Chapter 2

Experimental: Materials Synthesis and Characterization Techniques

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2.1 Materials

- For **Chapter 3,** PTMG with a number average molecular weight (Mn) of 2900 g/mol (terathane) was purchased from Sigma–Aldrich. Chemicals, namely 2,4-toluene diisocyanate (TDI), 4,4′-methylene diphenyl isocyanate (MDI), 1,6-hexamethylene diisocyanate (HDI), isophorone diisocyanate (IPDI), and solvent dimethylformamide (DMF), were used as received from Merck. Additionally, the anticancerous drug (paclitaxel) bioreagent ≥98% (Sigma) was obtained from Sigma–Aldrich (USA), and henceforth, paclitaxel will be denoted PTX.
- For **Chapter 4,** Poly (tetramethylene glycol) (PTMG) (number average molecular weight, Mn = 2900 g/mol) was purchased from Sigma–Aldrich. It was kept in vacuum oven at 70°C for at least 24 hours to remove residual moisture before synthesis. 2,4-toluene diisocyanate (TDI), catalyst, dibutyltin dilaurate (DBTDL), and solvent, dimethyl formamide (DMF), triethylamine (TEA) were used as received from Merck. L-Tryptophan (TRP) purity ≥ 99% and anti-cancerous drug (Paclitaxel) bioreagent ≥ 98% (Sigma) were obtained from Sigma Aldrich USA. HPLC-grade water and Acetonitrile were purchased from Merck, and anticancer drug PTX from Sigma–Aldrich.
- For **Chapter 5,** Poly (tetramethylene glycol) (PTMG) (number average molecular weight, M_n = 2900 g/mol) was purchased from Sigma–Aldrich. 1,6-hexamethylene diisocyanate (HMDI), catalyst, dibutyltin dilaurate (DBTDL), and solvent, dimethyl

formamide (DMF), triethylamine (TEA), were used as received from Merck. L-Cystine dihydrochloride (CYS), purity ≥ 99%, and anti-cancerous drug (Paclitaxel) bioreagent ≥ 98% (Sigma) were obtained from Sigma Aldrich, USA. Henceforth, L-Cystine will be referred to as CYS. Tryptic-soya broth (TSB) and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) were purchased from Thermofisher Scientific, India.

