Supporting Information

Hydrogel Nanotube with Ice Helix as Exotic Nanostructure for Diabetic Wound Healing

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Experimental Section:

Materials and methods

Acrylic acid (AAc), 99% pure, and N-[3-(dimethylamino) propyl] methacrylamide (DMAPMA) 98% pure were purchased from Aldrich (St. Louis, MO, USA). Ammonium persulphate (APS) and 2-butanone used in the experiment were purchased from Qualigen fine chemicals, India. N,N,N',N'-Tetramethylethylenediamine (TEMED) was purchased from SRL Pvt. Ltd., India. India Pharmacopoeia, 1996, II, standards were followed to prepare buffer solutions. All the reactions and preparations were carried out in double deionized water.

Synthesis

Poly(AAc-co-DMAPMA) (PADP) was synthesized in a two neck round bottom flask (degassed with inert gas) having one syringe inlet with nitrogen balloon and one syringe outlet. The setup was placed on a magnetic stirrer. DMAPMA was added using Hamilton syringe followed by a drop by drop addition of AAc with continuous stirring on an ice bath. This monomer mixture was stirred for 10 mins followed by the addition of water, APS, and TEMED (**Table S1**) and kept on stirring for 15-20 min, undisturbed. The prepared solution was poured into cylindrical molds and was sealed. These molds were kept at 40 ± 2°C for 24 h. A similar synthetic procedure was adopted for the development of PADP 60 and PADP 80 at 60 ± 2°C and 80 ± 2°C respectively. After the completion of the reaction, prepared samples were removed from the molds and kept for washing under running water for few days.

Morphological Characterisation

Transmission Electron Microscopy (TEM) images were taken on a JEOL, JEM-2100F electron microscope at an acceleration voltage of 200 kV. Samples were prepared by drop casting the material onto a carbon-coated copper grid followed by drying at room temperature. The X-ray diffraction (XRD) patterns were recorded in the 2θ range at 5–45 °C by using Cu Ka radiation M/S Philips X'pert Pro. The samples were first swollen in phosphate buffer solutions (PBS) of pH 7.4 followed by lyophilization in freeze drier (Ilshin Lab., Co., Ltd., Korea) for 24 h. The morphology of HNTs and PADP 60 and 80 were examined by using Scanning Electron Microscopy (SEM; ZEISS EVO 50). Lyophilized PADPs were stored in vacuum desicator until further use. For SEM analysis, the samples were fixed on aluminium stub by using double-sided adhesive conducting carbon tape and were gold coated in a sputter coater (PolaronE 5100 Gold Sputter Coating unit).

Chemical characterisation

FTIR spectra were performed on ATR-FTIR model, Nicolet-5DX FTIR spectroscopy equipped with a temperature controller using KBr pellets. The measurements were recorded in the wavenumber range of 4000-500 cm^{-1} at a fixed resolution of 4 cm^{-1}. Raman spectra were recorded on a Raman Spectrometer attached with a Microscope (Varian 7000 FT-Raman and Varian 600 UMA).

Thermal Analysis

Differential Scanning Calorimetry (DSC) measurement was performed on Pyris 6 DSC instrument (Perkin Elmer) at 10°C min^{-1}. Tg was determined from the thermogram of a second heating cycle wherein the midpoint value of the heat increment indicates the Tg. Thermogravimetric analysis (TGA) studies were performed using a Mettler-Toledo, TG850 in a N_2 atmosphere (flow rate = 50 ml min^{-1}) in the temperature range 25–800 ± 1°C (heating rate = 51°C min^{-1}).

Rheology

The rheology studies on the gel samples were performed using small-amplitude oscillatory shear experiments on a AR 500 Rheometer (TA Instruments, Surrey, England) by applying a fixed stress of 1.0 Pa. Measurements were carried out using a cone-plate geometry (2°) and silicone oil with wet sponges was used as a solvent trap. The frequency dependence of storage and loss modulus studies were done at room temperature.

Swelling Study

Swelling ratio was determined by the gravimetric method. The PADPs of diameter ~7 mm and thickness ~2 mm were weighed (Wo) and kept in PBS solution having pH 1 and 7.4 at room temperature. The ionic strength of the buffer solution was kept constant by adding 1M KCl solution. The PADPs were taken out from the solution at a fixed interval of time, surface water was gently soaked and the swollen weight (Wt) was recorded till equilibrium.

