Chapter 2

Materials and Methodologies

Chapter 2

MATERIALS AND METHODOLOGIES

2.1. INTRODUCTION

In this chapter, chemicals, reagents, and cell lines used for this work and experimental techniques followed to characterize and analyze the samples have been explained in detail.

2.2. MATERIALS

Glycine (98%, Qualigens), L-Phenylalanine methyl ester (Sigma), Triethylamines (≥99.5%, Merck), 1, 4-dioxan (99%) extra pure (Merck), Magnesium sulphate anhydrous (SRL), Potassium Hydroxide (KOH), Hexadecane anhydrous (HD) (99%, Sigma), SDS (sodium dodecyl sulfate) (90%, Merck), Divinyl Benzene (DVB) (Alfa Aesar), Acryloyl chloride stab with 400ppm phenothiazine (96%, Alfa aesar), Ethyl acetate, Diethyl Ether, Hydrochloric acid, 2,2'- Azo-bis-isobutyronitrile (AIBN, 98%, SRL), Dichloromethane, Sodium bisulphate, Toluene, Potassium Chloride, Potassium hydrogen phosphate, Magnesium chloride, Sodium sulphate, Sodium Chloride, , DMSO d_6, $CDCl_3$, Phosphate buffered saline (pH 7.4), Isopropanol, Phosphotungestic acid (Sigma), Triton-X, DNS (Sodium Chloride and Dextrose Injection IP (0.9% & 5% w/v)) (Jedux), Piperine (Sigma), Dihydroartemisinin (TCI), Calcium Chloride (Qualligen), Zinc Chloride (SRL), Gelatin (Sigma), Acetic acid (Merck), Tris Base (Ultra pure), Comassie brilliant blue 250 (himedia), Methanol (Qualligen), TEMED (Purgene), APS (Sigma), Acrylamide (Himedia), Methyl thiazoltetrazolium (MTT, >99.9%, Himedia), Dulbecco's modified Eagle medium (DMEM, Cell Clone), Penicillin streptomycin cocktail (Himedia), Trypsin-EDTA (Himedia), 4% Paraformaldehyde solution (Himedia), Thiopentone sodium (Abbott), 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine, 4-

chlorobenzenesulfonate salt (DiD) dye, 4%, Folic Acid (Sigma), Ethyl acetate, N-hydroxysuccinimide (SRL), Hydrochloric acid, 3-Dimethylamino-propyl)-ethyl-carbodiimide Hydrochloride (EDC.HCl), Triton-X (Thermo), Phalloidin (Invitrogen), BSA (Sigma), MitoSOX (Thermo fischer), Chloropromazine (Sigma), Nystatin (Sigma) and Amiloride Hydrochloride (Sigma), α-tubulin (Thermo fischer), FACS Kit (Invitrogen), DCFHDA (Cymann), Rhodamine 123 (Sigma), Fetal Bovine serum (FBS, Gibco), DABCO (Sigma), DMSO (Merck), Isoflurane (Abbott), Lidocaine (Abbott), White soft paraffin (SRL), Paraffin wax (SRL), Glycerine (SRL), Steryl alcohol (SRL), Liquid paraffin (SRL), Triethanolamine (Loba Chemie, 98%), Hematoxylin (SRL), Eosin (Himedia), Geltrex (Gibco), Calcein-AM (ThermoFischer Scientific) Triazole (Invitrogen), Chloroform (Merck), Diethyl polycarbonate treated water (DEPC-treated water, Himedia), PowerUp™ SYBR™ GREEN Master Mix (Invitrogen), KiCqStart® primers (sigma), Taq polymerase (TAKARA R001A) etc. Pure Lab Ultra water system (ELGA, High Wycombe, United Kingdom) was used to obtain Ultrapure water (18.2 ΩM) for sample processing. L929 (mouse fibroblast cells), A549 (Lung carcinoma epithelial cells) and MDA-MB-231 (triple negative breast cancer, TNBC), Raw 264.7 (macrophages), HUVEC (Human Umbilical Endothelial Cells), and PC12 (neural crest-derived cell) cell lines were acquired from NCCS-Pune repository, India.

2.3. METHODOLOGIES

2.3.1. Synthesis and Modifications of nanoparticles (NPs)

2.3.1.1. Synthesis of *N*-acryloyl glycine (NAG) monomer

Solution A: It was prepared in a 100 mL round-bottom flask, containing 20 mmol of glycine and 20 mL of aqueous 2 M KOH, which was maintained in an ice bath with continuous stirring at 600 RPM. **Solution B**: It was prepared in a 50 mL of round-bottom flask, containing 20 mmol of acrylol chloride and 5 mL of 1,4-dioxane in an ice bath with continuous stirring at 900 RPM.

Solution B was gradually added to Solution A over the course of one hour, ensuring continuous stirring to facilitate the reaction. Following the addition, the mixture was allowed to stir overnight at RT to ensure complete reaction. After 12h, the mixture was washed with 20 mL of diethyl ether for 3 times to remove unreacted materials and by products. The bottom layer was collected after each wash. The pH of the resulting solution was then adjusted to 2 by the dropwise addition of 5M HCl. Subsequently, the solution was saturated with excess NaCl, and the aqueous phase was extracted within 20 mL of ethyl acetate for 5 times to separate N-acryloyl glycine. The moisture was dried over $MgSO_4$ and the filtrate was then subjected to rotary evaporation to concentrate the solution, yielding white powder named as *N*-acryloyl glycine (NAG).

2.3.1.2. Synthesis of *N*-acryloyl-*L*-phenylalanine methyl ester (NAPA) monomer

Solution A: It was prepared in a 100 mL round-bottom flask containing 1.6g of L-phenylalanine methyl ester, 1.6 mL of triethylamine, and 60 mL of dry DCM, which was maintained in an ice bath with continuous stirring at 600 RPM. **Solution B**: It was prepared in a 50 mL round-bottom flask containing 0.9 mL of acryloyl chloride in 5 mL of DCM. Solution B was gradually added to Solution A over the course of one hour, ensuring continuous stirring to facilitate the reaction. This controlled addition was important for exothermic nature of the reaction and enhance the formation of the desired product. After the complete addition, whole mixture was stirred for 24h. Further, DCM was removed using a rotary evaporator at 30 °C, yielding a white residue. Then 100 mL of ethyl acetate was added to the residue, and the mixture was filtered using filter paper to remove insoluble impurities. The resulting solution underwent a series of wash, which include 40 mL of sodium sulfate ($NaHSO_4$) five times, followed by 40 mL of sodium bicarbonate ($NaHCO_3$) five times, and finally with 40 mL of brine solution three times. These washing steps are essential for the removal of impurities. Further, to ensure

moisture removal, $MgSO_4$ was added and filtered through a 0.45-micron filter. The filtrate was then subjected to rotary evaporation to concentrate the solution, yielding white powder named as *N*-acryloyl-L-phenylalanine methyl ester (NAPA).