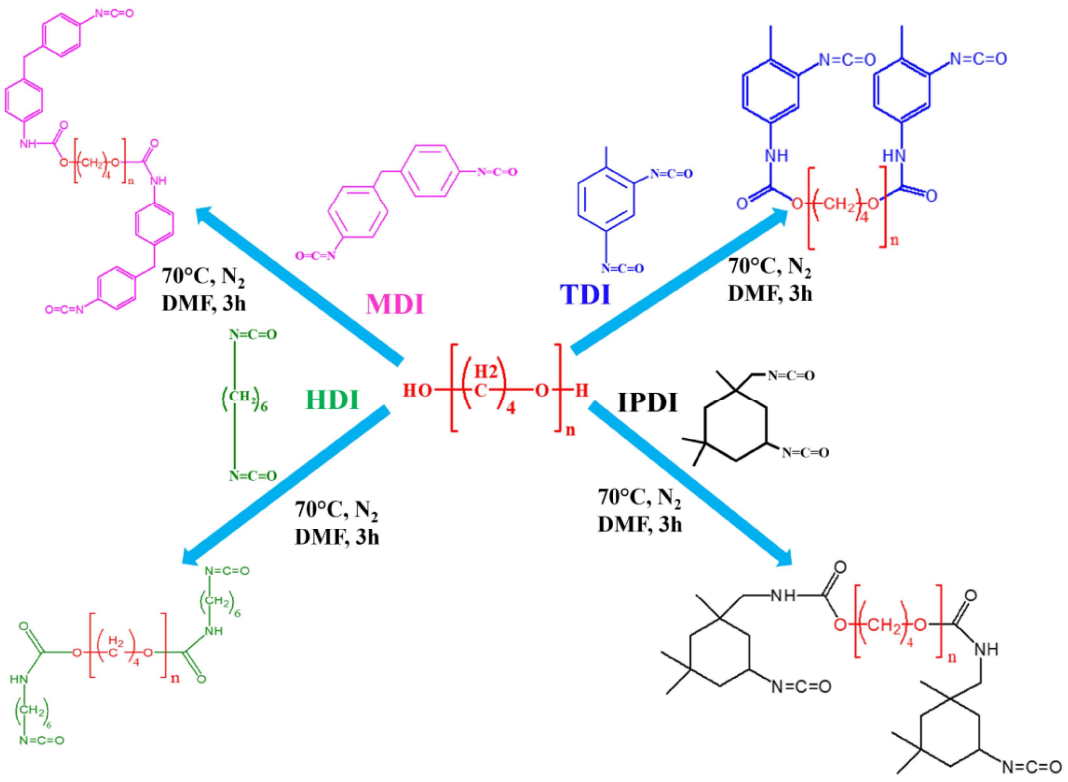
- For **Chapter 6,** Poly (tetramethylene glycol) (PTMG) (number average molecular weight, Mn = 2900 g/mol) was purchased from Sigma–Aldrich. 2,4- toluene diisocyanate (TDI), catalyst, dibutyltin dilaurate (DBTDL), and solvent, dimethyl formamide (DMF), triethylamine (TEA) were used as received from Merck. L-Glycine (Gly, G1) purity ≥ 99%, Glycine dipeptide (Gly-Gly, G2), Glycine tripeptide (Gly-Gly-Gly, G3), and anti-cancerous drug (Paclitaxel) bioreagent ≥ 98% (Sigma) were obtained from Sigma Aldrich USA. From this point forward, all the grafted PU will be referred to as PU-G1, PU-G2, and PU-G3.

2.1.1 Synthesis of different Polymeric architectures for Controlled Drug Release

2.1.1.1 Synthesis of various Polyurethanes by varying the diisocyanate units

Polyurethane (PU) synthesis involves blending diol and diisocyanate. Initially, PTMG and various diisocyanates (TDI, MDI, HDI, and IPDI) were mixed in a molar ratio of 1:1.05 at 70 °C for 3 h. The reaction was performed using DMF as the solvent. This resulted in the formation of an isocyanate-terminated prepolymer (PP) within a three-necked round-bottom flask, equipped with a mechanical stirrer and nitrogen purging inlet, placed in a silicon oil

bath. Subsequently, the entire reaction mixture was poured into deionized water (nonsolvent). The PU was separated by filtration, and consecutively washed to maintain a neutral pH and to remove any unreacted diisocyanate. The final material was kept overnight in a vacuum oven under reduced pressure at 70 °C. A schematic representation of this strategy is shown in detail in **Scheme 2.1.11.**