The equilibrium swelling ratio (ESR) at the particular time‘t’ was calculated as-:

(Wt – Wo)/Wo x 100

where Wo is the initial weight of the dry HNTs and Wt is the final weight of the swollen HNTs.

In vitro release

The benzalkonium chloride (BZC) loaded HNTs were immersed in 25 ml phosphate buffer solution of pH 7.4 as a dissolution medium for 48 h at 37°C, stirred at 75 rpm in a shaker incubator. At a fixed time interval, 3 ml of the aliquot was pipetted out and the same amount of fresh medium was added. The concentration of the drug was determined using UV- Visible spectrometer at 262 nm. The release study was performed in triplicates and the mean value was plotted on the graph.

Mechanical Properties

Dried HNTs (5.77 mm height; 18.48 mm diameter) were swollen in PBS buffer of pH 7.4 for 24 h at room temperature for prior to mechanical measurements. The compression testing was carried out on Universal Testing Machine (UTM) model H5KS (Tinius Olsen, England) with QMAT 5.37 professional software. The swollen HNTs were gently wiped with tissue paper to remove surface water before performing the compressive test. Furthermore, each sample was placed between a pair of compression plate (diameter 50 mm) with a load of 1000N up to the required strain at 1mm min^{-1} .

Cell culture

Cytotoxicity test was performed using CT26 mouse fibroblast cell line. Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 10 mg ml^{-1} penicillin-streptomycin cocktails in 25 cm^2 tissue culture flask (Corning) was used for routine culture and maintenance of the cell line.

HNT extract preparation

The cytotoxicity of HNTs was performed according to ISO 10993-5 (1992). HNTs were swollen in PBS with pH 7.4 and autoclaved at 121°C for 20 mins. 1 ml of culture media was added to the sample and incubated at 37° C for 48 h with 5% CO_2. The obtained extract was used for cytotoxicity assay.

In vitro cytotoxicity, hemocompatibility

The cytotoxicity assay of the HNTs was carried out according to ISO 10993–5 (1992). Swollen HNTs, in PBS buffer at pH 7.4, were cut into uniform discs and sterilized by autoclaving at 121°C for 20 mins. In a 24-well plate (Corning), two HNT discs of each kind were incubated with 1ml of culture media at 37 °C for 48 h with 5% CO_2. The test samples (50 µl per well in triplicates) were added to 96-well tissue culture plates (Corning). The cells were further suspended after trypsinization in the culture media and the density was determined by using a haemocytometer. 50 µl of cell suspension (~2800 cells) was added in each well containing the sample. Culture media free from cells was taken as blank and culture media with cells served as the negative control. After a period of 72 h, cytotoxicity was analysed using 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (**MTT**) (Sigma). After 4 h of incubation, the readings were taken using ELISA plate reader (Anthos HT 1) at 570 nm. The percentage survival was calculated as:

$$\frac{(Absorbance\ of\ test) - (Absorbance\ of\ blank)}{(Absorbance\ of\ control) - (Absorbance\ of\ blank)} \quad X\ 100$$

The investigated study was approved by "Institutional Animal Ethics Committee" in accordance with the guidelines of "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA, New Delhi, India). Wistar albino rats of either sex weighing about 280-300 g were used for this experiment. The animals were maintained in the "Institutional Animal House". Rats were given *ad libitum* access to water and food and exposed to normal day and night cycles.

In vitro haemolysis assay of HNTs was examined for *in vitro* blood compatibility. Blood was withdrawn by retro-orbital blood collection method from a healthy rat in disposable syringes holding 4.9% citrate phosphate dextrose adenine solution. Standard (Triton X) and test (HNTs) samples were equilibrated to normal saline (NS) sample. Each sample tube received an equal amount (100 µl) of freshly isolated uncoagulated blood and kept for 1 hr incubation (5% CO_2, 37 °C temperature, 95 % humidity). Absorbance was taken at 450 nm. Calculation of Haemolytic rate % was done using the following formula -:

$$\frac{(Sample absorbance) - (Negative control absorbance)}{(Positve control absorbance) - (Negative control absorbance)} X 100$$

RBC's cell aggregation (hemagglutinin) assay

Freshly collected blood sample was subjected to centrifugation at 7000 rpm for 10 mins. Later the pellet was collected and re-suspended with normal saline in ratio 1:9 (v/v). Additionally, 600 ml of normal saline was mixed with 100ml of re-suspended solution. The test sample and standard (Triton X) were equilibrated to normal saline sample. The prepared solution was mixed with all the samples in equal volumes and kept for 1 hr incubation at 37°C. After incubation period slide was prepared by smearing the cell suspension and observed at 40 X. Triton X and normal saline were used as positive and negative control respectively.

