CHAPTER: 2

MATERIALS, METHODS, AND CHARACTERIZATION TECHNIQUES

2.1 . *INTRODUCTION.*

In this chapter, materials, methods used to synthesize the samples, and experimental techniques utilized to characterize the samples have been explained in detail.

2.2. MATERIALS AND METHODS

2.2.1. **Materials.** Glycine (98%, Qualigens), Rhodamine 6G (Rh 6G, >97.8%), L-Glutamic acid (Sigma-Aldrich), L-leucine Methyl ester hydrochloride (98%)(Sigma-Aldrich),, D-phenylalanine methyl ester (98%)(Sigma-Aldrich), Methyl thiazoltetrazolium (MTT, >99.9%, Himedia), Triethylamine (≥99.5%, Merck), hexadecane anhydrous (99%, Sigma –Aldrich), and Divinyl Benzene (DVB) (Alfa Aesar), Acryloyl chloride stab with 400ppm phenothiazine (96%, Alfa Aesar) Chloroform, Ethyl acetate, 1, 4-dioxan (99%) extra pure (Merck), 2,2- Azo-bis-isobutyronitrile (AIBN, 98%, SRL), SDS (90%, Merck). Neurobasal Medium SFM (Thermo); NGF (Thermo), B-27 Supplement (Thermo), Fetal Bovine serum (Gibco), HBSS 1X (Thermo); penicillin streptomycin cocktail; gentamicin, Poly-L-Lysine (Sigma). All the chemicals were used without further purification. Anti-beta actin antibody, Anti-mouse-HRP secondary antibody, Anti-POLR2A antibody etc.

2.2.2. **Synthesis of N-acryloyl glycine and N-acryloylglutamate monomers .** N-acryloyl glycine (NAG) monomer was synthesized according to our previously reported method (Patent No.: 419638, Granted on. 30-01-2023) and the steps involved in the

synthesis process are shown in Scheme 3.1.1. In brief: solution A: 20 mmol of glycine was dissolved in 20 mL of 2 M KOH in an ice bath. Solution B: 20 mmol of acryloyl chloride was dissolved in 5 mL of 1, 4-dioxan in an ice bath. Then the solution B was added drop wise into the solution A under vigorous stirring (for 1 h in an ice bath) followed by overnight (12 h) at room temperature (25 oC) with continuous stirring. Then the reaction mixture was washed with diethyl ether (3 times, with 20 mL for each time) followed by acidification (~pH 2 adjusted by adding 5 M HCl drop wise) and was saturated by adding NaCl. Then the aqueous solution was extracted with ethyl acetate (5 times, with 20 mL each time) and dried by adding anhydrous MgSO4. Then the filtered reaction mixture containing NAG monomer was concentrated under reduced pressure using rota evaporator and lyophilized.

Note : N-acryloylglutamate[22] were prepared by using a similar method as like N-acryloylglycine.

2.2.3. **Preparation of poly(N-acryloylglycine-acrylamide) co-polymeric hydrogel.** Brief of the copolymer synthesis method has been shown in Scheme 3.1.1. Poly(NAG-b-A) co-polymeric hydrogel was prepared by a free radical mini-emulsion polymerization approach. 800 mg of NAG was dispersed in 8 mL of 1,4 dioxane for 30 min under bath sonication (bath temperature, 25 oC) with 5 mins of pulse. Then 40 mg of hexadecane (HD) was added followed by the addition of 40 mg of di-vinyl benzene (DVB) and addition of dissolved acrylamide (200 mg of acrylamide in 1000 μL of water) and then 20 mg of azobisisobutyronitrile (AIBN) within continuous stirring. Further, dissolved sodium dodecyl sulphate (SDS) (44 mg in 300 μL water) was added into the reaction mixture and stirred for 30 min at RT. Then it was sonicated for another 7 mins (ultra-probe sonicator, 750W, 30% power, 45:15 cycles) at 25 ℃. Then the total reaction mixture was transferred into a 100 mL round bottom flask and stirred for 12 h at 75 °C (in oil bath) for the

conversion of the monomer into polymer. The prepared sample was washed with the (50:50) ethanol water for 8-10 times followed by washing with ethanol water mixture (30:10) for 3 times. Then the sample was lyophilized and kept for study. Detail method was reported in an Indian Patent (Patent No.: 419638, Granted on. 30-01-2023).

