CHAPTER 2: MATERIALS AND METHODOLOGIES

2.1 MATERIALS

The list of chemicals and reagents used in this work has been listed in Table 2.1.

Table 2.1: The following materials were used during the execution of this project work.

S. No.	Name of chemical and reagent	Molecular Weight	Purity (%)	Manufacturer
1.	Glycine	75.07	98.00	Sigma Aldrich, Germany
2.	Hexadecane anhydrous	226.445	99.00	
3.	KiCqStart® SYBR® Green primers			
4.	Acryloyl chloride (stabilised with 400 ppm phenothiazines)	90.51	96.00	Alfa-Aesar,
5.	Divinylbenzene	130.190		
6.	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Methyl thiazole tetrazolium (MTT))	414.32	99.90	Himedia
7.	Dialysis membrane (MWCO 12kDa)			
8.	TRIzol Reagent			
9.	Phosphate Buffer Saline			
10.	SDS	288.38	90.00	Merck
11.	Chloroform extra pure	119.38	99.00	
12.	DMSO	78.13	99.70	
13.	KBr	119.002	99.00	
14.	Ethyl acetate	88.11	99.50	
15.	1,4-dioxane	108.14	99.60	
16.	2,2-azobisisobutyronitrile (AIBN)	164.21	98.00	SRL
17.	AgCl	143.32	99.99	
18.	$NaBH_4$	37.83	98.00	
19.	Sodium Nitroprusside dihydrate	297.95	98.00	
20.	Glycerol	92.09	83-85	
21.	Petrolatum	209.29	98.00	
22.	Paraffin wax			
23.	Mineral oil			
24.	Isopropyl alcohol	60.10	98.80	
25.	Dulbecco's modified eagle medium			Gibco
26.	Fetal bovine serum (FBS)			
27.	Penicillin-streptomycin cocktail			
28.	Gentamicin			
29.	ZnO			Nice
30.	$SnCl_2.2H_2O$	225.64		
31.	Griess reagent			Invitrogen
32.	Taq polymerase			Takara R0001A
33.	Diethyl polycarbonate (DEPC) water			
34.	Ultrapure water			

2.2 METHODOLOGIES

2.2.1 Synthesis and Preparation of PNAG and SP NPs

2.2.1.1 Synthesis of N-acryloyl glycine (NAG) monomer

N-acryloyl glycine monomer (NAG) was synthesised through a modified approach [1-3]. In brief: 8 gm of glycine were dissolved in a cold 100 mL of 2M KOH solution by stirring in a round bottom flask in an ice bath (designated solution 'A'). 20 mL of cold 1,4-dioxane was mixed with 8 mL of acryloyl chloride (designated solution 'B'). Further, Solution 'B' was slowly added to cooled solution 'A' at 0 °C (ice bath) for 2 h, under vigorous stirring (RPM~600) followed by 12 h of incubation at room temperature(25 °C) with stirring. The pH of the mixture kept pH above 12 by gradually adding 2M KOH. After 2 h of continuous stirring at room temperature (25 °C), the mixture was rinsed thrice with 20 mL of diethyl ether. Then the mixture was saturated with NaCl after being acidified with 5M HCl to achieve pH 2, obtained aqueous solution extracted by 100 mL of ethyl acetate, followed by 3-4 times. The ethyl acetate layer was dried by adding anhydrous magnesium sulphate and then filtered, and the monomer is obtained by evaporating ethyl acetate solvent using a rotary evaporator at reduced pressure. From a cooled combination of diethyl ether: ethyl acetate (1:1), with melting temperature 132-134 °C, 10.2 gm of crude product (NAG) (yield ~63.75%) was crystallised out.

2.2.1.2 Preparation of polymeric NAG nanoparticles (PNAG NPs)

The polymeric NAG nanoparticles (PNAG NPs) were synthesised by the mini-emulsion radical polymerisation technique as described in our previous reports [1, 2] with modifications according to the need. Briefly, NAG monomer (500 mg) and hexadecane (45 mg) were dispersed in toluene (5 mL oil phase) to form an emulsion of monomer, where the monomer is present here as dispersed phase (droplets). To this emulsion, an organic soluble radical initiator, AIBN (10 mg), is added and sonicated for 3 minutes on

a bath sonicator (Elmasonics, S 30H, Germany), which allows radical polymerisation within the oil/monomer droplets. Further, cross-linking agent divinyl benzene (DVB) is added and sonicated to increase the stability of nanoparticles and to impart cross-linked net-like structure, porosity and elasticity of nanoparticles. Hexadecane (HD) is a long-chain alkane used as a co-stabiliser. In another vial, 6 mg sodium dodecyl sulphate (SDS) was dissolved in 1.6 mL H_2O using an ultrasonic bath sonicator at room temperature. Then the SDS solution was added dropwise under vigorous stirring.

Further, the reaction mixture was sonicated for another 5 mins using an ultra-probe sonicator (750W, 30% power, 45:15 cycles) at 25 ℃. The obtained emulsion was kept at 80–85 ℃ in an oil bath for complete polymerisation for 12 h with continuous stirring; stable nanoparticles were formed, and the residual toluene was removed by heating the mixture in the presence of an excess amount of water, a process sometimes referred to as hydrothermal evaporation. The nanoparticles suspension was separated using a cooling centrifuge at 14000 RPM followed by freeze-drying.