2.3.1.3. Synthesis of p(NAG) and p(NAPA) homopolymers, p(NAG-*co*-NAPA)$_{(x:y)}$ copolymer library NPs

The library of [p(NAG-*co*-NAPA)$_{(x:y)}$] copolymer NPs have been synthesized via free radical miniemulsion polymerization as authors reported in their earlier work.[1, 2] In brief: monomers by were dispersed in toluene. Then HD and DVB were added and sonicated for 5 minutes at RT. The mixture was stirred for 30 minutes. Then, water based SDS solution and defined amount of AIBN were added to the above reaction mixture and stirred at RT for 1 h. Then the reaction mixture was sonicated (Sonics, Vibra cell, 750 Watt, 45:15 sec., 40% power) for few minutes in an ice bath followed by polymerization under continuous stirring still the reaction finished. Finally, the residual toluene was evaporated. The prepared NPs were washed thoroughly with (1:1) ethanol and water mixture for 8-10 times followed by centrifugation. Then, the samples were freeze dried and stored for further studies. p(NAG) and p(NAPA) homopolymers were synthesized by following the same procedure. The different ratios of monomers used are mentioned in **Table 3.1.1**. The details synthesis method of this polymeric NPs library has been filed for Indian patent (***Application No. 202511034476***).

2.3.1.4. Folate conjugation of p(NAG-*co*-NAPA)$_{(1:4)}$ copolymer NPs

The synthesis of folate-p(NAG-co-NAPA)$_{(1:4)}$ NPs (fP4) is a two-step process. At first p(NAG-co-NAPA)$_{(1:4)}$ NPs (P4) has been synthesized as mentioned in our earlier report.[3] In second step, FA has been conjugated to P4 NPs by EDC.NHS coupling reaction. The method of folate conjugation with p(NAG-co-NAPA)$_{(1:4)}$ NPs was as follows:[4] At first, 5gm of FA

was dissolved in 100mL of dry DMSO and 2.5mL of trimethylamine and allowed to react with 2.6gm of N-hydroxysuccinimide in the presence of EDC.HCl (4.7gm) for 12h at RT in the dark. The byproduct was removed by filtration, followed by concentrating the DMSO under reduced pressure, precipitation in diethyl ether, washing repeatedly in anhydrous ether, lyophilization of NHS-Folate, and stored as a yellow powder. Further, 50mg of NHS-Folate and 500mg of P4 NPs were suspended in 10mL dry DMSO and bicarbonate buffer. Then, kept for stirring at 600RPM for 12h at RT in the dark. After incubation, the fP4 NPs were centrifuged at 15000RPM followed by dialysis in PBS for 3 days and then in water for another 4 days. Finally, it was centrifuged at 15,000 RPM for 10 minutes at 4 °C to collect pellet followed by freeze drying, and stored in an airtight amber colour tube for further studies.

2.3.1.5. Synthesis of p(NAG-*co*-NAPA)$_{wc}$ copolymer NPs

The synthesis approach of p(NAG-*co*-NAPA)$_{wc}$ random block copolymers was quite similar with the procedure followed for the synthesis of p(NAPA) and p(NAG) with modifications as reported earlier.[1, 2] In brief, the mixture of two monomers (NAG and NAPA) with 1:1 ratio was taken by maintaining the total weight to 500mg. Without DVB, keeping all other parameters fixed, probe sonication time was increased to 8.30minutes and polymerization was performed. Further the washing with 1:1 ethanol and water mixture, for 8-10times, centrifugation (15,000RPM at 4°C for 45minutes) and freeze-drying (48h) steps were followed to obtain the dried lyophilized white powder. Then the sample was stored in a sealed container for further studies.

2.3.1.6. Development of p(NAG-*co*-NAPA)$_{wc}$ nanoformulations (NFs)

The p(NAG-*co*-NAPA)$_{wc}$ NF was prepared by disseminating p(NAG-*co*-NAPA)$_{wc}$ NPs (1% w/w) with an oleaginous base to apply smoothly on the wounds. The composition of the

oleaginous base was given in **Table S3.3.2**. p(NAG-*co*-NAPA)$_{wc}$ nano-particles were initially dispersed on the ointment slab, followed by trituration with base by using a long, broad spatula. Glycerine was added last after complete trituration of the ointment with the base. Then, the p(NAG-*co*-NAPA)$_{wc}$ NF was stored in an open-mouth airtight container at RT.

2.3.2. Characterization Techniques of NPs

The chemical functionality, and structural analysis of OTDDNs were confirmed through ^{1}H NMR, ^{13}C NMR spectroscopy (500 MHz OneBay NMR Spectrometer, BRUKER BioSpin INTERNATIONAL AG) and FTIR Spectroscopy (Nicolet iS5, THERMO Electron Scientific Instruments LLC). Chemical shifts are plotted in ppm relative to the signals generated by deuterated solvents ($CDCl_3$, DMSO-d_6 and 1:1 mixture of $CDCl_3$ and DMSO-d_6 (co-solvency approach) for p(NAG), p(NAPA), and [p(NAG-*co*-NAPA)$_{(x:y)}$], respectively and plotted using MestReNova 14.1. UV-Vis Spectroscopy (Lambda 750 Spectrophotometer, PerkinElmer) was performed (λ=180-300nm). Matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectra were acquired using a Bruker Autoflex instrument using Dithranol in THF as matrix and the method followed for analysis was RN_900-4500_Da.par. Thermal stability and phase transition of NPs were studied through TGA (TGA-50, M/s Shimadzu (Asia Pacific)) from 2°C to 600 °C at 10 °C/min heating rate with 100 mL/min of N_2 gas flow and DSC (DSC-60 Plus, M/s Shimadzu (Asia Pacific)) from 25°C to 300°C at 10°C/min heating rate with 50mL/min of N2-gas flow, respectively. The crystallinity of NPs was evaluated using High Resolution X-Ray Diffraction (Rigaku SmartLab 9kW Powder type (without χcradle) at 2θ = 10°-90° equipped with Cu Kα X-Ray source with λ = 1.54 Å).

