# Supporting Information (SI)

## Nitric Oxide Releasing Novel Amino Acid-Derived Polymeric Nanotherapeutic with Anti-Inflammation for Rapid Wound Tissue Regeneration

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### 1. Synthesis of NAG Monomer

N-Acryloyl glycine monomer (NAG) was synthesized through a modified approach and converted into PNAG NPs. [1-3] In brief, 'X' gm of glycine was dissolved in a cold "Y" mL 2M KOH solution under stirring in a round bottom flask (in an ice bath) (solution A). "Z" mL of cold 1,4-dioxane was mixed with 8 mL of acryloyl chloride (solution B). Then solution B was slowly added to the cooled solution A at 0°C (ice bath) for two hours under vigorously stirring (600 RPM). The pH of the mixture was adjusted above 10 by adding 2M KOH, as needed. Then stirred at room temperature (25°C) and mixture was rinsed thrice with 20 mL of diethyl ether for each time. The mixture was then saturated with NaCl after being acidified with 5M HCl to achieve pH 2. Then 80 mL of ethyl acetate was added and the aqueous layer was extracted. This process was followed for 4 times. The ethyl acetate layer was then dried for 12 h with anhydrous magnesium sulphate and then it was filtered to collect the liquid part. Then the ethyl acetate solvent was evaporated using a rotatory evaporator at reduced pressure. From a cooled combination of diethyl ether: ethyl acetate (1:1), 10.2 gm of crude product (NAG) (yield 63.75%) was crystallized out. Further, the NAG monomer was evaluated for the free amino group using ninhydrin reagents.

### 2. Fabrication of SP NPs

**Step-I: Synthesis of PNAG NPs**

As reported in our prior work [2, 3] the mini-emulsion radical polymerization method was used to synthesize the polymeric NAG nanoparticles (PNAG NPs), after a few modifications as required. Briefly, NAG Monomer ('XX' mg) and hexadecane ('YY' mg) were dispersed in toluene (5 mL oil phase). Then, radical initiator, AIBN ('ZZ' mg), was added and bath sonicated (Elmasonics, S 30H, Germany) for 3 min. Then crosslinking agent divinylbenzene (DVB) was added and further probe sonicated for 5 min in ice bath. In another vial, sodium dodecyl sulphate ('XY' mg SDS) was dissolved in 1.6 mL $H_2O$ using a bath sonicator at 25 ℃ and was added drop-wise under vigorous stirring (600 RPM). After complete mixing, the mixture was kept at 80-85 ℃ in an oil bath for complete polymerization and formation of stable nanoparticles. Residual toluene was evaporated through the co-evaporation of water by adding a sufficient amount of water and unreacted reagents, and SDS was removed by the alcohol: water solvent extraction method. Finally, nanoparticles were washed 3 times with a mixture of 3:1 isopropanol and water. The nanoparticle suspension was separated by centrifugation 14000 RPM (at 4℃) followed by freeze-drying.

**Step-II: Loading of SNP loading into PNAG NPs and Elemental Analysis**

Sodium nitroprusside (SNP) was loaded in the PNAG NPs at room temperature (25 ±5°C). In brief, 'XP' g of SNP was dissolved in 1 ml Mili-Q® water. The SNP solution was then mixed with 'YP' mg of PNAG NPs, and incubated for 24h under stirring (100 RPM) under reduced pressure and in dark (protected from light). The SNP-loaded PNAG NPs (SP NPs) were recovered by centrifugation at (15,000 RPM). The SNP attached on the surface of nanoparticles removed by washing with water twice. Then SNP loaded PNAG was dried through lyophilisation and preserved for further evaluation.

Energy-dispersive X-ray spectroscopy (EDS) was employed to perform elemental analysis and chemical characterisation of the specimens. The Model EDS: 51N1000 – EDS System from Oxford Instruments Nano analysis was utilized for the elemental analysis of the samples.

The detail of the formation of the nanomedicine has been filed for the Indian patent (Patent Application No.: 02311051276, date of filing: 31-07-2023).