2.2.1.3 Preparation of sodium nitroprusside (SNP) loaded poly-(N-acryloyl glycine) nanoparticles (SP NPs)

SNP-loaded poly(N-acryloyl glycine) nanoparticles (SP NPs) were fabricated in two steps, firstly Poly (N-acryloyl glycine) nanoparticles (PNAG NPs) were synthesised from N-acryloyl glycine (NAG) monomer, and then sodium nitroprusside (SNP) was loaded into these NPs. The details of the formation of the nanomedicine have been filed for the Indian patent (Patent Application No.: 02311051276, date of filing: 31-07-2023). Loading methodology is described further in the section 2.6.1 of this chapter.

2.2.2 Synthesis of Metal Oxides Nanoparticles

2.2.2.1 Synthesis of Zinc oxide (ZnO) NPs

In summary, a solution was prepared by combining 10 mmol of $ZnCl_2$ with 50 mL of

alcohol while stirring continuously. Concurrently, an ice bath was employed to add 20 mmol of $NaBH_4$ to 10 mL of alcohol. Subsequently, a $NaBH_4$ solution was introduced dropwise at a rate of 1 drop/2-seconds into the $ZnCl_2$ solution, maintained under continuous stirring at 300 RPM. The observable transition in color from transparent to milky white signified the completion of reaction. The resulting mixture underwent centrifugation at 8000 RPM, followed by three washes with deionized (DI) water and subsequent drying overnight at 80 ℃. The resulting product is denoted as ZnO NPs [4].

2.2.2.2 Synthesis of Zinc-Copper bimetallic oxide (ZnO-CuO) NPs

In summary, a mixture comprising 10 mmol of both $ZnCl_2$ and $CuCl_2.2H_2O$ was introduced into 50 mL of alcohol while being continuously stirred (300 RPM) at room temperature (25 ℃). Concurrently, 50 mmol of $NaBH_4$ was dissolved in 20 mL of alcohol, initiating an exothermic reaction on an ice bath at room temperature. The resulting solution was then added dropwise at a rate of 1 drop/2-seconds to the initial mixture. The color transitioned from greenish to a dark green, resembling a shade of black indicates completion of reaction and formation of nanoparticles. Subsequently, the solution underwent centrifugation, followed by three washes with DI water, and was finally dried overnight at 80 ℃. This resulting sample is identified as ZnO-CuO NPs [4].

2.2.2.3 Synthesis of Zinc-silver bimetallic oxide (ZnO-Ag_2O/Ag) NPs

In summary, equal amounts of silver and zinc chloride salts were employed, following the above mentioned procedure. Upon the gradual introduction of a $NaBH_4$ solution, a noticeable transition in color from dark green to a greenish-black hue was noted, indicator of completion of reaction. The resulting solution underwent centrifugation, simultaneously underwent three washes with deionized water, and residue was subsequently dried overnight at 80 °C. The resulting product is referred to as ZnO-Ag_2O/Ag nanoparticles [4].

2.2.2.1 Synthesis of Zinc-Tin bimetallic oxide ($ZnO\text{-}SnO_2$) NPs

In summary, 10 mmol of zinc and stannous chloride salts were utilized, employing the identical procedure as above described earlier. Upon introducing the $NaBH_4$ solution, the initially milky transparent solution transitioned to a brownish hue and eventually to greyish an indicator of nanoparticle formation. Subsequently, the solution underwent centrifugation and three washes with DI water and residue was left to dry overnight in oven at 80 ℃. The resulting sample is identified as $ZnO\text{-}SnO_2$ NPs [4].

2.3 CHARACTERIZATION

2.3.1 Chemical Group Confirmation

The synthesized NAG monomer and PNAG NPs were chemically evaluated using ninhydrin test for the the free $-NH_2$ group which is initially present in glycine amino acid and also evaluated for free -COOH group available in PNAG NPs using litmus paper test.

2.3.2 Fourier Transform Infrared (FTIR)

The Fourier Transform Infrared (FTIR) spectroscopy experiment is a powerful analytical technique used to study the vibrational modes of molecules. NAG monomer and PNAG NPs have been prepared and mixed with IR-grade potassium bromide (KBr), and a pellet has been prepared. IR spectra were acquired at 25 ℃, from 4000 cm^{-1} to 400 cm^{-1} wavelength range, at a resolution of 16 cm^{-1} and 128 scans in % transmittance mode on FTIR (Nicolet iS5, THERMO Electron Scientific Instruments LLC.)

2.3.3 Nuclear Magnetic Resonance (NMR)

The Nuclear Magnetic Resonance (NMR) spectroscopy experiment is a powerful analytical technique used to study the nuclear environment of atoms in a sample. NMR spectra were acquired using an NMR spectroscope (AVH D 500 AVANCE III HD 500 MHz OneBay NMR Spectrometer, BRUKER BioSpin INTERNATIONAL AG) by dissolving NAG and PNAG samples in DMSO-D6 and $CDCl_3$, respectively. TMS was

used as an internal standard.

2.3.4 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) is a powerful imaging technique used to study materials' internal structure and morphology at the nanoscale. Ultra-thin samples (typically less than 100 nanometers thick) of PNG NPs and SP NPs and metal oxides were prepared, allowing electrons to transmit through the material. The sample was stained with phosphotungustic acid (PTA), which is a negative stain, used in electron microscopy. The sample is mounted on a copper TEM grid placed into the sample holder and loaded into the TEM column (Tecnai G2 20 TWIN, FEI Company of USA (S.E.A.) PTE, LTD). Imaging started with low magnification to locate the region of interest on the sample and moved forward to increase the magnification to acquire high-resolution images.