2.3.3. Colloidal stability and Morphological Studies of NPs

The colloidal stability of the OTDDNs were evaluated by measuring zeta potential (ζ) using a Zeta sizer (Malvern Zeta Sizer) at 25°C. For this, suspensions of 500 µg/mL of NPs in MilliQ were prepared and three acquisitions were recorded for each sample and average hydrodynamic diameter and ζ values were recorded. Circular dichroism (CD) experiments using Jasco J-1500 CD Spectrometer were performed in MilliQ at 250µg/mL at RT from λ= 190 to 300nm with 0.5nm data pitch and bandwidth 1.0nm. For HRTEM analysis, ~0.1mg OTDDNs were taken in 500µL of isopropanol with two drops of phosphotungstic acid and sonicated in regular interval upto 8h for uniform dispersion. Then a few drops of suspension were taken on the C-coated Cu grid (200 mesh size). Images and EDAX pattern were acquired using Tecnai G2 20 TWIN (FEI Company of USA (S.E.A.) PTE, LTD) equipped with EDS SYSTEM and Octane Plus SDD Detector. For FESEM analysis ~0.1 mg of NPs was taken in isopropanol and sonicated in regular intervals for uniform dispersion. Then a few drops of copolymer samples were drop-casted over the cleaned silicon wafer and dried. Images were acquired using Nova NanoSEM 450. For AFM, a sample was prepared similar to FESEM and images were acquired using NTEGRA Prima.

2.3.3.1. CMC Determination

The CMC was determined using 6-methyl coumarin as a hydrophobic dye based on a previously reported method with slight modification.[5, 6] Once the concentration reaches above to CMC, the dye could associate with the hydrophobic domain of selected NPs and emit strong fluorescence. A stock solution of 6 µM solution of dye was prepared in dry DCM, and then from this stock 50µL of the solution was added to each amber color micro centrifuge tube and kept for 30minutes in the dark for drying. Further, 400µL of different NPs in MilliQ water with different concentrations (1000µg/mL to 0.488µg/mL with 2-fold serial dilutions) was

added and stirred in the dark at 25°C for 20h. Then, the emission spectra were recorded using a microplate reader (Biotek) with 285nm excitation and 410nm emission. Further, from the emission vs intensity plot a tangent was drawn to determine the CMC.

2.3.4. Biodegradation Studies of NPs

The biodegradation study was performed for selected NPs only in simulated body fluid (SBF) at pH 7.4.[7] In brief: 4mg of selected NPs was suspended in 8mL of SBF solutions and then shaken at 37°C with 100 RPM for 7 days. The degraded NPs were centrifuged at 14,000RPM at 4 °C for 15minutes and lyophilized further for morphology observations
through HRTEM. 0.2mg/mL of sodium azide was used to prevent any type of microbial contamination.

2.3.5. Computational Studies of NPs

2.3.5.1. Network Pharmacology

To find out the targeted proteins and genes, a Network Pharmacology study was performed. At first, the ADME parameters of drugs were determined in SwissADME (http://www.swissadme.ch/). 3D conformers of the drugs were retrieved from pubchem and .sdf files were subjected to SwissADME (http://www.swisstargetprediction.ch/) to find the enzyme targets of the compound with similarity index <0.7. The enzyme list was saved and processed in DisGeNET (https://www.disgenet.com/) to find protein targets related to TNBC. Further, based on NCBI (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE41678), GEPIA 2 (http://gepia2.cancer-pku.cn/)) and recent literatures,[8-10] more targeted proteins were listed and placed in STRING database (https://string-db.org/) to generate ‘x.tsv’ file. The ‘x.tsv’ files were run through Cytoscape 3.9.1 to generate the map for interrelated proteins for TNBC. Bioinformatics & Evolutionary Genomics site was used to draw the Venn diagram.

Additionally, pathways related to the targeted genes were drawn using Shiny GO 0.80 (http://bioinformatics.sdstate.edu/go/).

2.3.5.2. Molecular Docking Assessment

2.3.5.2.1. Preparation of Target Proteins and ligands

3D crystal structures of targeted proteins were retrieved from Protein Data Bank (PDB) and pre-processed by removing water molecules, ligand present, identical chains, and other heteroatoms listed in (**Table S3.1.1**). After that, polar H-atoms and Kollman charges were added followed by saving in 'x.pdbqt' format. Ligand structures (DHA (3000518), Piperine (638024) and Paclitaxel (36314)) were downloaded from the PubChem database in SDF format and energy minimization was performed using Avogadro Software (Universal force field) to ensure optimal conformation for docking study and saved in PDB format.

2.3.5.2.2. Docking Procedure

The docking studies were conducted in two steps: (1) single docking (protein with ligand) and (2) sequential docking (proteins with piperine followed by DHA and proteins with DHA followed by piperine). Blind docking was performed using AutoDock Tools 1.5.6. For single docking, the grid box centered on protein with a dimension to cover the complete protein. For sequential docking, one ligand was docked with protein and saved as a complex with 'x.pdbqt' format and further docked with another ligand by following the same steps. The parameters set here were GA run 50, population size 300 and saving the output file by selecting Lamarckian Genetic Algorithm (4.2) in 'protein.dpf' format. Further, 3D structures of the protein-ligand complex were visualized in Pymol and all types of interactions (polar hydrogen bonding and hydrophobic nonpolar interactions) between ligands and proteins were listed from the 'lig plot'.