## 3. Characterization

The chemical functionality of the NAG monomer, PNAG NPs and SP NPs were characterized through the FT-IR spectroscopy (Nicolet iS5, 4 Thermo Fisher Scientific Inc. USA) using KBr pellet and an $^{1}H$ NMR Spectrometer (500 MHz) (One Bay NMR Spectrometer, Bruker Bio Spin International AG) using DMSO-D6 solvent and tetramethylsilane as an internal standard in NMR For FT-IR spectra, a total of 32 scans per spectrum were performed in the 4 $cm^{-1}$ resolution regions between 4000 and 500 $cm^{-1}$. HRTEM (Nova Nano SEM 450, FEI, USA) was used to examine the particle size and morphology of the nanoparticles. The crystallinity of NAG monomer, PNAG NPs, SNP and SP NPs were checked through the XRD (Rigaku SmartLab, RIGAKU Corporation, Japan). The mean particle size and zeta potential (ζ) of SP NPs were measured by dispersing in PBS (pH 7.4) using dynamic light scattering (DLS: Nano-ZS ZEN3600 Malvern, UK) at 25 °C (n=3). Freeze-dried SP NPs were reconstituted in PBS (pH 7.4) for size measurements, and suspensions were filtered through a 0.45 μm membrane before the analyses. The investigations were performed in three experiments using reusable folded capillary zeta cells with a 10 mm path length in PBS (pH 7.4) solution at 25°C and a fixed angle of 173°.

## 4. *In vivo* study, ethics and approval

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 $kg^{-1}$) was injected intraperitoneally to anaesthetize rats. The absence of pedal and corneal responses confirmed anaesthesia. At $0^{th}$, $12^{th}$-, and $24^{th}$ h following surgery, tramadol hydrochloride (20 mg $kg^{-1}$) was administered intraperitoneally as an analgesic.

# Supporting Tables

**Table S1. Formulation of SP nanoformulation**

| 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. |

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

**Table S2. Qualitative evaluation of SP Nanoformulation**

| S. No. | Quality Parameter | Required quality | Formulation base | SP-nanoformulation |
|---|---|---|---|---|
| **1** | Colour | Same as API's colour or white/ off white | Colourless/ White | Colourless/ White |
| **2** | Appearance | Homogenous | Homogenous | Homogenous |
| **3** | Consistency/ Homogeneity | Good | Good | Good |
| **4** | Phase Separation | No | No | No |
| **5** | Odour | Same as API's odour/ Odourless | Odourless | Odourless |

Qualitative characterization of formulation base and SP-nanoformulation on the basis of observational parameters

**Table S3. Skin irritation evaluation (Draize Scoring system)**

| 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 |

Dermal irritability grading system according to Draize [4, 5]

Erythema and edema were graded on a scale of 0–4, with 0 denoting no symptoms and 4 denoting severe. The average irritation score per time point was calculated for each animal by summing the cutaneous response scored at 1 h, 24 h, 48 h, and 72 h following the removal of the test substance and dividing the result by four group. The results for PNAG nanoformulation induced skin irritation were compared with the basic formulation and with a positive control. The dermal irritation index (PDII) was calculated using the following equation.

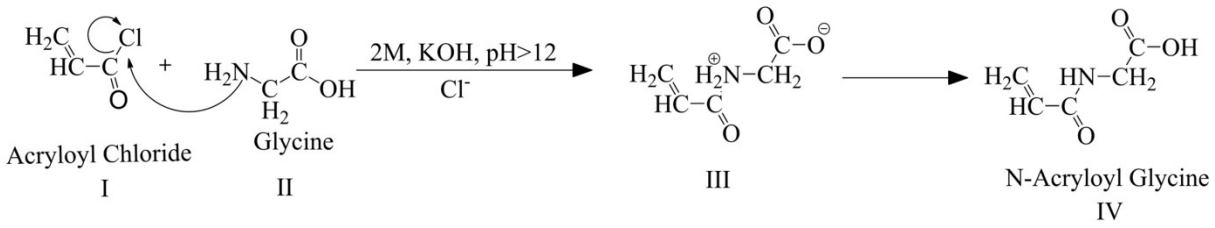
$$PDII = \frac{PDI}{4}$$

## Table S4: Elemental mapping results of PNAG NPs and SP NPs showing in weight percentage and atom percentage

| Element Name | PNAG NPs | | SP NPs | |
|---|---|---|---|---|
| | Weight (%) | Atom (%) | Weight (%) | Atom (%) |
| **Fe** | 0.21 | 0.05 | 14.15 | 3.6 |
| **Na** | 0 | 0 | 7.4 | 4.57 |
| **O** | 4.84 | 3.7 | 2.92 | 2.6 |
| **N** | 2.04 | 1.78 | 0.42 | 0.42 |
| **C** | 92.9 | 94.47 | 75.1 | 88.81 |