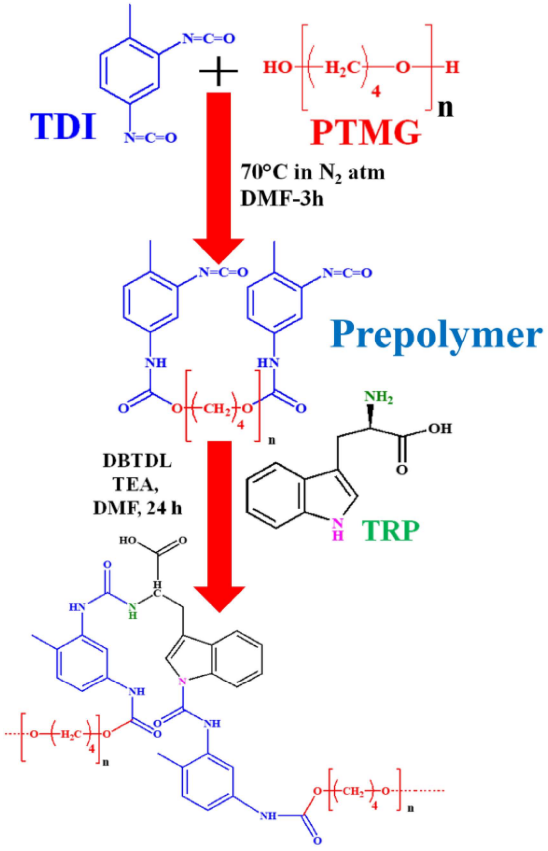


Scheme 2.1.1.1 Reaction Scheme Showing Different Diisocyanates React with PTMG forming Urethane Chains to Form Various Prepolymers (PU-T, PU-M, PU-H, and PU-I Using TDI, MDI, HDI, and IPDI as Diisocyanates, Respectively)

2.1.1.2 Synthesis of Tryptophan-based Polyurethanes

Polyurethane (PU) synthesis was done by a two-step methodology by varying the relation between the hard & soft segments from 30-70%. The strategy details are given in the **Scheme**