2.3.5.3. Molecular Dynamics Simulation (MD)

Molecular Dynamics simulations study was performed using GROMACS-2020[11] to understand the micro-structure and dynamics of the p(NAG-*co*-NAPA)$_{wc}$ at different conditions. CHARMM36[12] force field was used to model the bonded and non-bonded interactions between the various atoms of the atomistically modelled copolymers and water molecules using the following equation:

$$U_{nb}(r_{ij}) = \sum \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} + \sum_{i=1}^{N} 4\epsilon_{ij}\left(\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - 2\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right), \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where the non-bonded interactions between i^{th} and j^{th} atoms, having partial charges q, well depth ϵ, distance r, and radius in the Lennard-Jones (LJ) 6-12 term used to treat the van der Waals' interaction σ. The bonded interaction energy contributions are bonds, valence angles, dihedral angles, improper dihedral angles, selected Urey–Bradley along with torsional correction map (CMAP) terms which are further expressed as eqn. no. (2):

$$U_b = \sum_{bonds} k_b(b-b_0)^2 + \sum_{angles} k_\theta(\theta-\theta_0)^2 + \sum_{UB} k_{UB}(r_{1-3} - r_{1-3;0})^2$$
$$+ \sum_{dihed}\sum_{n=1}^{N} k_n(1 + \cos[\![(n\varphi]\!] - \delta_n)) + \sum_{imp} k_\varphi(\varphi-\varphi_0)^2$$
$$+ CMAP \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

500 molecules of the co-polymers were randomly placed within the simulation box considering dimensions of 12 nm × 12 nm × 12 nm. The copolymer molecules were solvated with water, modelled as TIP3P[13] and adequate ions were added to attain 0.15 M NaCl concentration and maintain electroneutrality. To obtain a reasonable starting configuration, the energy of the systems was minimized using the steepest descent algorithm with F_{max} <1000 kJ mol^{-1} nm^{-1}. The short-range interactions cut off for electrostatics and van der Waal's forces of interaction was taken as 1.2 nm, and the long-range electrostatics were treated using the particle mesh

Ewald (PME) method.[14] Here, similar experimental temperatures (298 K, 310 K and 315 K) and pressure of 1 atm were maintained during simulations. The temperature and pressure were maintained during simulations using the velocity rescale thermostat with a coupling time of 0.1 ps and the Parrinello-Rahman barostat with a coupling time constant of 2 ps, respectively. The equation of motion was integrated using a leap-frog algorithm in which the updated position was calculated using a Verlet algorithm[11, 12] eqn. no. (3) and eqn. no. (4),

$$r(t+\delta t) = 2r(t) - r(t-\delta t) + \frac{f(t)}{m}(\delta t)^2 + O(\delta t^4) \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

and the updated velocity was calculated at the half-integer time step;

$$v\left(t+\frac{\delta t}{2}\right) = v\left(t-\frac{\delta t}{2}\right) + \frac{f(t)}{m}(\delta t) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

with a timestep 'δt' of 2 fs.

The production runs were performed for 100 ns, and configurations were stored in every 2 ps for further analysis of the structure dynamics. All these analyses were performed in GROMACS 2023.3-Homebrew software. For all the systems, thermodynamic equilibrium has been achieved, and the properties calculated are ensemble-averaged over the last 5-20 ns of the trajectory. During the production run, the variation of different thermodynamic and structural parameters is shown against time, as shown in **Figure S3.3.11**. The properties of interest for describing the copolymer system include radial distribution function (RDF), hydrogen bonding (HB), solvent-accessible surface area (SASA), mean square displacement (MSD) and number of contact groups. The visual inspection of the simulation results was carried out using VMD 1.9.4.[15] To ensure the reliability of the obtained results and statistical significance, all the simulations were run three times. The topology file for the copolymer system has been provided in **Figure S3.3.6**. The description of the systems is shown in **Table S3.3.1**.

2.3.5.4. Image processing tools

2.3.5.4.1. Processing of microscopic images through GLCM

The microscopic images acquired at predefined time intervals were analysed using image processing tool. The three channel based RGB format images were converted to single-channel grayscale format, where the pixel intensities are ranging from 0 to 255. To enhance the quality of the images, a Gaussian blur filter was used to remove the noises. Then, the blood vessels were segmented from the background by Gaussian adaptive threshold algorithm (eqn. no. 5) that contains only two intensity levels, 0 and 255 representing the blood vessels and background, respectively.

$$\text{Threshold}(\boldsymbol{x}, \boldsymbol{y}) = \frac{\mathbf{1}}{\boldsymbol{N}} \sum_{(\boldsymbol{x}', \boldsymbol{y}') \in \text{"}\boldsymbol{Neighborhood}\text{"}} \boldsymbol{I}\,(\boldsymbol{x}', \boldsymbol{y}') - \boldsymbol{C} \quad \text{.....(5)}$$

Where, (*x, y*) represents the pixel coordinates, N: number of pixels in the neighborhood around (*x, y*), I (*x′, y′*): the intensity value of the pixel at coordinates (*x′, y′*) within the defined neighbourhood and C: a constant that is subtracted from the average intensity of the neighborhood and the considered value of C here is 10.[16]

2.3.5.4.2. Quantitative measurement of angiogenesis using GLCM

GLCM texture based features were implemented on binary images to compute 7 different parameters related to the structure of blood vessels.[17, 18] We have employed the following mathematical equations to compute the angiogenesis caused by p(NAG), p(NAPA), and p(NAG-*co*-NAPA) NPs *in ovo* model and tube formation assay.[19-21]

1. Number of pixels in the blood vessels: It is the total number of black pixels in the segmented image that correspond to the blood vessels.

2. Entropy: Measures the randomness or complexity of the texture. An increase in blood vessel density and complexity typically to higher entropy and can be represented as,

   $\text{Entropy} = -\sum_{i=0}^{N-1}\sum_{j=0}^{N-1} P_{ij}\log(P_{ij})$ ... ... (6)

   Where, P_{ij} represents the probability (or normalized frequency) of the co-occurrence of pixel intensity values 'i' and 'j' at a certain spatial relationship defined by the distance and angle parameters.

3. Energy: Measures the textural uniformity or smoothness of the image. Higher blood vessel density may decrease the energy, indicating a less uniform and more complex texture. The energy can be represented as,

   $\text{Energy} = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} P_{ij}^2$ .... (7)

4. Contrast: Measures the amount of local variations in the image. An increase in contrast generally correlates with a higher density of blood vessels and can be represented as,

   $\text{Contrast} = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} (i-j)\,P_{ij}$ .... (8)

5. Mean: Represents the average gray level in the GLCM. In binary images, the mean might not change significantly with an increase in the blood vessel density, but spatial distribution might shift. The mean can be represented as,

   $$\mu_x = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} i \cdot P_{ij} \quad \text{..... (9)}$$
   $$\mu_y = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} j \cdot P_{ij}$$

   Where μ_x and μ_y represent the mean intensity of the pixel values corresponding to the first and second dimension (or axis) in the joint distribution, respectively.