## Synthesis Schemes

**Scheme S1. Synthesis of NAG Monomer**




# Supporting Figures

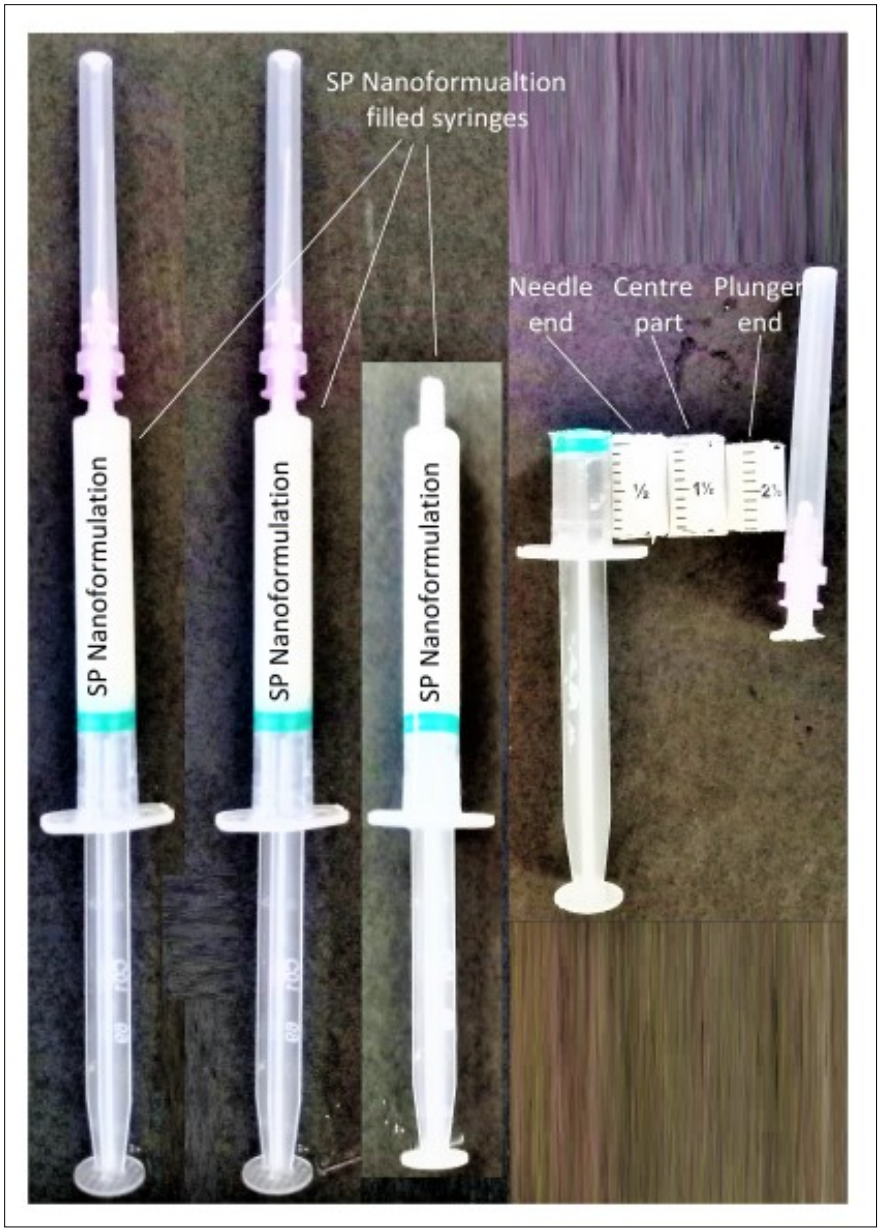



Figure S1. **Content uniformity test.** SP nanoformulation filled syringe tubes, tube was cut into pieces and content was evaluated for uniformity.