2.1.1.2. In the first step, PTMG and TDI were mixed in a molar ratio of 1:1.05 at 70°C for 3 h to yield isocyanate-terminated prepolymer (PP) (in this manuscript PP will be termed as pure PU) in a round bottom flask having three necks, equipped with mechanical stirrer & nitrogen purging inlet in a silicon oil bath.



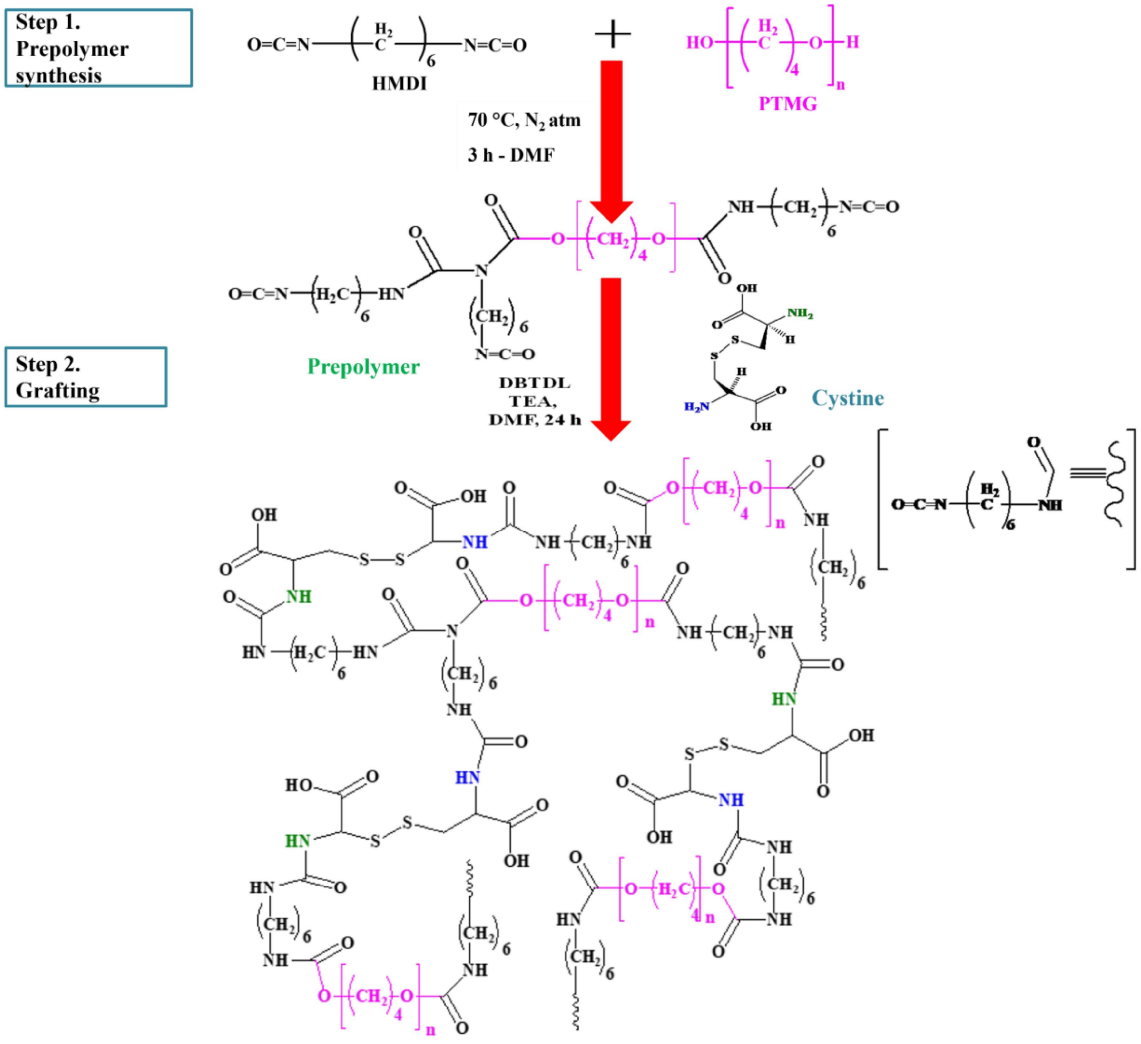
Scheme 2.1.1.2: The reaction scheme illustrates the architecture of PU via urethane chains, followed by tagging with Tryptophan linkages to form a superstructure, and the variation will be called PU-TRP.