2.3.5 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is a powerful technique used to study the surface morphology and composition of materials at the micro- to nanoscale. The methodology written below has been adopted for conducting an SEM experiment:

Method: SP NPs were dispersed in isopropyl alcohol stained with PTA and fixed onto a stable substrate or mounted on a conductive stub such as silicon wafers. Coating has been done with a thin layer of conductive material (e.g., gold, palladium) using a sputter coater to prevent charging during imaging. The sample is loaded by mounting on the specimen stage secure by using appropriate holders or clamps and finally inserted into the SEM chamber (Nova Nano SEM 450, FEI Company of USA (S.E.A.) PTE, LTD). Imaging started with low magnification to locate the region of interest on the sample and moved forward to increase the magnification to acquire high-resolution images. Energy Dispersive X-ray Spectroscopy (EDS: 51N1000 – EDS Syste, Oxford Instruments Nanoanalysis.) is also conducted to analyse the elemental composition of SP NPs.

2.3.6 Dynamic Laser Scaterring (DLS) and Zeta Potential Estimation

Dynamic Light Scattering (DLS) and zeta potential measurements are commonly employed techniques for characterizing polymeric nanoparticles. PNAG NPs and SP NPs were dispersed in PBS (pH 7.4) and diluted (0.1mg mL^{-1}) to ensure an optimal scattering intensity for DLS and zeta potential measurements conducted at 25 ℃ using zeta sizer instrument (Nano ZS series, Zetasizer, Malvern, UK).

2.3.7 X-ray Diffraction (XRD)

Benchtop X-ray diffraction (XRD) is a powerful technique used for analyzing the crystallographic structure of materials. The following is a general methodology that has been adopted for conducting XRD experiments on NAG, PNAG NPs, SNP, SP NPs and metal oxides.

Methodology: Finally divided sample of NAG, SNP, PNAG, SP NPs and metal oxides was loaded on a sample holder with the help of clean tools after the stabilization of the XRD instrument (Rigaku Miniflex 600 Desktop X-Ray Diffraction System, RIGAKU Corporation). Measurement parameters, including the range of 2θ angles (5-80°) and scan speed (2°/min) were set accordingly.

2.4 *IN VITRO* CELL CULTURE STUDIES

2.4.1 Cell Viability: MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a widely used colourimetric assay to measure cell viability and proliferation. Here is a general methodology for conducting an MTT assay:

Cell Culture and seeding: Cell lines (L929, HUVEC and PC12) have been cultivated in an appropriate culture medium (DMEM+FBS) under standard conditions (37 °C, 5% CO_2, humidified atmosphere). The cells were passaged to maintain the logarithmic growth phase. To obtain accurate and precise results the experiments were conducted in

the exponential growth phase. Cells were seeded into 96-well plates or other well number plates at a predetermined density ($1x10^3$ or $1x10^5$) based on the cell type and experimental requirements and allowed to adhere and proliferate for a specified period, typically 24 h.

Treatment and incubation: The cells were treated with experimental compounds such as NAG monomer, PNAG NPs, SNP, SP NPs and metal oxides or other substances (positive or negative controls) as needed, also appropriate controls were included such as untreated cells and vehicle controls. The cells were then incubated for the required time.

MTT solution preparation and incubation: MTT stock solution is prepared in phosphate-buffered saline (PBS) or complete cell culture medium at a concentration of 5 mg mL^{-1} and sterilized using a 0.22 μm syringe filter and stored protected from light. Culture medium from each well was removed and added MTT solution to each well at a final concentration usually ranging from 0.2 to 0.5 mg mL^{-1}, and then cells were incubated for a specified period (typically 2-4 h) at 37 °C.

Formazan crystal formation: After inoculation the MTT solution was removed carefully, ensuring not to disturb the formazan crystals formed by viable cells and optionally, the cells were washed with PBS to remove excess MTT.

Formazan solubilization: The formazan crystals solution was made by solubilizing in dimethyl sulfoxide, and DMSO to each well which dissolved the formazan crystals. The plates were mixed thoroughly on a shaker or by pipetting to ensure complete dissolution.

Spectrophotometric reading: The absorbance of the formazan solution was measured at a wavelength typically between 540 nm and 590 nm using a microplate reader and a blank well with only culture medium and MTT have been included to correct for background absorbance. The following Equation 2. 1 is used to estimate the viability of cells

$$Cell\ Viability\ (\%) = \frac{OD\ Treatment}{OD\ Control}\ x\ 100 \qquad \textit{Equation 2. 1}$$

2.4.2 Cell proliferation and migration: Scratch wound assay

The scratch wound assay, also known as the cell migration assay or wound healing assay, is a widely used technique to study cell migration and wound closure *in vitro*. The following general methodology has been adopted for conducting a scratch wound assay using cell culture (Section 2.4.1).

Cell seeding: Cells were seeded into a culture dish or multi-well (6 or 12-well) plate at a density that allows them to reach confluence within 24 h. Either by using a culture insert or a template to create a defined scratch/wound area. This can also be achieved by removing a portion of the cell monolayer.

Wounding: After cells have reached confluence, carefully the culture inserts or template was removed or a scratch was made with the help of a sterile pipette tip to create a straight, consistent scratch/wound. Optionally, the cells were washed with PBS to remove detached cells and debris.

Treatment and incubation: The cells were treated with the desired dose of experimental compounds such as NAG monomer, PNAG NPs SP NPs, or other substances (metal oxides) as needed, also appropriate controls were included such as untreated cells and vehicle controls. The cells were then incubated for the required time.

Image acquisition (Time 0): Immediately, after treatment, the images of the scratch/ wound area were captured at time zero (right after wounding) using a phase-contrast microscope or an automated imaging system and a marked reference point has been used to ensure consistent image acquisition across different time points.

Cell culture: Again, the cells returned to the incubator for the desired duration, typically up to 24 or 48 h and standard cell culture conditions were maintained.