Wound healing study on Rat model

Male Wistar rats of 200-220 g were obtained from Animal House Facility of Institute of Pharmacy, Amity University, Noida, India by the approved protocol of CPCSEA (New Delhi, India), and used for the present study. During the complete period of protocol, the animals were housed in separate cages and maintained in normal environmental conditions of institutional animal house (temp 20 ± 2°C, relative humidity 60 ± 10 %). All animals were allowed to access normal food and water *ad libitum*.

Induction of experimental diabetes mellitus

Diabetes was induced in rats *via* single intraperitoneal injection of streptozotocin (STZ) (50 mg/kg) dissolved in freshly prepared citrate buffer (pH 4.5). Measurement of blood glucose was estimated after 72 h using glucometer.

Formation of full-thickness excisional wound

The dorsal area of Wistar Rats was shaved by electrical clippers and exposed to cold wax strips. They were screened for any signs of irritation, infection or impairment on the skin for 24 h and anesthetized by intraperitoneal injection of ketamine (80mg/kg), and xylazine (10mg/kg) for the creation of wounds. The dorsum part of each rat was rinsed with 10% povidone-iodine solution followed by 70% isopropyl alcohol. A sharp cut of full-thickness (diameter 10 mm) in the interscapular region of the dorsal part was created by the help of a biopsy punch.

Experimental Design

In the present study, six Wistar Rats were involved in two experimental groups i.e negative control and positive control. The negative control group was observed for normal healing rate without any treatment whereas the positive control group was subjected to topical application of HNTs (0.5 mg on wound area per animal). Animals were treated with topical application of HNTs in respective groups once daily until healing was completely observed. All animals survived during the treatment period without any physical impairment or systemic inflammation further validating the biocompatibility of HNTs.

Estimation of Inflammatory Cytokines

Blood sample was collected from each animal and serum was isolated by centrifugation at 3,000 RPM for 10 mins. Release of cytokines were measured in all groups at day 5 and day 10 of wound. Estimation of pro-inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines was analysed from diabetic rats by performing enzyme linked immunosorbent assay (sandwiched ELISA kit) from Ray Biotech USA as per the kits manual.

Statistical Analysis

All results were statistically analysed using one-way ANOVA followed by Tuckey post-hoc test using Sigma Plot version 11.0 (Systat Software, Inc., San Jose California USA). All values were expressed as mean ± SD.

Histological Assessment

Wound skin was removed for histological assessment (fixation, dehydration, embedding, and cutting) on the 5^{th} and 13^{th} day and stained with haematoxylin and eosin to monitor structural changes. Photomicrographs were analyzed under an inverted microscope (COSLAB, INDIA).

Supporting Figures:

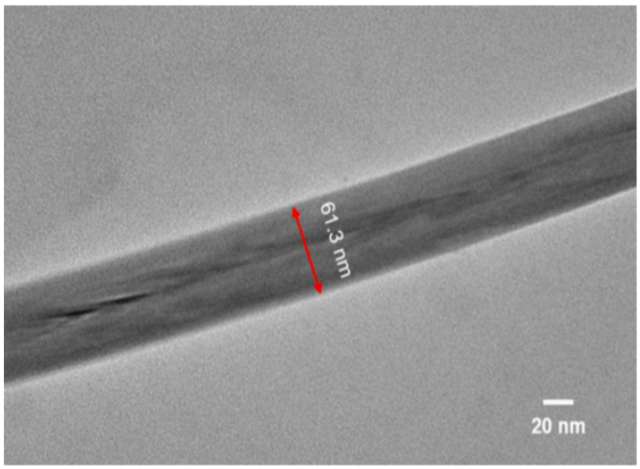

Figure S1. (a) TEM micrograph of HNT of width ~61.3 nm.