6. Dissimilarity: Higher dissimilarity means more variation between neighbouring pixel values. This can indicate more pronounced blood vessels and can be represented as,

   $\text{Dissimilarity} = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} |i-j|P(i,j)$ .... (10)

7. Variance: Higher variance means greater intensity variation in the image, which could correspond to more varied blood vessels. The variance can be represented as,

$$\text{Variance} = \sum_i \sum_j (i-\mu)^2 p(i,j) \quad \text{..... (11)}$$

2.3.5.4.3. Hardware and Software used for angiogenesis analysis

The entire procedure was coded using Python 3.11.5. Considering the experimental environment, the central processing unit (CPU) and random-access memory (RAM) specifications were an AMD Ryzen 3 5300U with Radeon Graphics, 2600 MHz, 4 cores, 8 logical processors, and 8 GB RAM. Jupyter Notebook was used to run Python, Excel for statistical analysis, and OriginPro 2021 for plotting the raw data obtained from features and to fit the B-spline curves.

2.3.6. Drug Loading and Release (%) Determination

2.3.6.1. Loading of Drugs (Encapsulation Efficiency % (EE%) and Loading Content% (LC%))

DHA and piperine were encapsulated into the pores of selected NPs by vacuum loading method. Briefly: 200mg of drugs (DHA and Piperine) and 200mg of NPs (1:1) were separately dispersed in 8mL of ethanol followed by drop wise addition under continuous stirring at 4°C. After complete addition, final mixtures were placed in rocker and shaker with RPM 300 at 4°C for 24h. In between 24h, 4 cycles of vacuum (20:10 seconds i.e; on-off cycle) was given to achieve maximum loading efficiency. Further NFs were centrifuged at 14,000RPM for 5minutes at 4°C and then supernatants were collected and stored for calculating drug entrapment efficiency (EE (%)) and loading efficiency (LE (%)). Further, pellets were washed with MilliQ twice and centrifuged at 14,00RPM for 5minutes at 4°C to remove unloaded drug molecules, lyophilized and stored in airtight amber colour tubes at 4°C. The supernatant was

accessed for DHA (λ_{max}= 20nm) and Piperine (λ_{max}= 343 nm) loaded in the NPs by UV-Vis spectroscopy. The equations attached below (eqn. no. 12 and 13) were used to calculate EE (%) and LE (%) for respective NFs. Further, TGA was studied to confirm the loading of drugs in the NFs.

$$\mathrm{EE}(\%) = \frac{\text{Weight of entrapped drug}}{\text{initial drug weight}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (12)$$

$$\mathrm{LE}(\%) = \frac{\text{Weight of entrapped drug}}{\text{Weight of formulation}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (13)$$

2.3.6.2. *In vitro* release study

The dialysis bag method was followed to study the *in vitro* drug release % of NFs. In brief: 4mg of prepared NFs were added to 2mL of 1×PBS (pH 7.4) and solutions were placed in a 10,000kDa dialysis bag and immersed in a beaker containing 50mL of 1×PBS (pH 7.4). The beakers were placed in an orbital shaker with 100 RPM at 37 ±2°C for a period of 30 days (720h). At predefined time intervals, 3mL of supernatants were collected from beaker for UV-Vis analysis at λ_{max}=205 and 343 nm for DHA and piperine, respectively. Simultaneously, beakers were replenished with equal amount of fresh 1×PBS (pH 7.4). Release studies were also performed at pH=6.8, since the pH of the tumor microenvironment is in a similar level. All the release studies were performed in triplicates for statistical significance.

2.3.6.3. Mathematical Modeling

To predict the release kinetics, six different mathematical models were used to fit the release data (see supporting Eq. S3.2.1-S3.2.17). The models can be listed as Zero order, First order, Higuchi, Korsemeyer-Peppas, Hixon Crowell and Weibull. Among all models, particularly, Korsemeyer Peppas model is used for polymeric system which suggest the fickian or non-

fickian diffusion of drugs from the carrier. This drug release kinetics study is required to understand, predict and optimize the therapeutic doses precisely.

2.3.7. *In vitro* studies of NPs

2.3.7.1. MTT assay (Cell viability or Cytotoxicity assay)

The cell viability or cytotoxicity of the homopolymers and copolymer NPs were determined using four healthy cells (L929, Raw 264.7, HUVECs and PC12) and two cancer cell lines such as A549 and MDA-MB-231. DMEM supplemented with 10% of FBS and 100 U of penicillin-streptomycin antibiotic was used to culture the cells and maintained in incubator at 37°C with 5% CO_2. 1×10^4 cells/well were cultured in 96 well plate for 24h followed by addition of selected concentrations of NPs and NFs. After incubation, samples were removed and to each well 5 µg/mL of MTT reagent was added and kept for 4h. Further, MTT reagent was removed and 100µL of DMSO was added per well and incubated for 20minutes in the dark. Then the cell viability was estimated by recording absorbance at λ_{max} = 570nm using a microplate reader (Biotek, SYNERGY H1M) followed by using following eqn. no 14.

$$\%\ \text{Cell viability} = \frac{\text{OD (treatment)}}{\text{OD (control)}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(14)$$

2.3.7.2. Evaluation of IC_{50} and combination index (CI)

The IC_{50} values of developed NFs were determined using MTT assay. In case of Co-NPs, a ratio based concentration has been considered for the MTT study. Further, combination index for Co-NPs were determined by using Chou and Talalay method.[22] The synergistic or antagonist effect of Co-NFs on MDA-MB-231, have been studied using eqn. no. 15.

$$CI_x = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(15)$$

Where, $(D)_1$ and $(D)_2$ represent the concentrations of NF1 and NF2, respectively in Co-NPs with IC_{50} values and $(D_x)_1$ and $(D_x)_2$ represent IC_{50} values of individual NF1 and NF2, respectively.