Image acquisition (Later time points): The additional images of the scratch/wound at predetermined time points (e.g., 24 h or 48 h) using the same settings as in the previous

step were captured.

2.4.3 Live/dead assay

A live/dead assay using fluorescent dyes like acridine orange (AO) and propidium iodide (PI) is a common method to distinguish between live and dead cells based on their membrane integrity. By adopting the above protocol of cell culture (Section 2.4.1), the live/dead assay on the L929 cell line in *in vitro* condition was conducted using acridine orange (AO) and propidium iodide (PI) method [5] to verify the viability of cells in the presence of NPs. Cells were incubated with AO (1.5 μM) and PI (31.5 μM). Following a 30-minute incubation period, specimens underwent a meticulous PBS washing procedure. Subsequently, cellular scrutiny and imaging were conducted utilizing a state-of-the-art fluorescent microscope (Nikon, A1, Tokyo, Japan), capturing high-resolution images.

2.5 HAEMOCOMPATIBILITY STUDY

2.5.1 Haemolysis assay

A haemolysis assay is a laboratory test used to assess the ability of a substance to cause the rupture of red blood cells (haemolysis). The following general methodology for conducting haemolysis assay has been adopted.

Methodology: Preparation of RBCs suspension: Blood was collected in blood collection tubes containing appropriate anticoagulants such as heparin or ethylenediaminetetraacetic acid (EDTA) to prevent blood clotting, from rats using sterile procedures. RBCs from whole rat blood was separated by centrifugation method. The collected whole rat blood was centrifuged at a low speed (e.g., 200-300 g) for 10 minutes to separate red blood cells (RBCs) from plasma. Plasma was discarded and the RBCs were washed two to three times with PBS to remove any remaining plasma or anticoagulant. RBSc suspension was prepared by resuspending the them in PBS or an appropriate buffer to achieve a 2-5% hematocrit. Hematocrit was determined using a

haemocytometer or automated cell counter.

Positive control preparation: Positive control, such as distilled water or 0.1% Triton X-100, is used to induce complete haemolysis (100% haemolysis) of RBCs.

Treatment and incubation: The samples (PNAG, SP NPs and metal oxides) to be tested is mixed with the RBCs suspension and incubated for desired time period (24 h) and shake the samples gently if needed to ensure proper mixing. Appropriate positive control (containing distilled water or Triton X-100) and negative control (containing only RBCs and buffer) were also included in the study.

Centrifugation: After incubation, the samples were centrifuged at low speed to pellet intact RBCs and any cell debris and supernatant was collected which contains haemoglobin released from lysed RBCs. This supernatant will be subjected for absorbance measurement in subsequent step.

Absorbance Measurement: The absorbance of the supernatant was measured at a wavelength around 540 nm using a spectrophotometer. A higher absorbance indicates greater haemolysis. The percent haemolysis was calculated using the Equation 2. 2.

$$Haemolysis\ (\%) = \frac{OD\ treatment - OD\ (-ve)\ control}{OD\ (+ve) control - OD\ (-ve)\ control}\ x\ 100 \quad \textit{Equation 2. 2}$$

2.5.2 Blood Coagulation Study

Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) assays are laboratory tests used to assess the intrinsic and extrinsic coagulation pathways, respectively. These assays are commonly employed to evaluate clotting function and monitor anticoagulant therapy. The impact of NPs on the blood coagulation cascade was assessed by evaluating 2 blood coagulation pathways. After incubating plasma with NPs (1 to 200 μg mL^{-1}), the activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured independently. The plasma fraction was separated from whole blood by centrifugation (2500 RPM, 10 minutes, 25 °C). NPs were incubated with

isolated plasma for 30 minutes at 37 °C with constant stirring. Incubated samples were taken on a coagulometer (Hemostar XF 2.0, Tulip Diagnostics Pvt. Ltd. India), followed by the addition of phospholipid calcium thromboplastin (200 µL) to trigger clot formation and then the prothrombin time (PT) for the sample was calculated. To determine the APTT, 100 µL of cephalin was added to the previously placed sample in the coagulometer. After incubating, it was mixed for 2 min, and then 100 µL of calcium chloride was added under stirring to stimulate the clot formation, followed by monitoring the clotting time (APTT). The process was repeated three times.

2.6 NITRIC OXIDE LOADING AND RELEASE DETERMINATION

2.6.1 Loading of gas releasing molecules in to PNAG NPs

Sodium nitroprusside was loaded into PNAG NPs as NO gas releasing molecule under the vacuum assistance. The weighted quantity of PNAG NPs was dispersed in a saturated and known concentration aqueous solution of SNP and stirred for 24 h by protected from light at a temperature of 2-8 °C.

2.6.2 Entrapment Efficiency (EE, %) and Loading capacity (LC, %)

The entrapment efficiency (EE, %) of SNP was measured through UV-Vis spectroscopy (550 nm) of supernatant recovered after SNP loading using Griess Reagent using the following Equation 2. 3. Loading capacity (LC, %) was measured by dispersing 1 mg of lyophilised SP NPs in 5 mL of DMSO and incubating for 30 min at 37 °C. After centrifugation at 18,000 rpm, NO amount was measured by UV-Vis spectrometer and the percentage of SNP loaded was determined using Equation 2. 4.

$$EE\ (\%) = \frac{Weight\ of\ SNP\ in\ NPs}{Weight\ of\ SNP\ added\ to\ solution}\ x\ 100 \qquad \textit{Equation 2. 3}$$

$$Drug\ loading\ (\%) = \frac{Weight\ of\ SNP\ in\ NPs}{Weight\ of\ SNP\ loaded\ NPs}\ x\ 100 \qquad \textit{Equation 2. 4}$$