2.2.4. **Synthesis of p(NAG-Ac-NAE) nanohydrogel**. Free radical, mini-emulsification polymerization techniques were used for the synthesis of p(NAG-Ac-NAE) nanohydrogel. 400 mg of N-acryloylglycine and 400 mg of N-acryloylglutamate were dispersed in 8 mL of 1,4-dioxane for 20 minutes with continuous stirring. Then completely dissolved 200 mg of acrylamide in cold water was added. Then 40 mg of hexadecane (HD) was added followed by the addition of 40 mg of di-vinyl benzene (DVB) and then 20 mg of azobisisobutyronitrile was added (AIBN) under continuous stirring for 10 min followed by sonication for 5 minutes in a water bath sonicator. Then 44 mg sodium dodecyl sulfate (SDS) in 300 μL water solution, was added to the reaction mixture and was kept for stirring for another 20 minutes. Further, the suspended mixture was sonicated for 7 min with a 45:15 on-off cycle at 30 % of amplitude and then the emulsion was transferred to 50 mL of round bottom flask and stirred for 12 hours at 70 ℃ till the precipitate was formed. The polymerized product was washed with a 70:30 ethanol-water mixture followed by 50:50 (ethanol: water) 8 times with proper sonication to remove the unreacted monomer and SDS. And further swelled hydrogel was freeze-dried to obtain nanohydrogel.

2.2.5. **Characterization of NAG, p(NAG-b-A) and p(NAG-Ac-NAE) co-polymeric hydrogel**. Functional groups and structural analysis were confirmed through FTIR, 1H NMR and 13C NMR spectroscopy. FTIR spectra for NAG with KBr pellet and for co-polymer nanohydrogel (poly(NAG-b-A)) with ATR were recorded (Thermo Electron Scientific Instruments LLC/ model Nicolet iS5). 1H NMR and 13C NMR spectroscopic measurements were conducted by dissolving 10 mg of NAG monomer in DMSO-d6 and

p(NAG-b-A) in CDCl3 (AVH D 500 AVANCE III HD 500 MHz spectrometer). The molecular weight of p(NAG-b-A) was calculated by MALDI-TOF (MS: Bruker AutoFlex Speed MALDI ToF). Thermal stability was studied with TGA (a TGA-50 TGA instrument), (from 25 ℃ to 500 ℃, at heating rate of 10 ℃ min-1 in N2-gas environment, with gas flow rate of 100 mL min-1). Phase transition of polymer was studied through DSC (DSC-60 Plus), (sample holder in aluminum pan, reference Indium, and heating rate was 10.0 ℃ min-1, in N2-gas environment, experiment range -140 ℃ to 550 ℃). X-Ray diffraction (XRD) patterns of the samples were recorded in between 2θ = 5 to 90° using a HR-XRD (Rigaku Smart Lab 9 kW Powder type) equipped with Cu Kα X-Ray radiation source of λ = 1.54 Å).

2.2.6. **Morphological evaluation. Morphology and 3D structural analysis of p(NAG-b-A)** co-polymeric hydrogel was characterized by field- emission scanning electron microscopy (FESEM) (Model: Nova Nano SEM 450) with the accelerating voltage of 15 kV and with High-resolution transmission electron microscopy (HRTEM) (model: Technai G2 20 TWIN). Energy-dispersive X-Ray Spectroscopy (EDXS) analysis was performed and chemical compositions were estimated using a TEM EDS SYSTEM with Octane Plus SDD Detector, which is attached with the HRTEM. For the FESEM analysis, ~0.1mg lyophilized sample (p(NAG-b-A)) was dispersed in 500 µL of isopropanol and drop casted the sample on silicon wafers and coated with Au-Pd for 30 sec. Similarly, for HRTEM analysis, ~0.1 mg of lyophilized p(NAG-b-A) was dispersed in 500 µL of isopropanol along with two drops of 2% uranyl acetate (UA) and the suspension was kept for 6 h. Then few drops of the dispersed sample were casted on the C-coated Cu grid (200 mesh size) and HRTEM images were acquired after proper drying.