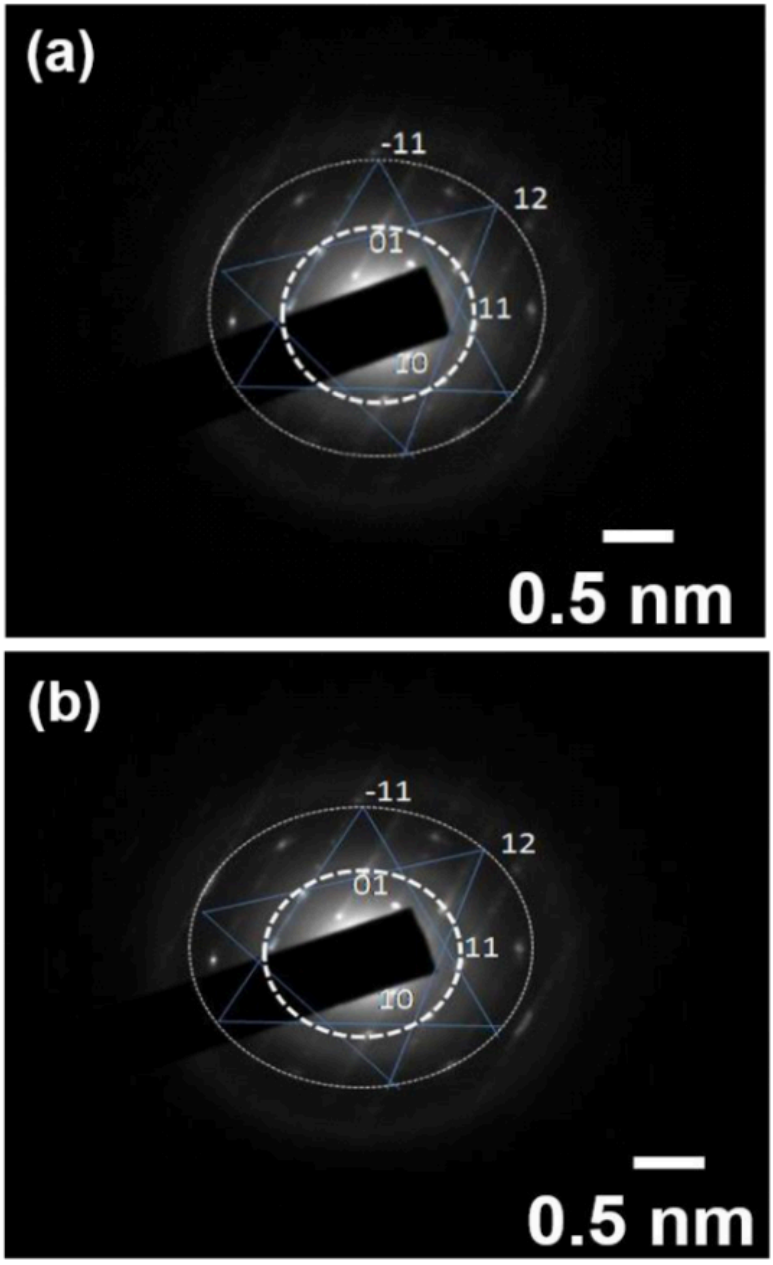

Figure S2. (a) and (b) Indexed hkl planes from SAED patterns of HNTs.