2.6.3 Determination of NO content and NO release profile

NO content was determined for all the batches of SP NPs. Briefly, 1 mL of DMSO and

Griess reagent (1:1) was used to dissolve 5 mg of SP NPs, which were then sonicated for 1 h. After centrifugation at 18,000 RPM, the supernatant was diluted, and the amount of NO was determined by measuring absorbance at λ = 550 nm (UV-Vis spectrophotometer: Jasco; V-730, JASCO International Co., Ltd., Japan). NO release was investigated using the dialysis method at a pH of 7.4. The release pattern of NO from the prepared SP NPs was studied for 24 h. Briefly, 5 mg of SP NPs in 5 mL of PBS (pH 7.4) was dispersed and poured into a dialysis bag and simultaneously immersed in 25 mL of dissolution media (PBS pH 7.4, 37 ±2 ℃, 100 RPM), and release was conducted for 24 h. At a predetermined time interval, samples were withdrawn in sink condition, and the amount of NO released was measured by taking absorbance at λ = 550 nm. Cumulative drug release (%, CDR) was calculated.

2.7 NANOFORMULATION DEVELOPMENT AND EVALUATION

2.7.1 Preparation of nanoformulation and qualitative evaluation

Figure 2.1 Apparatus for measurement of the spreadability of nanoformulation

NPs were used to prepare nanoformulation for the smooth application on the wound. NPs (5% w/w) were dispersed into an oleaginous ointment base prepared (Table 2.2) as

mentioned in ointment bases [6]. Briefly, the accurately weighted quantity of NPs was taken on an ointment slab and triturated with a small amount of base ointment (already prepared through the melt fusion method) with the help of a spatula having a long and broad blade. After proper trituration with base ointment, glycerine (5% v/w) is incorporated. Finally, the nanoformulation's pH (6.8–7) was adjusted using triethanolamine (98%) and gently swirled to combine.

Table 2.2 Table showing the ingredients (in percentage) used for the preparation of oleaginous ointment base

S. No	Ingredients	Weight (%)
1.	Glycerine	5
2.	Paraffin Wax	10
3.	Liquid Paraffin	15
4.	Stearyl Alcohol	25
5.	White soft Paraffin	45
6.	Triethanolamine (98%)	q.s.

Both nanoformulation and ointment base (formulation base) were evaluated for parameters such as appearance, odour, colour, and homogeneity by visual inspections and measurable parameters such as spreadability by "slip & drag" approach (Figure 2.1) using Mutimer's recommended apparatus [7]. Spreadability was computed using the following Equation 2. 5.

$$S = \frac{MxL}{T} \qquad \textit{Equation 2. 5}$$

where S is spreadability, M is the total weight of the container or beaker, L is the distance travelled by the glass slide, and T is the amount of time (in sec) needed to separate the slides from one another.

2.7.2 Physicochemical Evaluation of SP Nanoformulation

(i) Nitric Oxide content determination

The USP method (USP 40-NF35, 2017) was employed to evaluate content homogeneity. Briefly, 100 mg of SP nanoformulation was taken from the barrel's top (plunger end), centre, and bottom (needle end) parts of SP nanoformulation filled syringes (Figure 2.1). They were combined with 5 mL of PBS (pH 7.4) and Griess reagent (1:3). Then the

mixtures were homogenised at 25 °C for 15 mins. with 5000 RPM in shield condition. Then it was filtered using a 0.45 nylon filter. NO content was calculated using the Griess reagent method after diluting the samples for examination.