2.2.7. **Swelling behavior of poly(NAG-b-A) co-polymeric hydrogel and p(NAG-Ac-NAE) hydrogel.** To find out the dynamic swelling behavior of the prepared p(NAG-b-A)

nanohydrogel, a defined amount of hydrogel was taken and then incubated in the PBS buffer of different pH such as 3, 5, 6, 7.4 and 8 at room temperature (25 oC). After the specified time interval, both the weight and volume of the swelled hydrogel were recorded. The Mass Swelling Index (MSI) and Volume Swelling Index (VSI) was calculated by the eq. (2.1) and eq. (2.2):

$$\text{MSI} = \frac{Ws - Wi}{Wi} * 100 \ldots\ldots\ldots (2.1)$$

$$\text{VSI} = \frac{Vs - Vi}{Vi} * 100 \ldots\ldots\ldots (2.2)$$

Where W_s = weight of swollen hydrogel, W_i = initial dry weight of hydrogel, V_s = swelled volume of hydrogel and V_i = initial dry weight of hydrogel. All the experiments were performed in triplicates.

2.2.8.**Rheological behavior of hydrogel.** Rheological characterization of the hydrogels was performed using an Anton Paar MCR 102 Rheometer with parallel plate (30 mm diameter). Each sample was handed out on the preheated/cooled rheometer plate while it was in the swelled (semisolid) state. The test geometry was lowered to the desired gap height of 0.5 mm, and the excess hydrogel was discarded. The samples were allowed to equilibrate at 25 °C for 2 min before each test. The continuous shear tests with shear rate range from 10^{-6} to 10^{2} s^{-1} were performed to determine the relationship between the viscosity and the shear rate with the various concentrations of hydrogel samples at 34 ^{o}C, 37 ^{o}C and 43 ^{o}C. Preliminary amplitude sweep tests were confirmed with 5% strain (within the linear viscoelastic region of all samples). The frequency sweep tests were conducted to evaluate the change in the viscoelastic modulus as a function of angular frequency (0.1-100 rad s^{-1}) with an oscillating strain of 5%. The temperature ramp test was carried out to determine the relationship between viscoelastic modulus and loss

modulus (G″), and storage modulus (G′) and the complex viscosity as a function of angular frequency and temperature.

2.2.9.**In vitro cytotoxicity assay.** The cytotoxicity and neurotoxicity of p(NAG-b-A) copolymers were assessed through the MTT assay on PC12, Hek293, and HepG2 cell lines. All cell lines were acquired from NCCS, Pune repository, India. For all the experiments, 1 $x10^4$ cells were seeded in 96 well plate in 100 μL of DMEM media supplemented with 100U of penicillin-streptomycin cocktail, 15% FBS and was incubated for 24 h at 37 ℃ with 5% CO_2. After 24 h, the media was replaced with different concentrations of hydrogels in complete DMEM media (200 μL) and was incubated for another 24 h. Then the media was replaced with MTT reagent (5 μg mL^{-1}) and was incubated for 4 h to form the formazone crystal. Then the formazone crystal was dissolved in 200 μL of DMSO. Then, after 30 min of incubation in the dark, the absorbance was measured at λ= 570 nm using a microplate reader (Bioteck). Similarly, to evaluate the material response under the stress conditions, PC12 cells were treated with different concentration (10, 20, 40, 80 and 160 μM) of H_2O_2 and were treated with 100 μg mL^{-1} and 250 μg mL^{-1} concentrations of hydrogel (in presence of stress and by removal of stress) and cell viability was measured using MTT assay. Details of the method have been presented in the subsequent section.

2.2.10. **Animal Ethics Permission.** Animal work involved in this project was approved by the Institute Animal Ethical Committee of IIT (BHU), Varanasi, Uttar Pradesh, India (Regd. No. 2123/GO/Re/S/21/CPCSEA (IAEC Approval Number: IIT(BHU)/IAEC/2022/079). All the experimental procedures and handling of the animals were followed as per the ethical guidelines.

2.2.11. **Cortical neuron isolation and primary culture.** Primary cortical neuron was isolated from Wister rats (day 1 pups). In brief, rat brains were separated and

collected in 1X HBSS solution. By removing the meninges, the cerebellum has been collected. Then, the collected cerebellum was transferred to the serum free neurobasal media. Trituration process was conducted by aspirating and passing the brain tissue using a 20 mL syringe followed by 10 mL, 5 mL and 2 mL syringe. Further, the rat brain cortex was enzymatically treated with 0.25% trypsin for 10 min with incubation at 37 ℃. Then 10 mL of complete media was added to stop the trypsinization process. The cell containing media was passed through a 70 µM strainer. Then, the filtrate cells were centrifuged at 1600 RPM for 10 min at room temperature (25 ℃). After removal of media, the cells were dispersed in complete neurobasal media and 1 x 10^6 cells were seeded in p(NAG-b-A) hydrogel. Then the cells were incubated and cultured in CO_2 cabinet for 24 h at 37 ℃ with 5% CO_2. After 24 h, the media was changed with freshly prepared 1X B27 supplemented and 50-100 ng NGF supplemented complete neurobasal media.