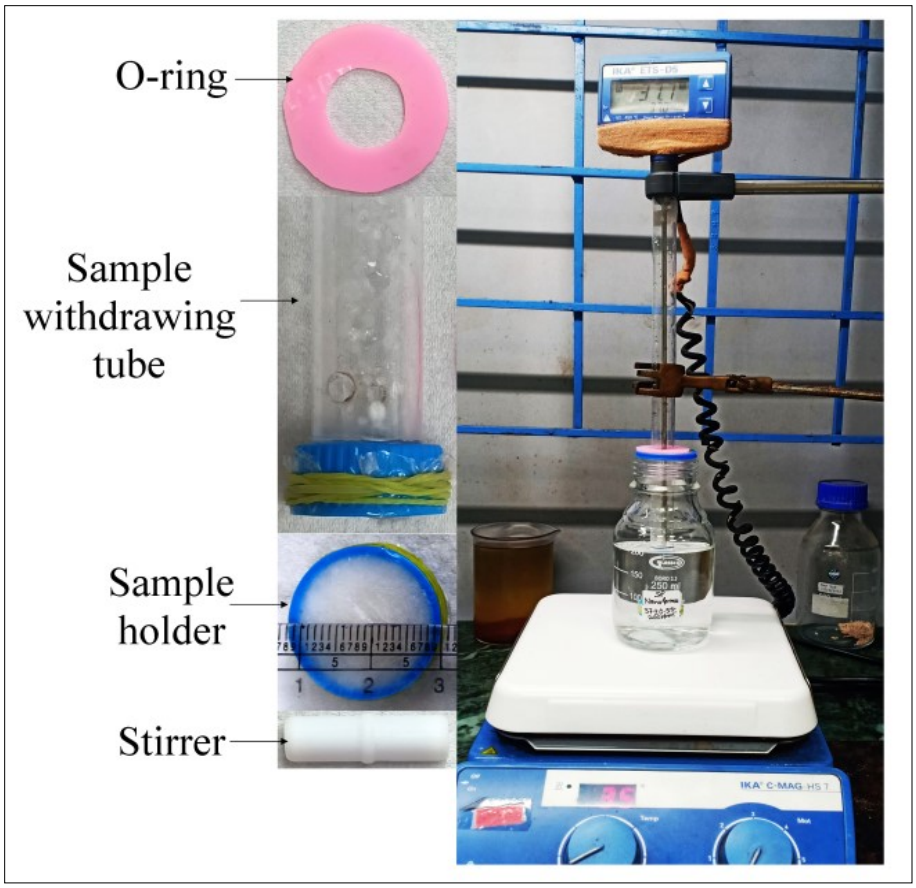



Figure S2: *In vitro* dissolution of SP nanoformulation

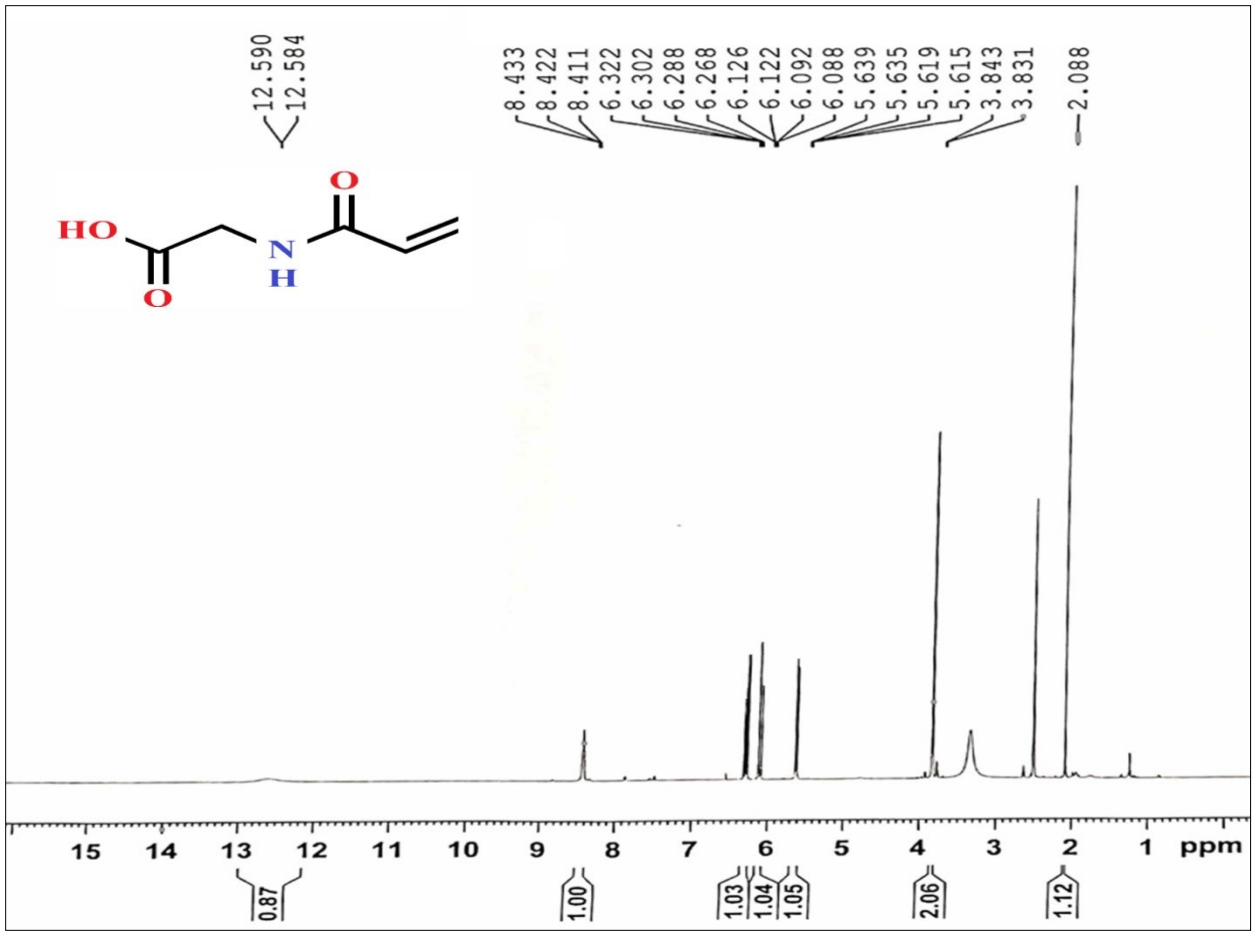



Figure S3. $^{1}$H NMR spectra of NAG monomer: The characteristic peaks are assigned as $^{1}$H-NMR (DMSO-D6): 12.587 (1H, s), 8.41 (-CONH, t) 6.26 and 6.09 (-CONH-, 1H, cis, t), 5.63 and 5.61 (-CONH-, 1H, trans, t), 3.844 and 3.832 (-$CH_2$, CH-, 2H, vinylic hydrogen, d), 3.5 (-$CH_2$-, 2H, d) 2.56 (=CH-, 1H, t) are the corresponding ppm values.