2.3.7.3. Hemolysis Study

Hemolysis was conducted on RBCs collected from rat whole blood. Initially, RBCs were collected and suspended in DNS. To determine the hemolytic effect of copolymer NPs, 5×10^6 RBCs were incubated with all samples separately with different concentrations for 2h and 8h at 37°C with 100RPM. Incubation with distilled water and DNS were considered as positive and negative control, respectively. After incubation, samples were centrifuged at 3500RPM for 5minutes at RT. Then the supernatants were collected and hemolysis was calculated by recording absorbance at λ_{max} = 540nm followed by using the below mentioned eqn. no. 16 Experiments were performed with the same rat blood in three distinct tubes for each concentration of NPs.

$$\text{Hemolysis (\%)} = \frac{\text{OD (treatment)} - \text{OD (negative control)}}{\text{OD (positive Control)} - \text{OD (negative Control)}} \times 100 \dots\dots\dots\dots\dots (16)$$

2.3.7.4. Cell migration and proliferation (Wound Scratch assay)

The migration inhibitory effect of different NPs and NFs were performed with MDA-MB-231 cells by considering without treatment as negative control. Cells were cultured taking 2×10^5 cells per well with 800µL of complete media in 12 well plate and kept for 24h for 70-80% confluency with adhesion. Then a linear scratch wound was generated in each well by using a 20µL sterile tip followed by washing with 1X PBS three times. Then desired concentration of NPs (350µg/mL) was added to the wells. Further, images were acquired at 0 and 24h. Fiji ImageJ software was used to quantify change in width (%) and rate of migration (%).

2.3.7.5. Cell Cycle Study Through FACS

The cell cycle analysis was performed according to an earlier study with slight modification.[23] 2×10^5 cells per well of 6 well plate were cultured and kept for 24h. Then, the selected NPs and NFs at a desired concentration were added to the cells and incubated for 24h. Then, cells were trypsinized in PBS followed by centrifugation at 200×g for 5minutes. After discarding the supernatant, cells underwent fixation in 70% ethanol (-20°C) and incubated for 20minutes. Further, cells were centrifuged at 200×g for 5minutes and discarded the supernatant. 500µL of RNase A (200µg/mL stock) was added to each tube and incubated for 30 minutes. Then 500µL of PI (50 µg/mL stock) was added to each tube and incubated for 30minutes in dark (in ice). Finally, the samples were mixed thoroughly and analyzed through the Flow cytometer.

2.3.7.6. Apoptosis/ Necrosis Assay Through FACS

The apoptosis study was performed using annexin-v/PI kit (Cat. No. BMS500F1-100, Invitrogen). 2×10^5 cells per well of 6 well plate were cultured and incubated for 24h. Then, selected NPs and NFs at a desired concentration were added to the cells and incubated for 24h. Then, cells were trypsinized in PBS followed by centrifugation at 200×g for 5minutes. After discarding the supernatant, 5µL of annexin-V and 10µL of PI were added to each tube, mixed thoroughly and incubated in dark for 30minutes. Finally, 400µL of binding buffer was added to each tube and performed Flow Cytometer for apoptosis study (CytoFlex B53000, Beckmann Coulter).

2.3.7.7. Determination of Intracellular ROS generation and Effect On Mitochondrial Membrane Potential (MMP) Through FACS

Flow cytometry analysis was also conducted to quantify the intracellular ROS and change in

mitochondrial membrane potential. After 24h of treatment with NPs and NFs at desired concentration and untreated as control, the adhered cells were collected (1200RPM) and washed with 1×PBS for 3 times. Then cells were stained with 50µM solution of DCFH-DA and incubated at 37℃ for 30minutes in dark. Further, the supernatant was removed and washed with 1×PBS for two times and suspended in 500µL of 1×PBS. The shifting of bands and intracellular ROS generation were analyzed. Similarly, for mitochondrial membrane potential measurement, instead of DCFH-DA, Rhodamine-123 was used and all other steps were followed similar to the measurement procedure of ROS.

2.3.7.8. Cellular Internalization Mechanism Through FACS

To investigate the quantitative cellular uptake and NP's internalization mechanism, endocytosis inhibitor assays were performed using standard endocytosis inhibitors such as Chloropromazine (30µM), Nystatin (25µM) and Amiloride Hydrochloride (1.5mM). $10x10^5$ MDA-MB-231 cells were seeded on a 6-well plate for 24h. Then, cells were treated with desired concentration of inhibitors for one hour and without any treatment were considered as positive control. After the treatment, cells were further treated with 50 µg/mL of rhodamine B loaded P4 NPs for 6h. Then, cells were washed, trypsinized and collected in PBS. Finally, the uptake was quantified using Beckmann Coulter BD flow cytometer.

2.3.7.9. Cellular Uptake Through Confocal Microscopy

The cellular uptake of different NPs and NFs by MDA-MB-231 cells was assessed qualitatively using Confocal microscopy. $1×10^5$ cells per well were cultured over the microscopic cover glass (BLUE STAR) with 800µl of complete media in a 12-well plate and incubated for 24h for 70-80% confluency and adhesion. Then, samples of 280µg/mL were added to the designated wells, and cells were re-supplemented with fresh media. After 24 h of

incubation, cells were fixed with 400µl of 4% paraformaldehyde solution per well and left for 30 minutes. Then, cells were treated with 0.1% Triton-X for permeabilization for 30 minutes followed by blocking with 5% BSA for 30 minutes. Further, cells were incubated with 1° antibody (α-tubulin, 1:3000) for 12h at 4°C and 2°antibody (Phalloidin, 1: 30,000) for 1h 4°C. Next, cells were stained with DAPI, and MitoSOX (2µl per well from 10mg/mL) and finally, slides were prepared using DABCO as a mounting agent. Note that, in each transition step, cells were washed three times with 1X PBS (1 mL per well). The slides were placed used to acquired images of the Cells (Zeiss Confocal microscope (LSM 80, Axio Imager Z2)) and the images were analyzed using Fiji image j software.