In the next step chain extension was done using TRP keeping the ratio 1:0.5, added to prepolymer flask in presence of DMF (10-15 ml depending upon the viscosity) solvent and DBTDL catalyst (0.1 ml of 1 wt.% toluene solution) dropwise to complete the polymerization reaction with rapid stirring at 70°C for 24 h. At this point of time, an appropriate amount of triethylamine (TEA) is added to the reaction vessel to keep the medium slightly basic. For filtration and material collection, follow the steps described in the earlier section 2.1.1.1.

2.1.1.3 Synthesis of Cystine-based Polyurethanes

In the first step, PTMG and HMDI were (1:1.05) mixed; here we have used Cystine for

chain extension in the second step (1:0.5). Other things are same as already discussed in sections 2.1.1.1 and 2.1.1.2. **Scheme 2.1.1.3** represents the strategy details.

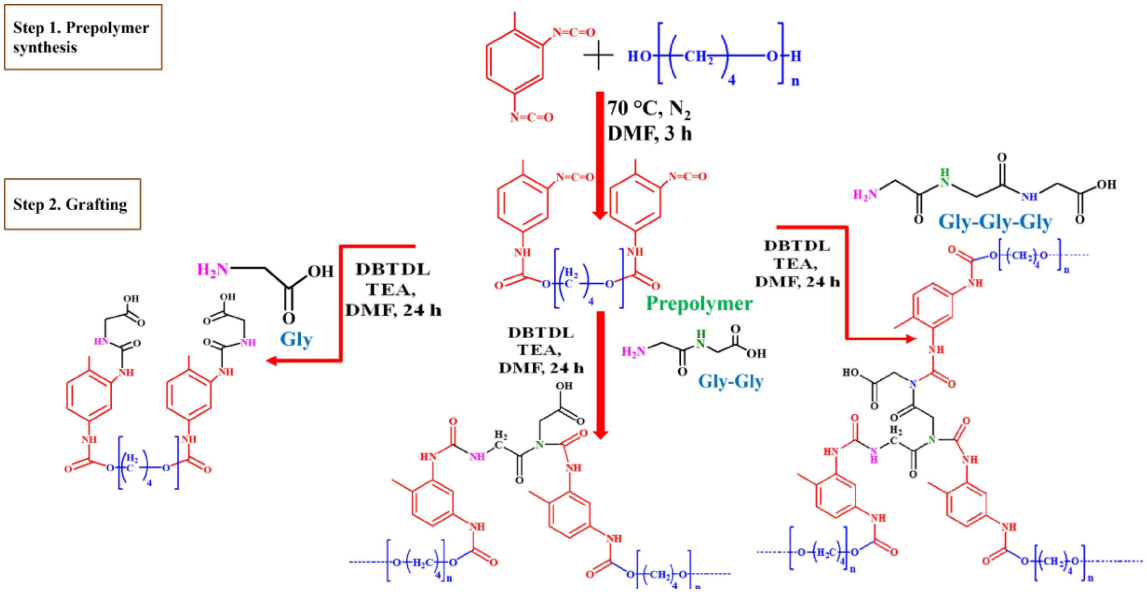


Scheme 2.1.1.3: The reaction scheme illustrates the architecture of PU via urethane chains, followed by tagging with Cystine linkages to form a superstructure, and the variation will be termed 'PU'.

2.1.1.4 Synthesis of Glycine and its homopeptides-based Polyurethanes

Polyurethane (PU) preparation was conducted using a two-step methodology. This section is already described in section 2.2.1.2. In 2nd step, chain extension was done using Glycine

amino acid and its homopeptides at a ratio of 1:0.5 in presence of DMF (5-10 ml, depending on the viscosity) solvent, and DBTDL catalyst (0.1 ml of 1 wt.% toluene solution) was added dropwise to complete the polymerization. **Scheme 2.1.1.4** represents the strategy details.



Scheme 2.1.1.4: The reaction scheme illustrates the architecture of PU via urethane chains, followed by tagging with G1, G2, and G3 linkages to form a superstructure, and the variation will be called PU-G1, PU-G2, and PU-G3.

2.2 Characterization Techniques

2.2.1 ^{1}H and ^{13}C

NMR spectroscopy (**Figure 2.2.1**) is based on the principle that some atomic nuclei have a property called spin and behave like tiny magnets. Placing these nuclei in an external magnetic field causes them to align with it, and it also excites them with radiofrequency radiation. ^{1}H, ^{13}C NMR were performed on an AVH D 500 AVANCE III HD 500 MHz OneBay NMR Spectrometer (BRUKER BioSpin INTERNATIONAL AG, Germany) at 0-20 ppm and 0-200 ppm spectral width, and 500 scans with DMSO-d6 (99.98%) as a solvent.

Figure 2.2.1: representation of NMR Machine

2.2.2 FTIR

Fourier-transform infrared spectroscopy is a powerful, non-destructive technique for analyzing the chemical composition. The principle underlying this method is to irradiate the sample with infrared radiation. Molecules with specific functional groups, usually covalent bonds that have a dipole moment, absorb specific wavelengths of the IR radiation due to resonance effects. The absorption causes the functional groups to oscillate at characteristic frequencies. FTIR yields a spectrum by measuring the intensity of absorbed IR light at different wavelengths (wavenumbers). The spectrum, which plots absorbance or transmittance against wavenumber, identifies the presence and types of functional groups in the sample, providing much information about its chemical structure. In this investigation,

transmission spectra were recorded in ATR mode by using (FTIR-4700 JASCO with a resolution of 2 cm^{-1} in the spectral range of 4000 to 400 cm−1.

2.2.3 UV-VIS

Light absorption is utilized as a tool for both qualitative and quantitative analysis of specimens. This technique involves exposing the specimen to electromagnetic radiation in the UV-VIS range, measuring electronic transitions. The UV-visible absorption (**Figure 2.2.3**)[119] spectra of pristine and functionalized membranes were recorded using a Jasco V-650 spectrometer, scanning wavelengths from 200 to 800 nm at a constant rate of 200 nm/min.