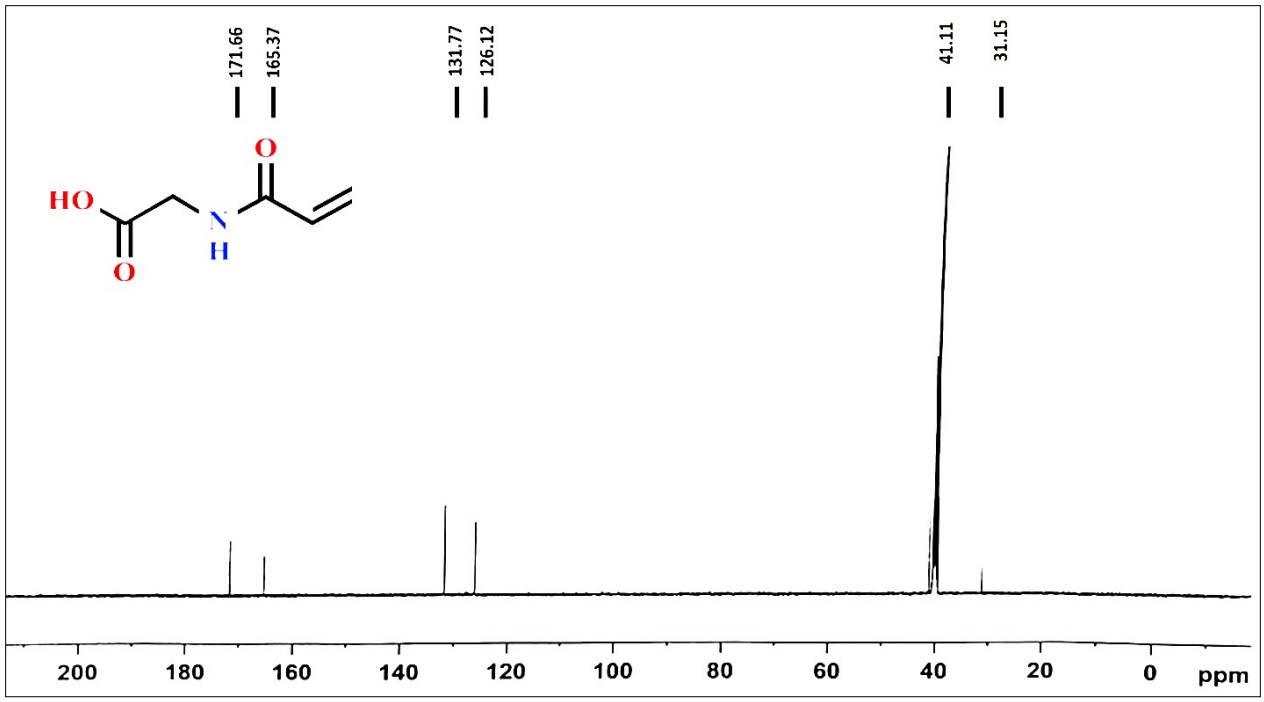



Figure S4. $^{13}$C-NMR Spectra of NAG monomer: The characteristic peaks are assigned as $^{13}$C-NMR (DMSO-D6): 171.66 (-CO-OH), 165.37 (-CO-NH), 131.77 and 126.12 ($H_2C$=CH-), and 41.10 (-N-$CH_2$).

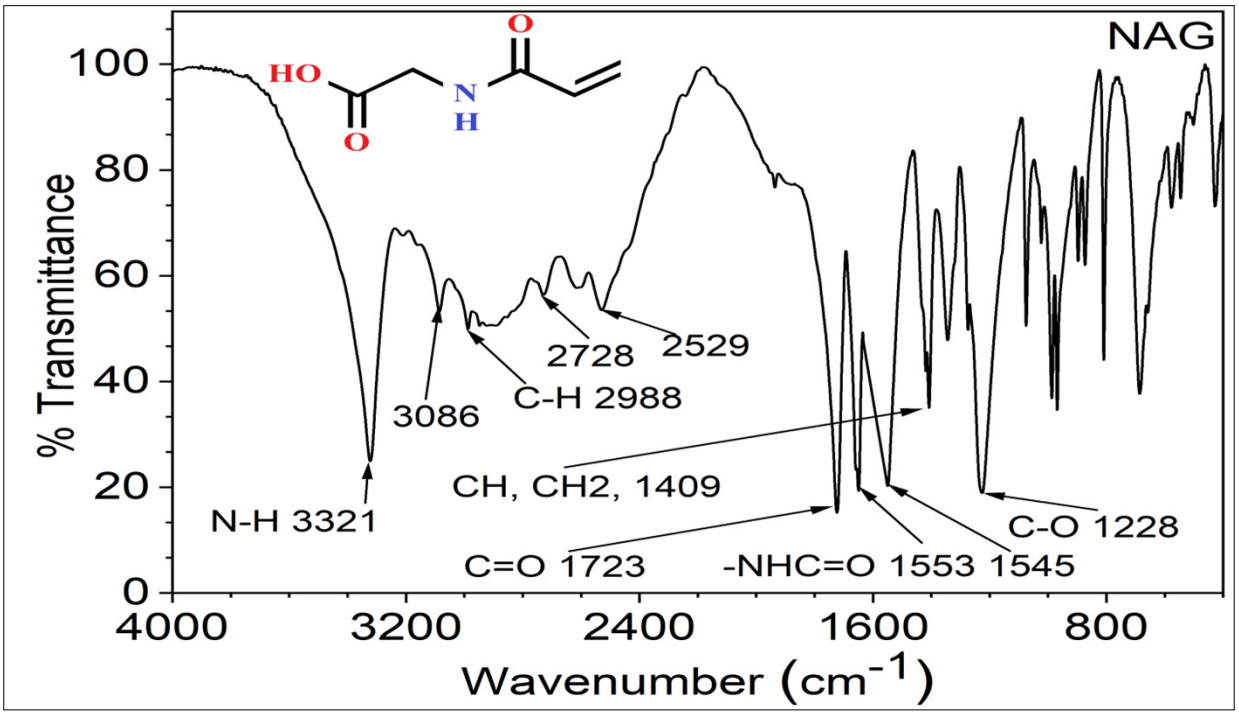



Figure S5. FTIR of NAG monomer: FTIR spectra of NAG monomer show the characteristic bands at 3321 $cm^{-1}$ for secondary amine (-NH-), 3086 $cm^{-1}$ for alkene (-CH=CH-), 2988 $cm^{-1}$ for alkane (-$CH_2$-), 2728 $cm^{-1}$for carboxylic group (-COOH), 2529 $cm^{-1}$ for hydroxyl group (-OH), 1723 $cm^{-1}$ for carbonyl ketone group attached to an acid group, 1274, 1228 $cm^{-1}$ for C-O stretching due to ester - COOR group, 1553 $cm^{-1}$ for carboxylate ion.