2.3.7.10. Estimation of extracellular MMP-2 and MMP-9 expressions through Gelatin Zymography

Gelatin zymography study was performed to estimate the matrix metalloproteinases (MMP-2 and MMP-9) expressions in extra cellular matrix protein by using gelatin as a substrate. The experiment is followed as per the earlier reports.[24] In brief: 10×10^5 MDA-MB-231 cells were seeded and treated with NPs and NFs at a desired concentration for 24h. Then, the supernatant or conditioned media was collected and 30µg of each sample was loaded on SDS-PAGE containing gelatin co-polymerized with 7.5 % polyacrylamide based resolving gel. After complete running, gels were rinsed thrice with washing buffer followed by incubation in incubation buffer for 20h at 37 °C. Then, gels were stained with 1% CBBR-250 staining solution for 1h followed by destaining until digested regions of MMP-2 and MMP-9 are appeared as a white band against a black background. Images were captured in Chemi-DOC system (G:BOX, SYNGENE).

2.3.7.11. Reverse Transcriptase polymerase chain reaction (RT-PCR)

To check the relative gene expression responsible for TNBC, RT-PCR experiment has been performed for all samples at desired dose. After 24h of incubation of MDA-MB-231 cells with samples, cells were collected from each well. Total RNA were isolated using 1 mL of TRIZOL followed by 200µl chloroform and 500µl isopropanol. The RNA pellets were washed with 70% ice cold ethanol for 2 times and suspended in DEPC-treated water. Afterward RNA was quantified. Then 1µg of RNA sample was used for cDNA synthesis using oligo dT sequence and reverse transcriptase polymerase as per the manufacturer's protocol.

RT-PCR (QuantStudio 5, Applied Biosystems) was setup using diluted cDNA samples (1:100) in a final volume of 20 µL using PowerUpTM SYBRTM GREEN Master Mix (Invitrogen) and different optimized concentrations of KiCqStart® primers (sigma) for biomarker target genes such as MKi67IP, STAT3, DNMT3B, EGFR, BcL2, CDK2, CASP9 and GAPDH as housekeeping gene using Taq polymerase (TAKARA R001A). The primer sequences of the selected genes are listed in **Table 2.1**.

Table 2.1. List of primer sequences of selected genes for RT-PCR study of MDA-MB-231 cells treated with NPs.

Gene Name	Primer Sequence
MKi67IP	5'-TACAAAAGGCCAGGTTTTAC-3' 3'-CTTTTAGAGTTCACCGACTT-5'
STAT3	5'-GGTACATCATGGGCTTTATC-3' 3'-CTAAGTCACTTTCGTCGTTT-5'
DNMT3B	5'-CTTACCTTACCATCGACCTC-3' 3'- CTGTCAAGTCTCATAGTCCTA-5'
EGFR	5'-AGTGCCTGAATACATAAACC-3' 3'- CGTCTCTGGGTGTGATG-5'
BcL2	5'-GATTGTGGCCTTCTTTGAG-3' 3'- CCTACGGAAACACCTTG-5'
CDK2	5'-TGTTATCGCAAATGCTGC-3' 3'- CTGAGACTATCGGAAGAAC-5'
CASP9	5'-CTCTACTTTCCCAGGTTTTG-3' 3- GATTACGACAAAGCACTTT-5'
GAPDH	5'-TCGGAGTCAACGGATTTG-3' 3'-GAGACCATTTCACCTATAACAAC-5'

Firstly, 10minutes activation at 95°C, then 40 repetitive cycles of denaturation and annealing at 95°C for 15s and 55°C for 60s, followed by melting (95°C for 15s and 60s at 55°C). RT-PCR experiments were performed in triplicates for all samples with control. The fold change in selected genes expressions with respect to control was calculated using comparative Ct method.[25]

2.3.7.12. Tube formation assay

At first, 50µL of Geltrex, growth factor-reduced basement membrane matrix (Gibco, Cat. No. A14132) was used to coat a 96-well cell culture plate, which was subsequently incubated at 37°C for 30minutes to facilitate matrigel polymerization. Following this, isolated primary HUVECs (15×10^3 cells/well) (Himedia, Cat. No. CL002) were seeded onto the wells coated with matrigel. They were cultured at 37°C for 6h in the presence of p(NAG-*co*-NAPA)$_{(1:1)}$ (P1) NPs (1, 10, and 100µg/mL) to observe the tube formation. The formation of the tubular structures was monitored by calcein AM (ThermoFischer Scientific, Cat. No. C3099) stain. Calcein-AM (1:2000) was added to each well, incubated for 15minutes, and then observed using a fluorescence microscope (Evos FL Auto). Images were analyzed using Angiotool (ImageJ) and GLCM texture features based Image processing tool.

2.3.8. *In ovo* Studies of NPs

2.3.8.1. Chorioallantoic Membrane (CAM/CEMA) Assay

Fertilized chicken eggs were purchased from a trusted and certified vendor (Ramana Hatchery, Varanasi, Uttar Pradesh, India) and incubated in an egg incubator (37 °C, 50-55% RH) up to 4 days before the experiment. On the day of experiment, using a light-shadow approach, the eggs were tested to check whether embryo-genesis had occurred or not. Then, 1-

2 mL of albumen was removed for the detachment of the developed chick embryo chorioallantoic membrane, and a small window was created on the top of the egg-shell. Further different concentrations of desired NPs were prepared in PBS 7.41, and PBS as a control were added and examined upto 24 h. The images were captured at different time intervals (0, 2, 4, and 8h) using a stereo zoom microscope-mounted Magnus camera (Magcam DC Plus 10, Magnus Opto Systems India Pvt. Ltd.) at a resolution of 10 megapixels and analysed using the Angio tool and Fiji ImageJ software.

2.3.9 *In vivo* studies of NPs

2.3.9.1. Animal Handling and Ethics

All animal experiments were carried out following the guidelines of CPCSEA and approved by the Institutional Animal Ethical Committee (IAEC) of IIT (BHU), Varanasi, Uttar Pradesh, India (regd. no. 2123/GO/Re/S/21/CPCSEA). The animal ethical approval reference No. are IIT(BHU)/IAEC/2024/I/049 and IIT(BHU)/IAEC/2023/048.

