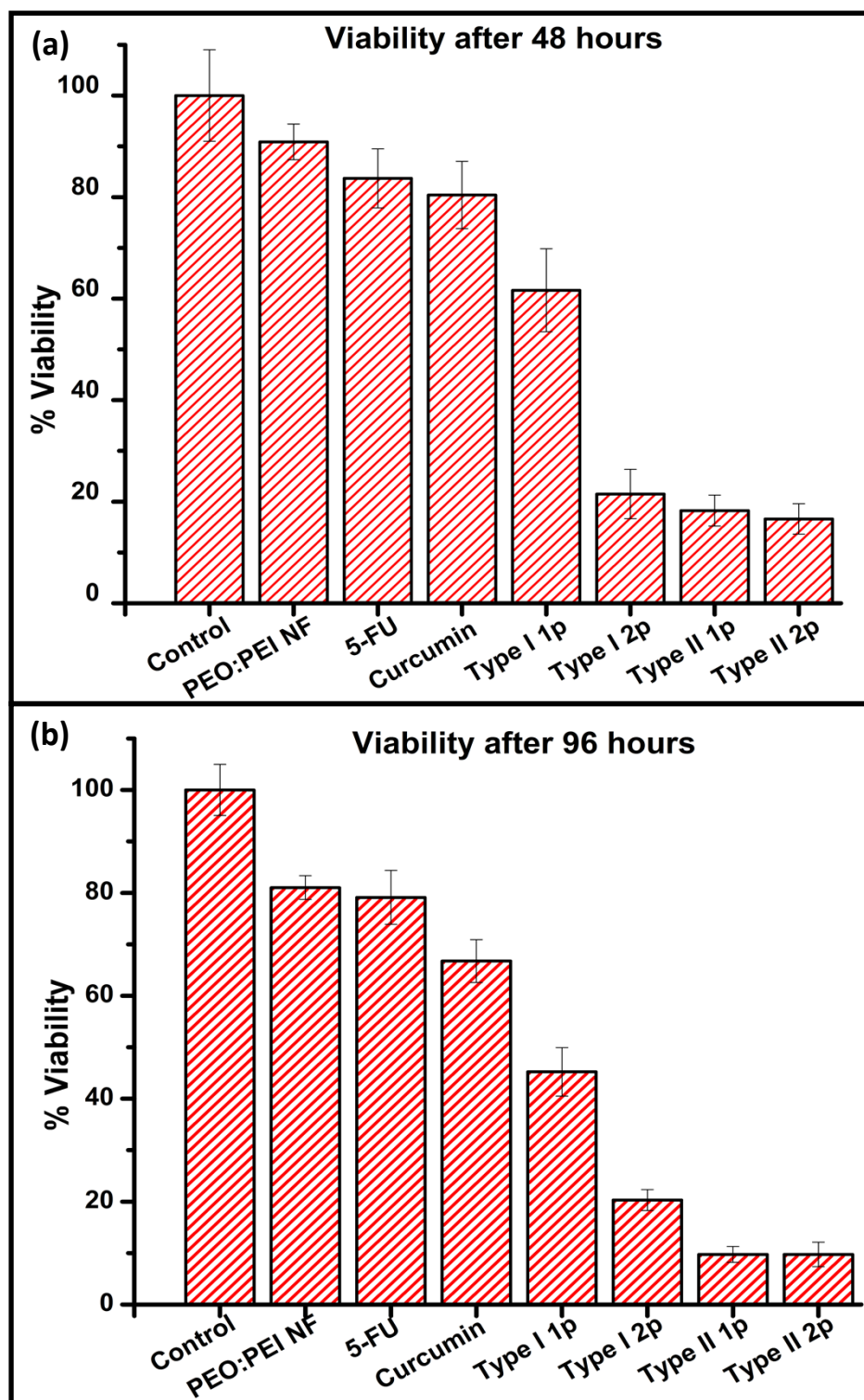


Fig. S4: U-87 MG cells viability assay (MTT assay) after seeding on type I and type II nanofibers at (a) 48 hours and (b) 96 hours. (NF-nanofibers)

**1p- Concentration of 5-FU 2.5 wt% of PEO. **2p- Concentration of 5-FU as 3.5 wt% of PEO.*