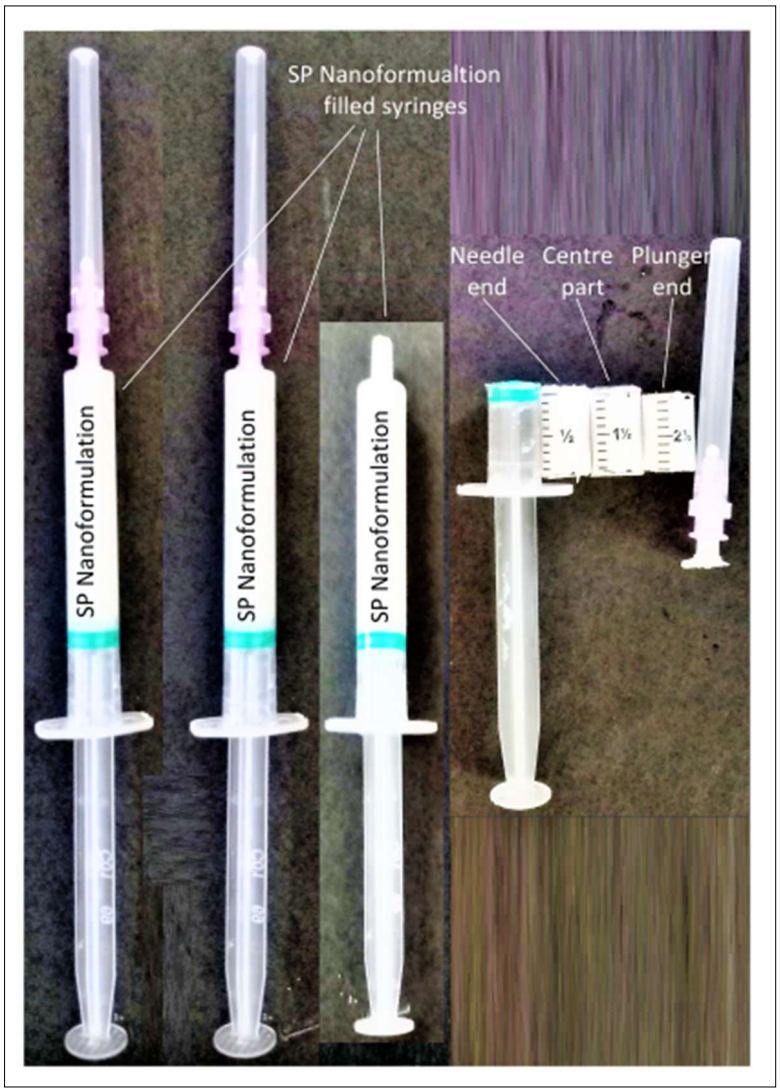

Figure 2.2: Content uniformity test. SP nanoformulation filled syringe tubes, tube was cut into pieces and content was evaluated for uniformity.

(ii) Nitric oxide release from nanoformulation

A modified USP Apparatus 2 (Figure 2.3) was employed to evaluate NO release from SP nanoformulation. Excess SP nanoformulation was placed in a unique sample holder having a 3.14 cm^2 exposed surface area. After flattening, smoothing, and removing extra material from the nanoformulation surface with a spatula, the precise loaded amount of nanoformulation was calculated by weight. The figure (Figure 2.3) shows that the sample holding cell was inverted on the static shaft connected to the burette stand. A pre-cut and

pre-wetted 0.22-µm pore-size syringe filter membrane (Millipore, Billerica, MA) was placed on top of the nanoformulation and tightened with a rubber band. To reduce shearing stress induced during sample preparation and its potential impact on drug release, the mounted sample holding cell was left stationary inside the dissolution vessel for 30 min.

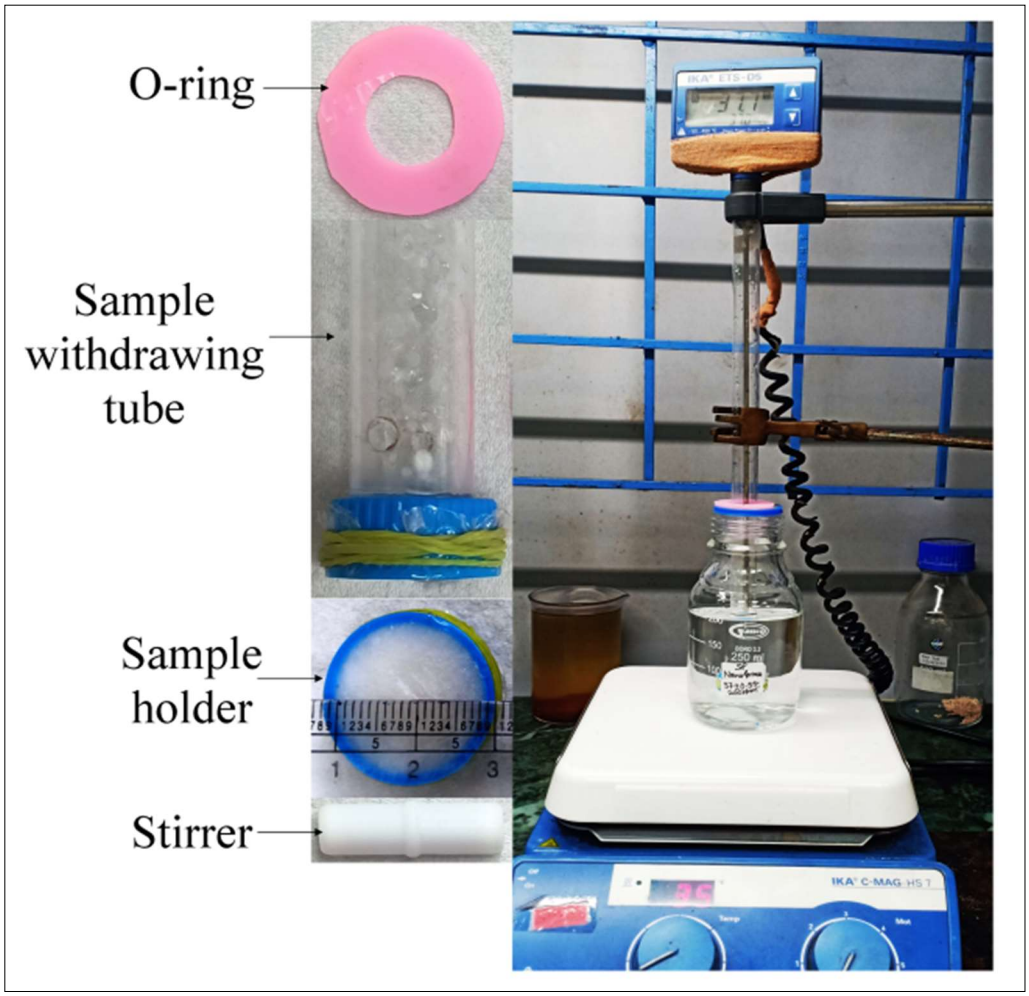

Figure 2.3: In vitro dissolution of SP nanoformulation

The dissolution test was started by adding 200 mL of pre-heated release medium (PBS, pH 6.8, 37 ±2 °C) to the vessel and stirred with 200 RPM. Using the Griess reagent method, aliquots of the sample (0.5 mL) were obtained at predefined intervals (15 min, 30 min, 1, 2, 4, 8, 10, 12 and 24 h), and the amount of NO released from SP nanoformulation was calculated.