2.3.9.2. Bio-distribution Studies

The *in vivo* biodistribution of all NPs were studied using DiD dye. Initially, 2µL of dye (1 mg/mL in DMSO) was incubated with NPs suspension (1×PBS) for 24h in dark with a constant stirring of 10RPM. The NPs suspension was centrifuged for 5minutes at 14,000 RPM followed by removal of supernatant, washing of pellet, and re-suspended in PBS for bio-imaging studies. Swiss albino mice (female) were divided into seven groups with n = 3 and were anaesthetized by I.P. injection of thiopentone sodium at a dose of 0.2 mg/kg of body weight, followed by injection of 200µL of DiD loaded NPs through the tail vein. After administration of NPs, images were acquired at 1, 6, 12 and 24 h by using IVIS imaging system (PHOTONIMAGER

OPTIMA, Biospace Lab, France) at excitation/emission maxima of 637/672nm in near infrared (IR) region.

2.3.9.3. Dermal Irritation Study

To check any type of irritation and erythematous reactions, p(NAG-*co*-NAPA)$_{wc}$ NF was applied on the Wistar rat's skin and observed for 48 h. For this study, ~4 cm^2 rat skin was shaved, and p(NAG-*co*-NAPA)$_{wc}$ NF was applied with formalin (a common irritant) considered as a control. The treated area was wrapped with a bandage and kept for 48 h. After 48 h, bandages were removed, washed with sufficient amounts of water, and visually examined for any kind of irritation and sensitization. Additionally, high-resolution images were captured and compared with images acquired before treatment. Further, examined rats were kept under observation for checking the edema and erythema. To calculate the skin irritation score, Draize scoring system[26-28] (**Table S3.3.4**) was used with the primary dermal irritation index (PDII) through eqn. no. (17). Further tissue samples were collected for histology.

$$PDII=PDI/4 \quad \text{.....(17)}$$

If, PDII is '0', then it indicates no irritation, 0.5–1.9 indicates modest discomfort, 2–4.9 indicates mild irritation and values above 5 indicates severe irritation.

2.3.9.4. Wound Healing Study

The treatment efficiency of p(NAG-*co*-NAPA)$_{wc}$ NF for wound healing was evaluated using Wistar rats. Initially, 3% isoflurane was used to anesthetize the rats, followed by the removal of hair at the back using a trimmer. Then, lidocaine, a local anaesthetic, was applied at the target site. An 8-10 mm full-thickness cutaneous wound was generated at the shaved site by using a sterile 8 mm biopsy punch. Cleaned the wound region with sterile cotton and topically treated with ~50-60 mg of p(NAG-*co*-NAPA)$_{wc}$ NF on each alternative day (0^{th} to 12^{th}, once in each

alternative day). Wounded regions were covered with a conventional sterile gauze bandage. The dressing was replaced with a fresh bandage after each treatment. Similar steps were followed for the control group for the base as well.

2.3.9.5. Histological Analysis

For histology study, on the day 15th of post-treatment, from cicatricial tissue, deep granulation tissue and cross-sectional full-thickness skin specimens were collected. Then specimens were fixed and embedded in formalin and paraffin wax, respectively. Blocks were further sectioned with a thickness of 5μm in the transverse plane. The sections were stained with hematoxylin and eosin, mounted on glass slides, followed by analysis using an optical microscope at 20x magnifications.

2.3.9.6. Immunochemical Parameters

For immunochemical analysis, the blood serum sample was isolated from rats. The blood samples were collected in three different days (0^{th}, 7^{th} and 14^{th} day), which represented distinct phases of the wound healing cycle and were stored at −80°C for further processing. Immune levels markers such as IL-1β, IL-6, CRP, TNF-α and IGF-1 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The relative protein concentrations were estimated by recording the absorbance at 450nm and compared with the control samples.

2.3.9.7. Reverse Transcriptase polymerase chain reaction (RT-PCR)

On the 14th day of post treatment, nearly 1 mm thickness of tissues were collected from excised wounds and also normal skins from all the groups, respectively. RNA were isolated by homogenizing tissue samples in 1mL of TRIAZOL reagent followed by chloroform extraction.

Then aqueous layer was collected and RNAs were precipitated in 1mL of isopropyl alcohol and centrifuged (12,000g) at 4°C for 15minutes. The RNA pellets were washed with 70% ice cold ethanol for 2 times and suspended in DEPC-treated water. Afterward RNA was quantified. Then 1µg of RNA sample was used for cDNA synthesis using oligo dT sequence and reverse transcriptase polymerase as per the manufacturer's protocol. RT-PCR (QuantStudio 5, Applied Biosystems) was setup using diluted cDNA samples (1:100) in a final volume of 20 µL using PowerUpTM SYBRTM GREEN Master Mix (Invitrogen) and different optimized concentrations of KiCqStart® primers (sigma) for biomarker target genes such as KDR, VEGFA, PECAM-1, IL-1β, TNF-α and GAPDH as housekeeping gene using Taq polymerase (TAKARA R001A) with 40 repetitive cycles of denaturation at 95°C for 25s, annealing for 30s at 60°C, extension at 72°C for 35s and final extension for 7minutes at 72°C. RT-PCR experiments were run in triplicate for all samples with control. The fold change in selected genes expressions with respect to control was calculated using comparative Ct method. The primer sequences of selected genes are listed in **Table 2.2**.

Table 2.2. List of primer sequences of selected genes for RT-PCR study of NPs treated tissue sample.

Gene Name	Prime Sequences
KDR	5'-AAACTGGATAAAATGGGCG-3' 3'-AGCCTTTTAGGTAGAGTCAG-5'
VEGFA	5'-CTCATCTCTCCTATGTGCTG -3' 3'-GATAGAGTATATCTTCAAGCCG-5'
PECAM-1	5'-AAAACCACAATTGAGTACCAG-3' 3'-ACTTAGCTTGACGTTCTTTG-5'
IL-1β	5'-GGATGATGATGATAACCTGC-3' 3'- CATGGAGAATATCACTTGTTGG-5'
TNF-α	5'-CTATGTCTCAGCCTCTTCTC-3' 3'- CATTTGCCAACTTGCCATCC-5'
GAPDH	5'-TCGGAGTCAACGGATTTG-3' 3'-GAGACCATTTCACCTATAACAAC-5'

2.4. STATISTICAL ANALYSIS

One-way ANOVA with independent Student's t-test was used to evaluate the *in vitro* and *in vivo* data statistically. The significance was calculated by considering $p<0.05$ and represented as a mean (±) SD or unless otherwise stated.

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