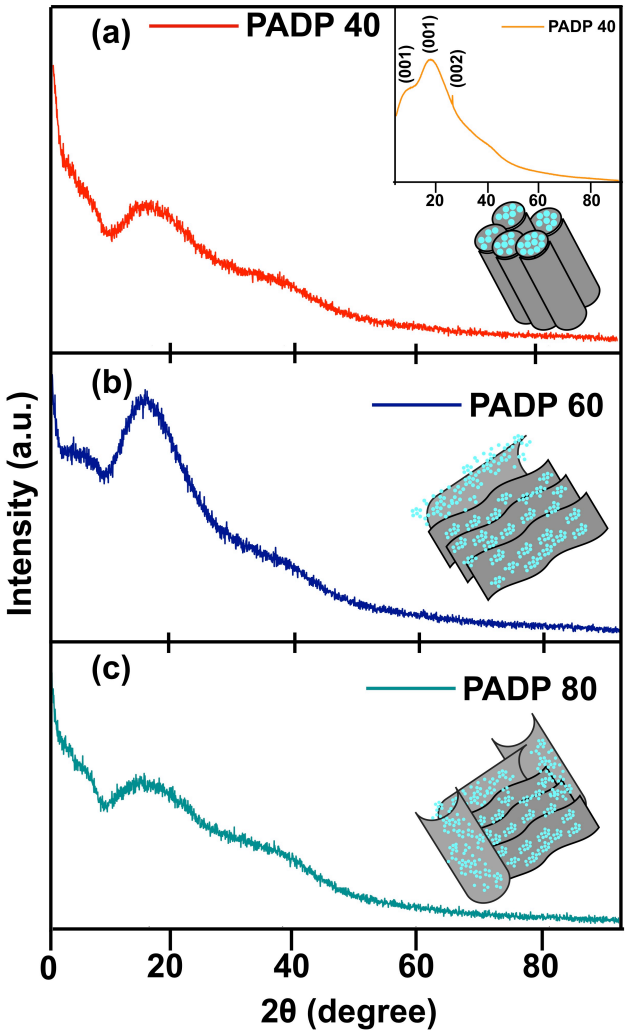

Figure S3. X-Ray Diffraction pattern of PADPs. (a) PADP 40 (HNTs). (Inset: Slow scan of HNTs) (b) PADP 60. (c) PADP 80.

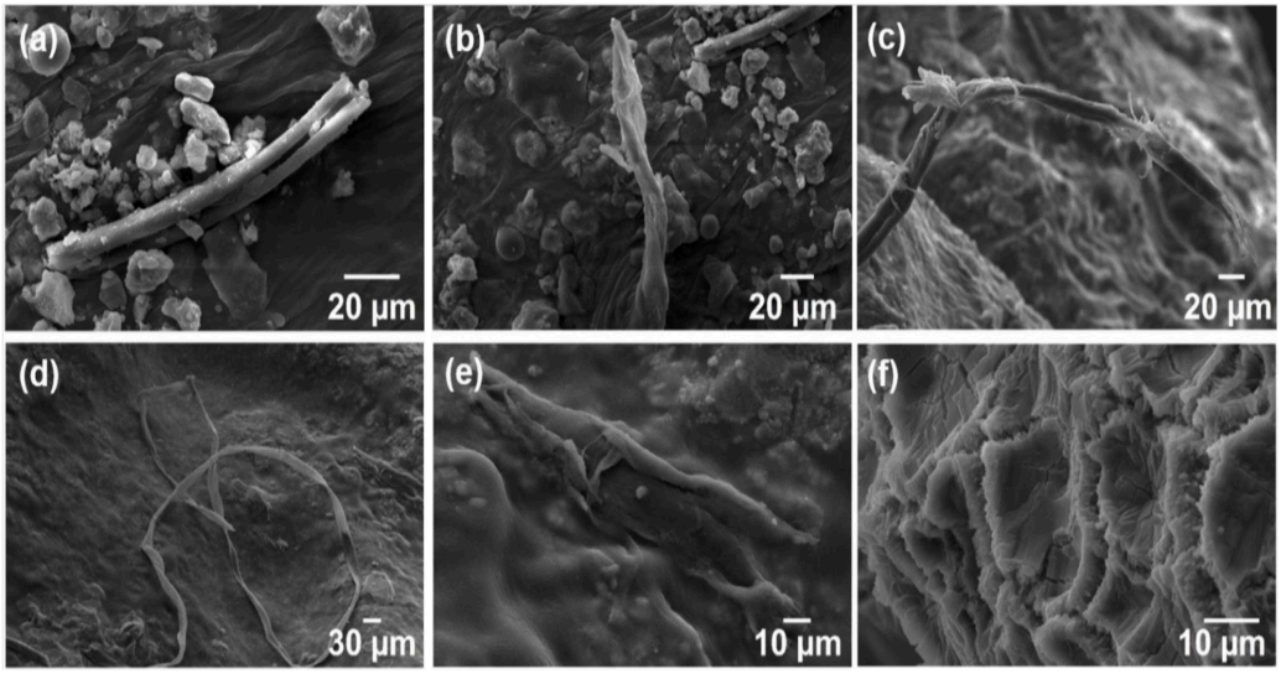

Figure S4. Scanning electron micrographs of (a-c) PADP 60 and (d-f) PADP 80. (a) Highly distorted tube ripped from one end. (b) Intertwined rolled sheets forming highly distorted tubular architecture. (c) Sheets ripped apart from irregular tubular structures. (d) Long, flexible flat-noodle like architecture. (e, f) Collapsed structure.