2.8 BIOLOGICAL EVALUATION OF NANOFORMULATION

2.8.1 Angiogenesis: Chicken Embryo Angiogenesis Assay (CAM assay)

The chicken embryo angiogenesis assay, also known as the chick chorioallantoic membrane (CAM) assay, is a widely used *in vivo* method for studying angiogenesis and assessing the effects of compounds on blood vessel formation. The following general methodology for conducting a CAM assay has been adopted:

Egg incubation and windowing: Fertilized chicken eggs were incubated for 5 days in a humidified incubator at 37.5 °C with regular rotation to ensuring embryonic development. On the day 5^{th} very carefully a small window in the eggshell to expose the CAM was created. A small drill or file is used to make a hole in the shell, taking care not to damage the underlying CAM.

Preparation of test compounds: The compounds or substances to be tested at the appropriate concentrations/ doses were prepared by dispersing them into sterilized suitable buffer system.

Treatment and incubation: The CAM is treated with test compound by applying a small sterile filter paper disc or using a gelating sponge or by administering them *in ovo* condition. Appropriate and adequate controls should also be included such as vehicle-treated CAMs.

Image acquisition (Time 0): Immediately, after treatment, the images of CAM were captured at time zero (right after wounding) using a phase-contrast microscope or an automated imaging system and a marked reference point has been used to ensure consistent image acquisition across different time points.

Incubation post-treatment: The eggshells window was closed with tape or paraffin film and eggs were returned to the incubator and incubate for an additional time points such as 2, 4 or 8 h or more as days to allow angiogenic responses according to the need of

experiment.

Image acquisition (Later time points) and angiogenesis assessment: The additional images of the CAM at predetermined time points (e.g., 2, 4 or 8 h) using the same settings as in previous step were captured. Assesment of angiogenesis for angiogenic responses, such as blood vessel density, branching, and overall vessel morphology was done with the help of images aquired or with the help of CAM collected at last day.

2.8.2 Animal Protocol and Ethics

All the animal studies were examined and approved (Approval No. IIT(BHU)/IAEC/2022/078, dated 03/05/2022) by the Institutional Animal Ethical Committee established at Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (BHU), Varanasi (Registration no. 2123/GO/Re/S/21/CPCSEA). The use of animals in this work was carried out in compliance with the IEAC's recommendations and guidelines. In a nutshell, the male Wistar rats (wt: 125-150 gm, age: 12-14 weeks) were purchased from Institute of Medical Sciences, BHU and Central Drug Research Institute, Lucknow. They were given unrestricted access to the laboratory by keeping them in cage and put on fed to commercial pellet meal and unlimited amounts of water. Prior to the start of the experiment, the animals have to spend 1 week becoming acclimatize to the laboratory environment. One day before to the commencement of the experiment, all rats had their skin cleansed with water and shaved with the help of an electric razor. The skin was then disinfected with a povidone-iodine solution or with 70% v/v ethanol solution, and kept apart in animal cages. The animals were given a once-over for general health on the day of the experiment, right before the application of test material, and the skin was inspected for any anomalies. Only when there was no visible evidence of prior skin irritation were experiments carried out. Pentobarbital sodium (50 mg/kg) was injected intraperitoneally

to anaesthetize rats. The absence of pedal and corneal responses confirmed anaesthesia. At 0^{th}, 12^{th}-, and 24^{th} h of following surgery, tramadol hydrochloride (20 mg/kg) was administered intraperitoneally as an analgesic.

2.8.3 Dermal Irritation Study

Dermal irritation studies hold paramount scientific significance in evaluating the potential adverse effects of substances applied on the skin. These studies contribute crucial data for cosmetic, pharmaceutical, and chemical industries, aiding in the formulation of safe products.

Table 2.3 Dermal irritability grading system according to Draize

Value	Erythema development	Value	Oedema development
0	No erythema	0	No oedema
1	Extremely minor erythema	1	Extremely minor oedema
2	Mild erythema (well defined margins)	2	Mild oedema (well defined margins)
3	Moderate severe erythema (Specified colour and erythema region)	3	Moderate severe oedema (Specified colour and oedema region)
4	Maximum possible erythema	4	Maximum possible oedema

By assessing the irritant potential, we can enhance consumer safety, comply with regulatory standards, and refine product development. Understanding dermal irritation also facilitates advancements in dermatology and toxicology, offering insights into skin barrier function and inflammatory responses. Ultimately, these studies play a pivotal role in ensuring the development and use of products that minimize the risk of skin irritation, promoting public health and well-being. Nanoformulation samples were applied to rat skin for a predetermined period to check for any irritation or erythematous reaction. Each rat had about 4 cm^2 of intact shaved skin, treated with nanoformulation and base formulation of about 1 gm, coupled with the positive control (1% formalin solution as a common irritant). The treated area was wrapped in an occlusive bandage and confined separately. The wrappings were removed after 24 h, and any leftover testing materials

were carefully removed and washed with water. At the end of the study, the skin of rats was examined visually for any indications of skin irritation and sensitisation and simultaneously imaged digitally. Further, rats under the experiments were observed for 7 more days to check for indications of oedema and erythema. The Draize scoring system (Table 2.3) was used to calculate the skin irritation score using the Primary Dermal Irritation Index (PDII), calculated using the following Equation 2. 6.