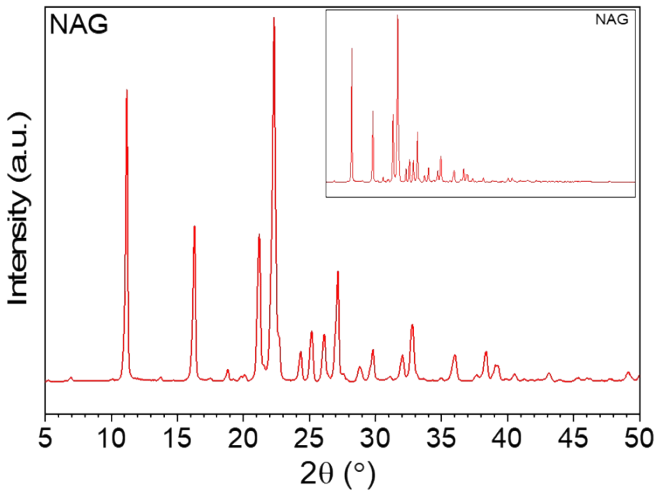



Figure S6. XRD pattern of NAG monomer: NAG monomer is highly crystalline in nature and shows approximately 81% crystallinity.

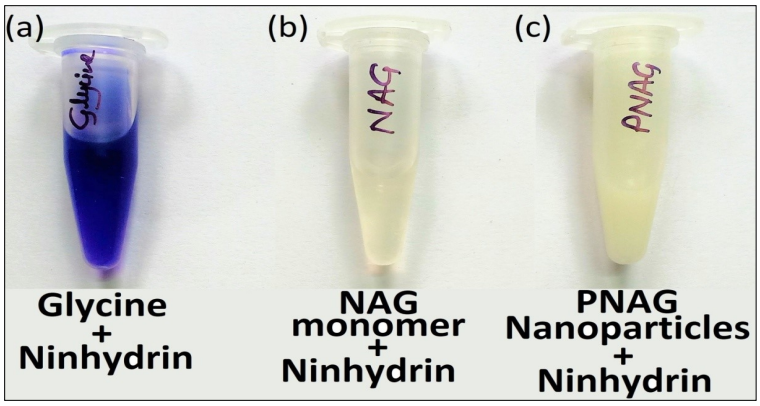



Figure S7. Ninhydrin test. (a) Glycine amino acid gives vibrant blue/violet colour, (b and c) NAG monomer and PNAG NPs does not respond to ninhydrin test, showing absence of free amino group ($-NH_2$).