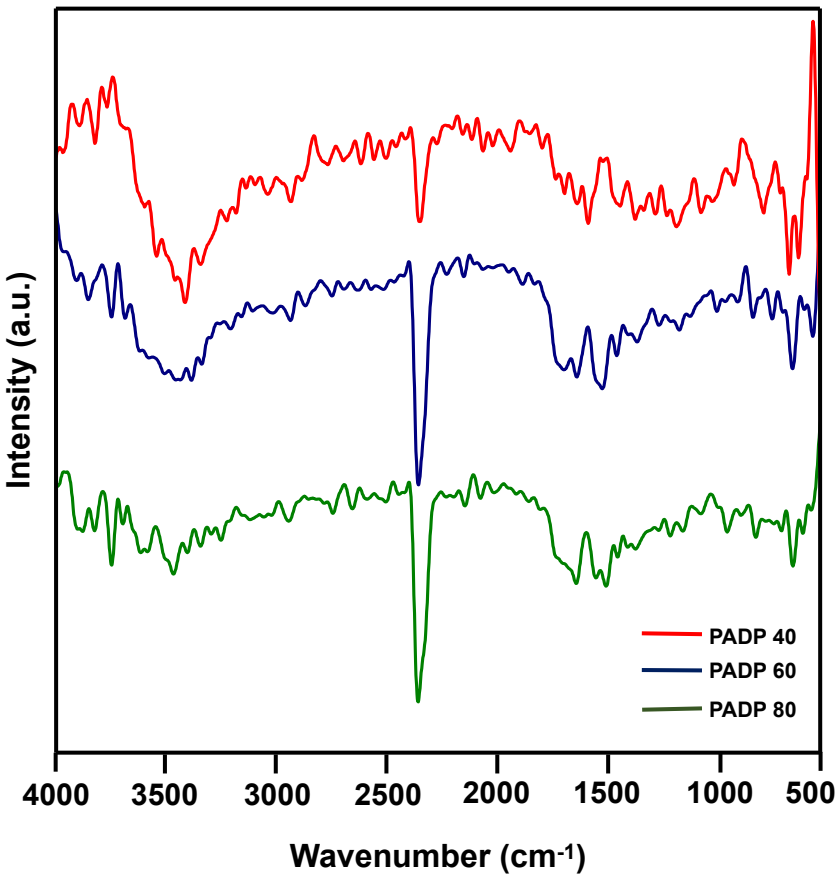

Figure S5. (a) FTIR spectra of PADP 40 (HNTs), PADP 60 and PADP 80.