$$PDII = \frac{PDI}{4} \qquad \textit{Equation 2. 6}$$

2.8.4 *In vivo* Wound Healing Study Protocol

Rat excisional wound splinting model [8] was used to evaluate the wound healing ability of formulations containing NPs. Splinting rings firmly cling to the skin around the wound, preventing wound closure brought on by skin contraction and enabling wound healing by granulation and re-epithelialisation. A 10-12 mm diameter full-thickness skin wound on the dorsal area was created using a sterile 10 mm diameter biopsy punch. Then, the rats were put on sterile sheets. To reduce contraction throughout the healing process, sterile silicone rings with an internal diameter of 12–15 mm were firmly clung to the skin around the wound. The wounds were dressed with self-adhesive waterproof tape gauze. After each procedure, rats were put on a heating pad until they had fully recovered from anaesthesia. The base formulation and PNAG nanoformulation were applied as soon as the wound was created, preventing the wound region from drying up and ensuring it was protected from the outside environment. On days 0, 1, 3, 5, 7, 9, 11, and 13th after wounding, 100 mg of base formulation and nanoformulation was applied to the wound lesions. The wounds were imaged digitally on days 0, 3, 7, 9, and 14th with proper scaling and at an identical height. Self-adhesive sterile gauze tape was used to cover each wound, and new dressings were applied after each treatment on alternate days. Rats were employed as a control group, receiving just formulation based. The therapy regimen was

evaluated using ImageJ software to quantify the wound area using Equation 2. 7, and the percentage of wound closure was calculated as.

$$A = \frac{W_t}{W_0}\ x\ 100 \qquad \textit{Equation 2. 7}$$

W0 is the wound area at starting time 0, Wt is the wound area at time t, and A is the percentage of wound closure.

2.8.5 Histological Analysis

Deep granulation tissue and cross-sectional full-thickness skin specimens from cicatricial tissue were acquired on the 14th-day post-treatment. Formalin-fixed paraffin-embedded blocks were sectioned in the transverse plane with a section thickness of 5 microns and stained with Mayer's Hematoxylin & Ethyl eosin and Mallory's Trichrome stain. Mounted sections were analysed with an optical microscope at different magnefications [9].

2.8.6 Immunochemical Analysis

As a whole, the body interacts constantly with its local tissues. If the local tissue is compromised in any way, the usual molecular changes in body markers will also be affected [10]. Therefore, blood plasma was collected at various intervals and kept at -80 °C until further processing to ascertain the extent of systemic change in wound healing indicators. The serum level of cytokines and chemokines were determined during various phases of wound healing. The enzyme-linked immunosorbent assay (ELISAs) was used to measure the levels of wound healing indicators (IL-6, IL-1β, TNF-α, CRP, and IGF-1) in serially collected blood samples on the 2nd, 7th, and 12th day following wounding. Commercially available ELISA test kits were used to check the antigen levels of IL-6, IL-1β, TNF-α, CRP, and IGF-1. In short, previously collected and stored blood (-80 °C) was thawed by storing it at 4 °C for the whole night (12 h); the serum was collected by centrifugation (3500 RPM, 15 min, 4 °C). After the preparation, 100 µL of each sample and standard were taken in the wells of a 96-well plate, then incubated for 90 min at 37

°C. After adding the antibodies and biotin in 100 µL aliquots to each well, the samples were kept for 60 min at 37 °C. After that, samples were washed five times with 0.01M TBS, and 100 µL of avidin-biotin complex was added to each well and kept at 37 °C for 30 min. To the above mixture, tetramethyl benzidine (TMB) was added at 37 °C in the dark, allowing the reaction to complete for 30 min. Then, TMB termination solution was added. The relative protein concentrations were estimated by taking the absorbance at 450 nm using a microplate reader.

2.8.7 Semi-Quantitative Reverse Transcription and Polymerase Chain Reaction (sqRT-PCR)

From the excised wound of the control group and treatment groups of day 2, 7 and 14, nearly 1-mm of tissue were collected. The RNA isolation was performed by homogenizing the tissue in 1ml of TRIzol reagent followed by chloroform extraction. Next, the aqueous layer was collected, and RNA was precipitated by using 1 ml of isopropyl alcohol and centrifuge at 12000g for 15 min, 4 ℃ temperature. The RNA pellet was washed for 2 times with 70% ice-cold ethanol and suspended in Diethyl Pyrocarbonate (DEPC)-treated water. After RNA quantification, 1µg of RNA was used for cDNA synthesis using oligo dT sequence and Reverse transcriptase polymerase as per the manufacturer protocol. Afterwards, PCR were conducted using KiCqStart® (Sigma) primer for biomarker target gene PECAM-1 (forward primer sequence 5’AAACCACAATTGAGTACCAG3’ reverse primer 5’ACTTAGCTTGACGTTCTTTG3’), VEGFA (forward primer sequence 5’GATAGAGTATATCTTCAAGCCG3’ and reverse primer 5’CTCATCTCTCCTATGTGCTG3’), KDR (forward primer sequence 5’AAACTGGATAAAATGGGCG3’ and reverse primer 5’AGCCTTTTAGGTAGAGTCAG3’) and housekeeping gene GAPDH (forward primer

sequence 5'TCGGAGTCAACGGATTTG3' and reverse primer 5'CAACAATATCCACTTTACCAGAG3') as reference by using Taq polymerase (TAKARA R001A) with 35 repetitive cycles of denaturation at 95 ℃ for 25 sec, annealing at 59 ℃ for 30 sec and extension 72 ℃ for 35 sec followed by final extension at 72 ℃ for 7 min. PCR products were run on 2% agarose gel and dosimetry calculation were performed.

2.9 STATISTICAL ANALYSIS

Using the Origin 2021 software (OriginLab Corporations, USA), the independent Student's t-test and one-way ANOVA with the Tukey test were used to statistically analyse the *in vitro* and *in vivo* data. Statistical significance was defined at a *P*-value < 0.05. The data are shown as mean ±SD.

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