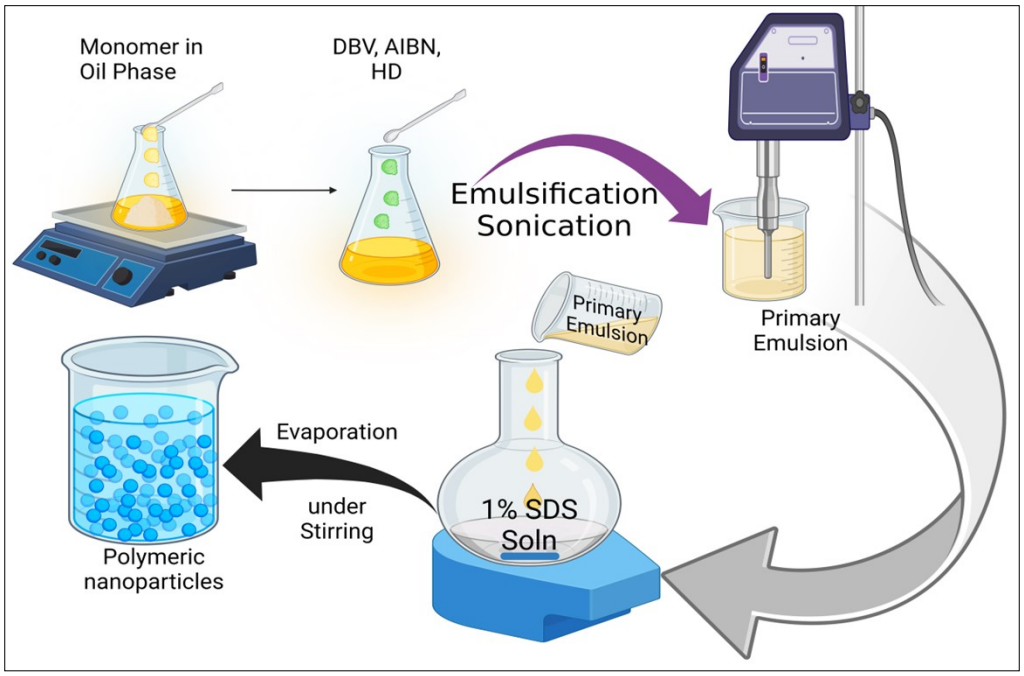



Figure S8. Schematic representation of the synthesis of PNAG NPs

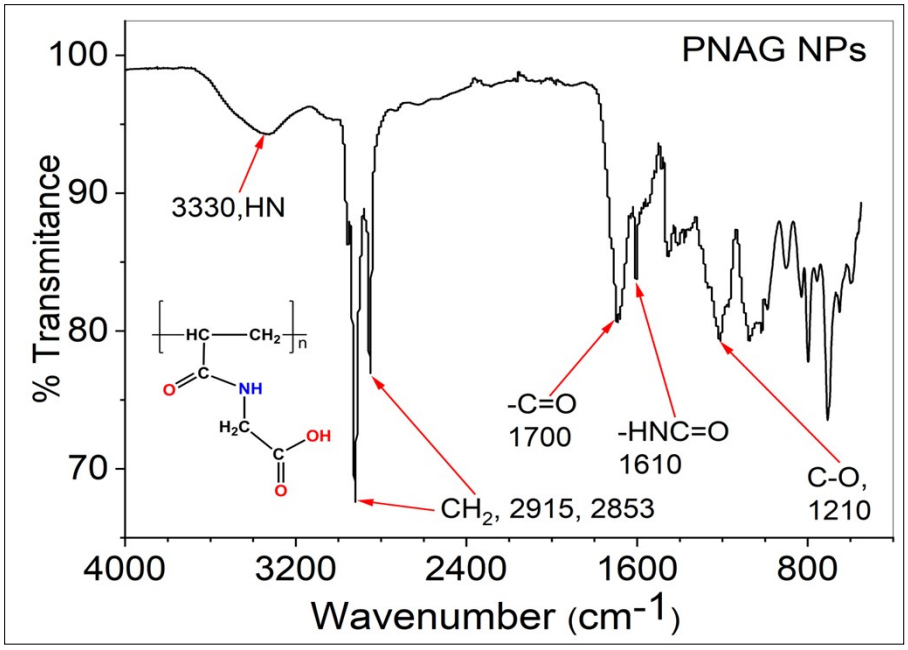



Figure S9. FTIR spectra of PNAG NPs: FTIR spectra of PNAG NPs show the characteristic bands 1610 $cm^{-1}$ carbonyl ketone attached to amide group (-NH bending), 1210 $cm^{-1}$ for C-O stretching due to ester -COOR group, 3330 $cm^{-1}$ for secondary amine (-NH-), 2915 $cm^{-1}$ for alkane (-$CH_2$-) and 2853 $cm^{-1}$for carboxylic group.

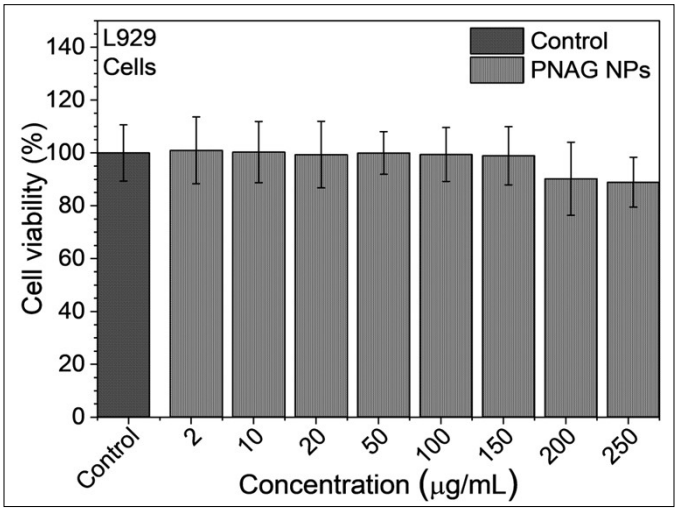



Figure S10. Viability with the PNAG NPs at the highest tested concentration (250 μg $mL^{-1}$) were found 88.92±9.43%.

**References**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