2.2.12. **Immunofluorescence Staining.** The 3 x 10^6 primary neuronal cells were loaded on the hydrogel as well as in poly(L-lysine) coated 12 well plate with neurobasal media supplemented with 10% FBS, 1X B27 and 50 ng NGF. After incubation of different time intervals as per the experimental design, the cells were fixed by 4% paraformaldehyde in PBS for 20 min followed by incubation with chilled methanol and then permeabilization was conducted for 20 mins with 0.1 wt % Triton X-100 in 1X PBS. Cells were blocked with 2% BSA for 1 h at room temperature. Further, the blocked cells were incubated for overnight at 4 ℃ with primary antibody β-tubulin III (1: 500) in 0.1% BSA followed by 1 h incubation with secondary anti-mouse antibody (1:1000), Rhodamine phalloidin and Hoechst 33258. It can be noted that in each step of transition the cells were washed with 1X PBS for three times. Then confocal microscopy images were acquired using a LSM780 Carl Zeiss confocal microscope (Make Germany) for 3D

visualization, and Z-stack images were then imported into Fiji J software[27] for 3D reconstruction, morphological analysis, and co-localization.

2.2.13. **Hemocompatibility of hydrogel.** Whole blood samples were obtained from the animal, RBCs were separated and 5 X 1010 were suspended in 5% dextrose solution (pH 7.4). Treatment with 5% dextrose solution was considered as negative control, while treatment with 0.1-1% Triton-X was considered as positive control. RBCs were incubated for 12 and 24 h at 37 °C/100 RPM with different concentrations (1000, 500, 250, 125 and 62.5 µg mL-1) of p(NAG-Ac-NAE) hydrogel and generated haemolysis were estimated by recording the absorbance at 540 nm.

2.2.14. **In-vitro recovery from oxidative stress.** To evaluate the material's response in stress condition, 1 x 10^4 PC12 cells per well were seeded on 96 well plate with FBS supplemented complete DMEM media and incubated in CO_2 incubator at 37 °C for 24 h. For the measurement of protective role, the cells were treated with different concentrations of H_2O_2 (10, 20, 40, 80 and 160 µM) and treated with 500 µg mL^{-1} of hydrogel and the cell viability was estimated using MTT assay. Similarly, for the evaluation of recovery status, after 24 h of incubation 1 x 10^4 cells per well, they were treated with different concentrations of H_2O_2 (10, 20, 40, 80 and 160 µM) and again incubated for 12 h in 5% CO_2 supplemented humidified environment. After 12 h, complete H_2O_2 treated media removed and cells were treated with 500 µg mL^{-1} concentration of hydrogel and cell viability was estimated using MTT assay. Further for macroscopic examination, 500 µg mL^{-1} (in 1X HBSS solution) of hydrogel was coated on sterile coverslip and placed in 12 well plate. 1 x 10^5 cells per well were seeded in a 12 well plate for 24 h and treated with 40 µM concentration of H_2O_2 for another 24 h followed by AO/EtBr staining. After, staining cells were fixed with 4% paraformaldehyde and

fluorescent macroscopic images were acquired. Similarly, to analyze the protective role of hydrogel on differentiated neurons, $1x10^5$ cells per well were seeded on 500 μg mL^{-1} hydrogel coated slide and differentiated by providing 50 ng NGF supplemented DMEM complete media for 3 days. Then differentiated neurons were treated with 40 μM concentration of H_2O_2 for 24 h and then Immunolabeling was performed as mentioned in the subsequent sections. After Immunolabeling, confocal macroscopic images were acquired and evaluated for the morphology. To elucidate the p(NAG-b-A) hydrogel assisted protective role, 500 μg mL^{-1} of p(NAG-b-A) hydrogel was treated with 40 μM concentration of H_2O_2 in 1.5 mL centrifuge tube for 24 h at 37 ℃. Further, hydrogel was centrifuged and lyophilized. Then the Raman and FTIR spectroscopic analysis were performed and the results were compared with the untreated hydrogel.