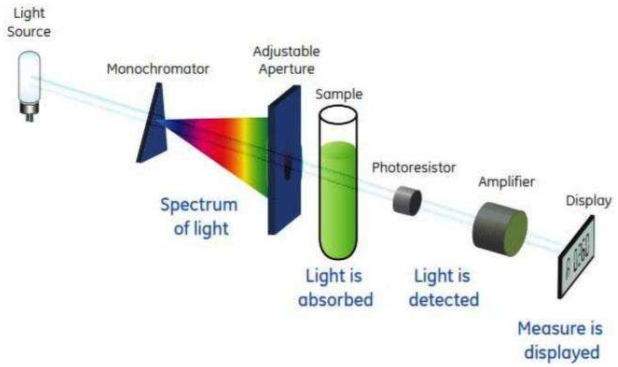


Figure 2.2.2: Schematic representation of UV-VIS spectroscopy

2.2.4 XRD

XRD is an analytical technique used to observe the atomic and molecular arrangement of crystalline materials. This is done by sending an intense X-ray beam onto a rotating sample, causing the diffracted X-rays to take place according to the particular directions dictated by the internal arrangement of the crystal. This diffraction yields a pattern of peaks that obey Bragg's Law and, by this means is analyzed by the researchers for the determination of

different phases in crystalline materials and the crystal structure of the material. XRD can distinguish between regions that are amorphous or crystalline because amorphous materials lack a well-defined structure and thus do not give well-defined diffraction peaks. A standard XRD setup consists of an X-ray source, sample holder, and detector to record the diffraction pattern.

For this study, a Rigaku Miniflex X-ray diffractometer with Cu Kα having λ = 1.54 Å in Bragg-Brentano configuration was utilized to obtain the XRD data, as presented in **Fig. 2.2.4a**. The instrument used had a graphite monochromator, and the X-ray wavelength applied was 1.5418 Å. The data obtained from the XRD was indexed and compared with the

Figure 2.2.4a: XRD machine

JCPDS database in order to confirm the phase purity of the samples. XRD is highly utilized non-destructive material characterization technique, which enables one to determine phase, purity, and crystal structure. The basis for this technique lies on Bragg's law of diffraction developed by W. H. Bragg and W. L. Bragg. According to Bragg's law, when X-rays reflect off a set of regularly spaced crystal planes, constructive interference is achieved only under two conditions: the angle of incidence must be equal to the diffraction angle, and the path length difference between the incident and diffracted X-rays must be an integer multiple of the X-ray wavelength. Mathematically, Bragg's law is expressed as:

$$2d \sin\theta = n\lambda$$

where λ is the X-ray wavelength, d is the interplanar spacing, θ is the angle of incidence, and n is the order of diffraction. A schematic diagram showing Bragg's law is represented in **Fig. 2.2.4b.**

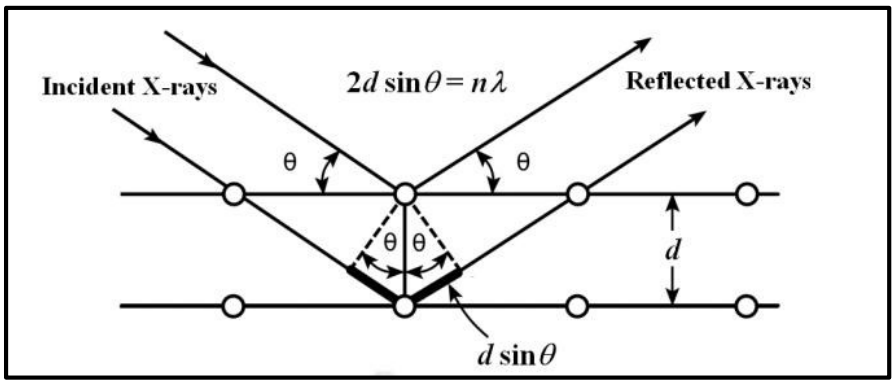


Figure 2.2.4b: Schematic diagram illustrating Bragg's law.

Strong Bragg peaks occur only at particular scattering angles where the condition of constructive interference is satisfied according to Bragg's law. The number of Bragg peaks that occur in a diffraction pattern varies with the crystal symmetry of the material. For

example, low-crystal-symmetry materials such as monoclinic crystals have many Bragg peaks because there are many lattice planes. High crystal symmetry such as in cubic and tetragonal structures will display fewer Bragg peaks since these structures have few lattice planes. Amorphous materials, having no symmetry, typically show a broad hump instead of distinct peaks. In addition, the dimensions and form of the particles affect the Bragg peaks.