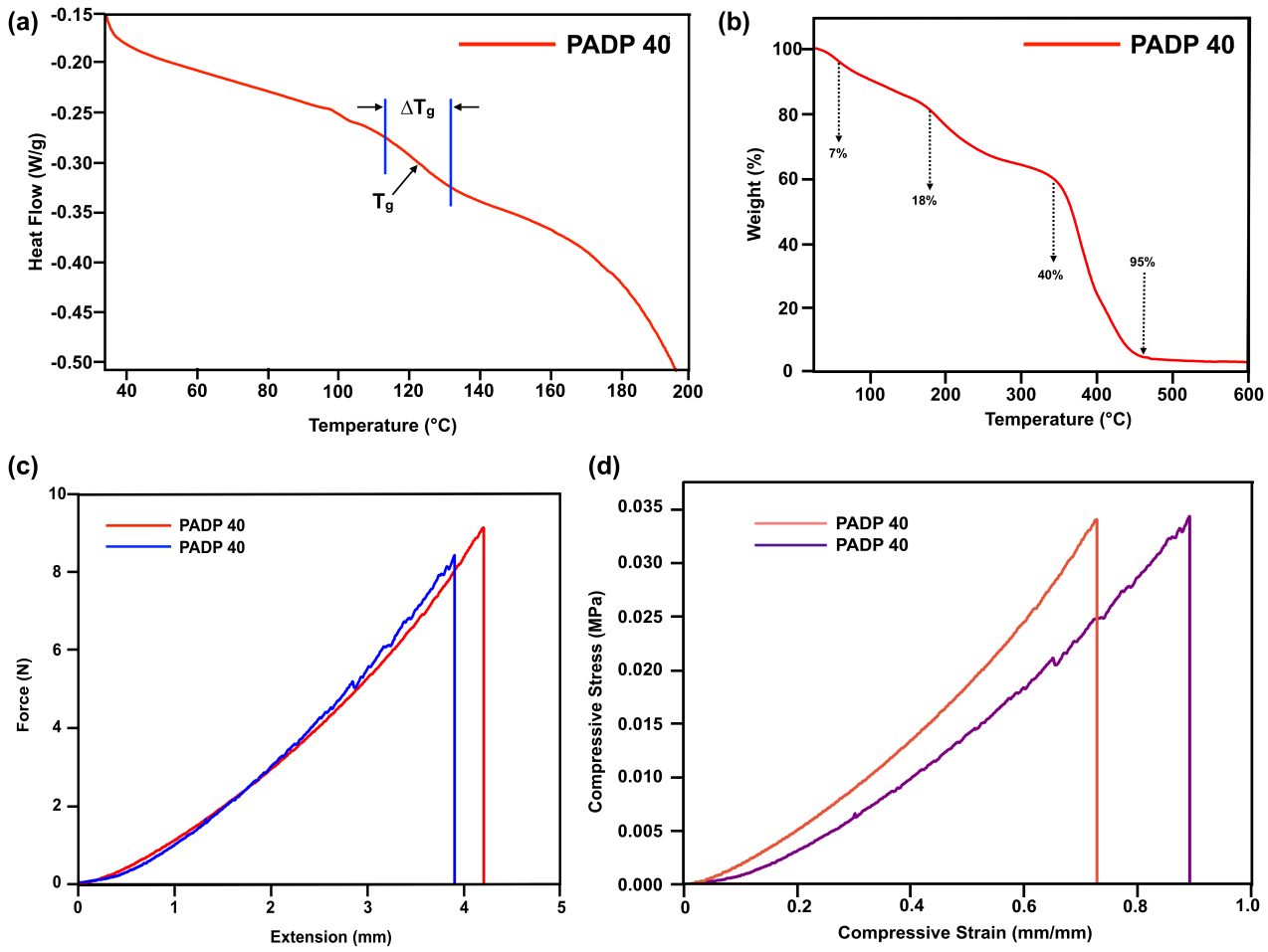

Figure S6. Thermal analysis and mechanical properties of HNTs. (a) DSC thermogram. (b) TGA trace. (c) Compressive testing. (d) Compressive stress-displacement curves.

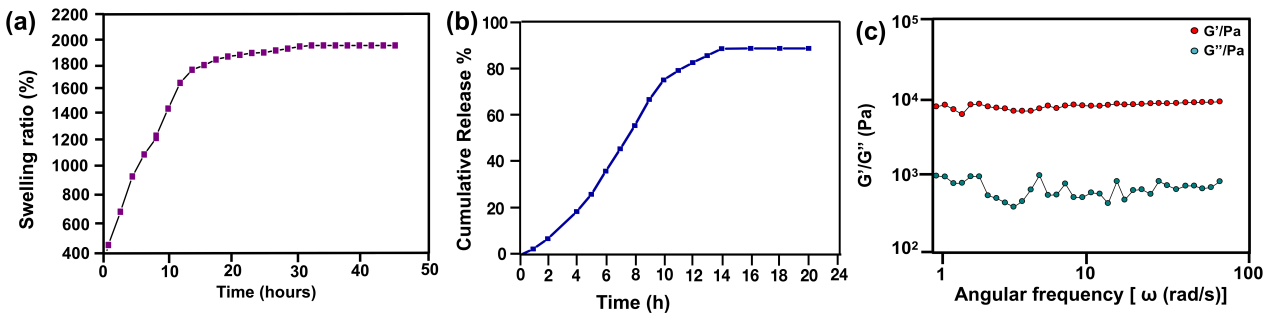

Figure S7. Equilibrium swelling, release profile and rheological tests of HNTs. (a) Swelling isotherm. (b) *in-vitro* drug release profile. (c) Storage and loss moduli

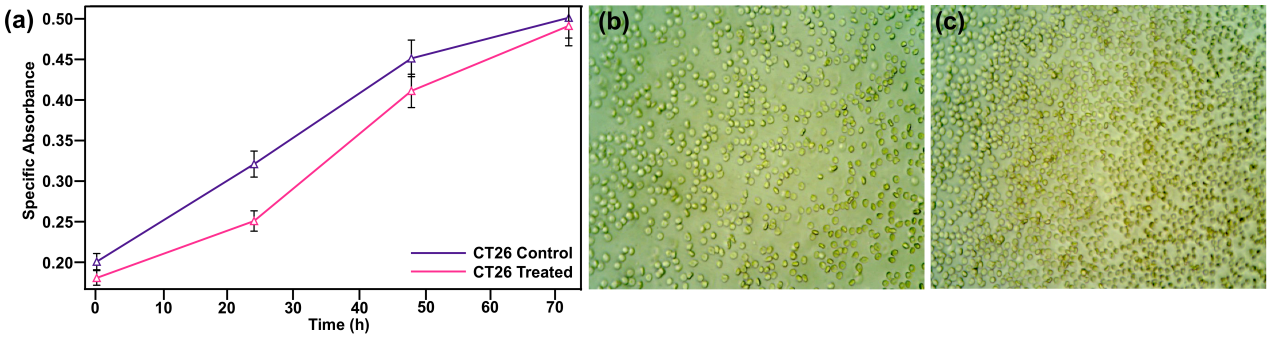

Figure S8. Cytotoxicity assessment of HNTs with CT26 cells. (a). Time-dependent cytotoxicity. (b-c) RBC Aggregation assay. (b) with saline. (c) with HNTs.