2.2.15. **Determination of intracellular ROS generation and effect on mitochondrial potential.** PC12 cells were seeded in 6 well plates and incubated for 24 h. After that, the cells were exposed to 20 μM and 40 μM of H_2O_2 and 250 μg mL^{-1} of p(NAG-Ac-NAE) hydrogel and p(NAG b-A) hydrogel separately for 24 h. Only 20 μM and 40 μM of H_2O_2 treated cells were considered as positive control and samples without treatment were considered as the negative control. Similarly, PC12 cells were also treated with 250 μg mL^{-1} of p(NAG-Ac-NAE) hydrogel and p(NAG b-A) hydrogel separately without exposure to H_2O_2, After 24 h of incubation with H_2O_2, p(NAG-Ac-NAE) hydrogel and p(NAG b-A) hydrogel, cells were harvested by treating the cells with 1 mM of EDTA for 20 min and washed with ice-cold PBS by centrifuging at 1000 X g for 5 min. The pellet was re-suspended in 500 μL of PBS and 50 μM solution of 2',7'–dichlorofluorescin diacetate DCFDA was added and incubated in 5% CO_2 incubator at 37 ℃ for 30 min. The ability of intracellular ROS production was measured by using a flow cytometer (Beckman coulter). Similarly, for determination of the effect on mitochondrial

potential, $1X10^4$ PC12 cells were seeded in per well of 48 well plate and the cells were exposed with 20 μM and 40 μM of H_2O_2 and with/without 250 μg mL^{-1} of p(NAG-Ac-NAE) hydrogel and p(NAG b-A) hydrogel separately. After 24 h of treatment, treated media was replaced with 5 μM Rhodamine 123 and 1 μg mL^{-1} of propidium iodide containing serum-free media. After incubation of 15 min at 5% CO_2 incubator at 37 ℃, fluorescent intensity was measured at an excitation of 488 nm to the emission of 520 nm and an excitation of 550 nm to the emission of 610 nm.

2.2.16. **Egg yolk Angiogenesis assay.** For the investigation of vascular sprouting CEA assay was performed with fertilized chicken eggs, which were purchased from certified poultry, Varanasi, Uttar Pradesh, India. Eggs were incubated at a humidified 37 ℃ incubator for 4 days. After that a small window was created and suspended particles of different concentrations1 μg mL^{-1}, 25 μg mL^{-1} and 100 ug mL^{-1} of hydrogel. Further images were acquired at different time intervals such as: 0, 2, 4 and 8 hours by fixing the Magnus Mag Cam DC-10-megapixel camera to the stereo zoom microscope. Images were then analysed using Angiotool Fiji image J software.

2.2.17. **Wound scratch assay.** Cell migration ability was analyzed by performing wound scratch test on LN229, MDA-MB-231 and L929 cell lines. The cells were seeded in 12 well plates and cultured till to get 80-90% confluent monolayer. Then scratched were made by using 200μL micropipette tip and then cells were washed two times using PBS. Further, three different concentrations of hydrogel, monomer and glycine were added separately and untreated as control and cultured in complete media for 48 hours. Then the images were acquired using inverted microscope at 24^{th} h and 48^{th} h. Then Image J analytical tool was used to estimate the percentage wound closer area at different time interval.

2.2.18. **Live and dead assay.** To examine the comparative cellular death after the treatment, MDA-MB-231 cells were incubated with glycine, NAG, and p(NAG-co-Ac) for 3 days. After 24 hours and 72 hours the cells were stained with acridine orange and ethidium bromide (100μg ML^{-1}) for 30 min. Afterword, the fluorescent macroscopic images were acquired at 20 X magnification.

2.2.19. **Apoptosis and necrotic assay.** After the 24 hrs of treatment with glycine, NAG, and p(NAG-co-Ac) hydrogel, the percentage viable apoptotic and necrotic cell death in LN229 cells ($1X10^5$ cells) were quantified by flow cytometry. After 24 hrs of treatment, cells were detached by using 1mM EDTA solution and collected after washing with PBS followed by staining with Annexin V/PI as per the manufactures protocol (BD Biosciences FITC Annexin-V apoptosis detection kit).

2.2.20. **Semi-quantitative reverse transcription and polymerase chain reaction (sqRT-PCR):**

In brief, $1x10^5$ PC12 cells and Raw264.7 cells were cultured in 6 well plate and followed with treatment p(NAG-Ac-NAE) hydrogel and p(NAG b-A) in presence of H_2O_2 as per the experimental design. After 24 h of treatment, cells were collected and homogenized in 500μL TRIZOL reagent and RNA isolation was performed by phenol: chloroform extraction method. Next, RNA was precipitated from the collected aqueous layer using 1ml of isopropyl alcohol and centrifuge at 12000g for 15 min, 4^o C. The RNA pellet was washed 2 times with 70% ice-cold ethanol and pallet was suspended in 50 μL DEPC treated water and quantified using nanodrop. After RNA quantification, 1μg of RNA was used for cDNA synthesis using oligo dT sequence and Reverse transcriptase polymerase as per the manufacture's protocol. Afterword, PCR were conducted using kicqkstart forward and reverse primer for biomarker target gene VEGFa, Kdr, HIF1α, TNFα, IL1β and housekeeping gene GAPDH as reference by

using Taq polymerase (TAKARA R001A) with 35 repetitive cycle of denaturation at 95^{o}C for 30 sec, annealing at 59^{o} C for 35 sec and extension 72^{o} C for 40 sec followed by final extension at 72^{o} C for 7 min. PCR products were run on 2% agarose gel and densitometry calculations were performed.

2.2.21. **Statistical analysis.** All data presented were confirmed using at least 3 replicates for each of the experimental groups. The results are expressed as the mean of the values ± standard error of the mean. One-way ANOVA was performed to determine the statistical significance ($p < 0.05$), unless otherwise stated.

2.2.22. **Protein ligand interaction screening.** Molecular docking approach was used to determine the nature of interactions between the NAG monomer and different unit of the polymeric units like dimer of N-acryloylglycine-N-acryloylglycine (G-G), N-acryloylglycine- acrylamide(G-A); trimer of N-acryloylglycine-N-acryloylglycine-N-acryloylglycine (G-G-G), N-acryloylglycine-N-acryloylglycine-acrylamide (G-G-A; G-A-G; G-A-A) and with GSK3β protein. Crystal structure of GSK3β was taken from the RCSB protein data bank with PDBID-1q5k (https://www.rcsb.org/structure/1Q5K) having a resolution of 1.94 Å. Further, the reference molecules such as TMV (N-(4-Methoxybenzyl)-N′-(5-nitro-1,3-thiazol-2-yl)urea), SB415286 (3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1H-pyrrole-2,5-dione) and SB216763 (3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione), which are the potent inhibitors of GSK3β, were retrieved from the PubChem. Macromolecule preparation and energy minimization were conducted on UCSF Chimera 1.10.2 with 100 steepest descent steps by applying AMBER ff14SB. Similarly, for the ligand structure, the energy was minimized using PyrX interface by applying MMff94 force field in AutoDock 4.2 tool. The receptors were prepared by removing the hetero atoms, and adding the polar hydrogen atoms and Kollman charges. Further, the solvation parameter

and 80 x 80 x 80 grid box were generated at ATP pocket. Further, computational rigid docking was performed by setting the genetic algorithms of 30 run and Lamarckian parameter. Finally, the analysis of data was performed by using AutoDock, Chimera software and ligplot software.

2.2.23. **Theoretical and computational methodology.** For the evaluation of physicochemical properties and bioactivity, random arrangements were designed using Chem Draw Ultra 8.0 software and categorized into: (1) Linear homopolymer (polymer of N-acryloylglycine (G) arranged in G, G2……G_n), (2) Linear hetropolymer (polymer of N-acryloylglycine (G) and acrylamide (A), GA (GA1), G-A-G……..(GA_n)), (3) cross-linked homopolymer (polymer of N-acryloylglycine cross-linked by using divinyl benzene (DVB), D1, D2……D6) and (4) arranged cross-linked hetropolymer (polymer of N-acryloylglycine and acrylamide and cross-linked by DVB (D) and final synthesized polymer termed as p(NAG-co-Ac) hydrogel. Firstly, bioactivities of all these structures were evaluated using molinspration software (https://www.molinspiration.com/). The predicted structures were determined using Avogadro software (version1.2.0) and then optimized using the Merck molecular force field (MMFF 94) at molecular mechanic level. Further, Density functional theory (DFT) calculation were carried out using ORCA 5.3.2 computational package. The computing efficiency was accelerated by using the resolution of identity approximation with basis sets B3LYP/6-311g*/def2-SVP level. Further, CPCM continuum solvation model was applied at water level ($\varepsilon = 80.4$).

All the other experimental sections have been described with subsequent chapters. Such as the experimental part for the siRNA related work has been explained with the **Chapter 3: Part IV.**