To evaluate the time-dependent change in metabolic activity, MTT assay was carried out with CT26 mouse fibroblast cells. The results showed reduction in metabolic activity at first 24 h which improved after 72 h. No significant difference was observed between test and control groups. Furthermore, HNTs were assessed for haemolytic activity of RBC by utilizing fresh blood against normal saline (negative control) and Triton X group (positive control). After 1 hr of incubation, Triton X treated sample unveiled more haemolysis in comparison to test and negative control groups. Haemolytic rate of HNTs was found to be remarkably lower than the permissible rate of 5%. The RBCs revealed insignificant aggregation and we could not observe any morphological changes establishing haemocompatibility of HNTs.

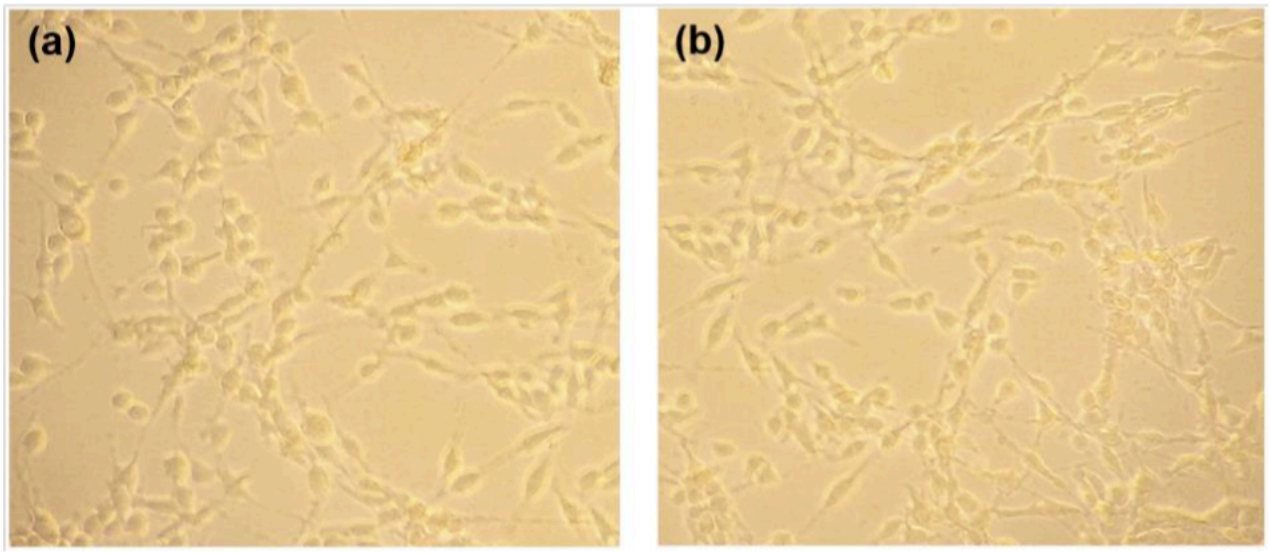

Figure S9. Cellular morphology of CT26 after treatment with HNTs. (a) control (b) treated

Table S1. Feed-ratio table for synthesis of copolymers.

Sample	AAc (mol %)	DMAPMA (mol %)	Water (mol %)*	APS (mol %)	TEMED (mol %)
HNT	70	30	380	0.025	0.3

Table S2. Compressive testing of HNTs.

Force (N)	Elastic Modulus (kPa)	Compressive modulus (kPa)	Deformation at Compression (%)	Deformation at Failure (%)	Compressive Strength (kPa)
9.14	47.3±1.52	126.1±1.28	27.0±0.03	72.0±0.96	34.0±0.10
8.44	38.6±2.43	319.8±3.12	10.7±0.05	89.0±0.24	34.0±0.40