2.2.5 POM images

Polarized optical microscopy (POM)[120] is a method used to analyze materials that display birefringence, a property where the material has varying refractive indices along different axes. Optical images of the PU samples, with a thickness range of 20 μm, were taken using an ACCU-SCOPE, EXC-120 series, with a resolution of 40×.

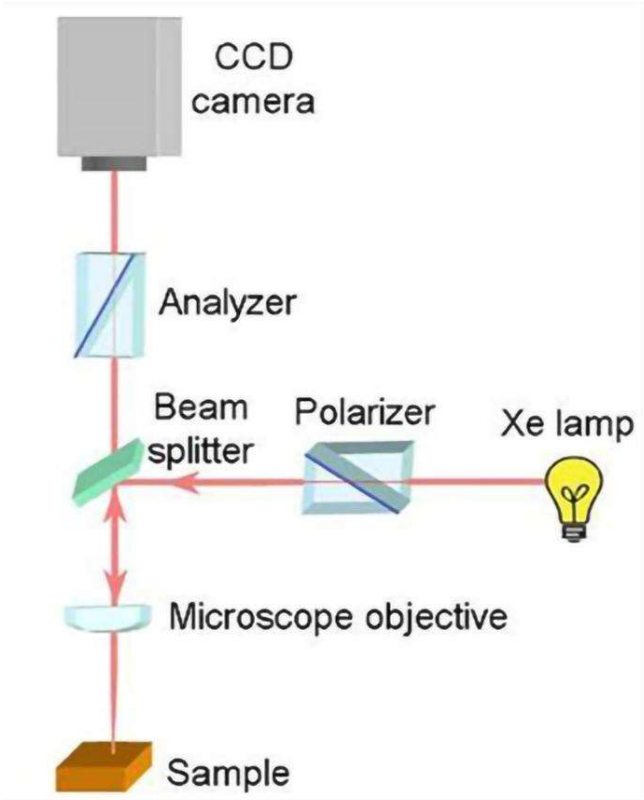


Figure 2.2.5: Schematic representation of Polarized Optical Microscopy

2.2.6 Thermal analysis

2.2.6.1 TGA-DSC

Thermogravimetric Analysis is simply the method of measuring sample mass as a function of temperature, which determines the thermal stability and capacity to detect mass changes, such as absorption, desorption, phase transitions, and thermo-decomposition. If sample weight remains constant through a certain temperature range, it is concluded that the material is thermally stable at this range. TGA also enables one to determine the calcination temperature at which the sample tends to break down. On the other hand, Differential Scanning Calorimetry is one of the thermal analysis techniques that varies between the difference in heat of a test sample and a reference sample at the same temperature by keeping them at a set temperature. A sample is usually packed in a small, very compact alumina crucible along with keeping them at a set temperature in an inert environment that exhibits no heat-related effects, and two such crucibles are heated at a constant rate; the temperature difference is plotted either over time or at specific temperature points. Changes occurring in the test sample, such as heat release or absorption, result in increasing or decreasing the sample's temperature compared with the reference temperature. These temperature changes are manifestations of exothermic or endothermic processes. The extent of these temperature changes can thus be used to quantify the amount of heat released or absorbed in a given physical or chemical transformation. In this experiment, the TGA analysis was conducted in a temperature range of 40–600 °C, with a heating rate of 20 °C/min under a nitrogen atmosphere. The melting and glass transition temperatures of the samples were determined by rapidly cooling them from room temperature to −50 °C at a rate of 10°/min, followed by heating to 200 °C at a rate of 10°/min using a Mettler 832 DSC. The

cooling process from 200 to −50 °C was conducted at the same rate as heating. The heat of fusion (ΔH) during melting was calculated from the area under the endothermic peak. Before use, the DSC instrument was calibrated using indium.

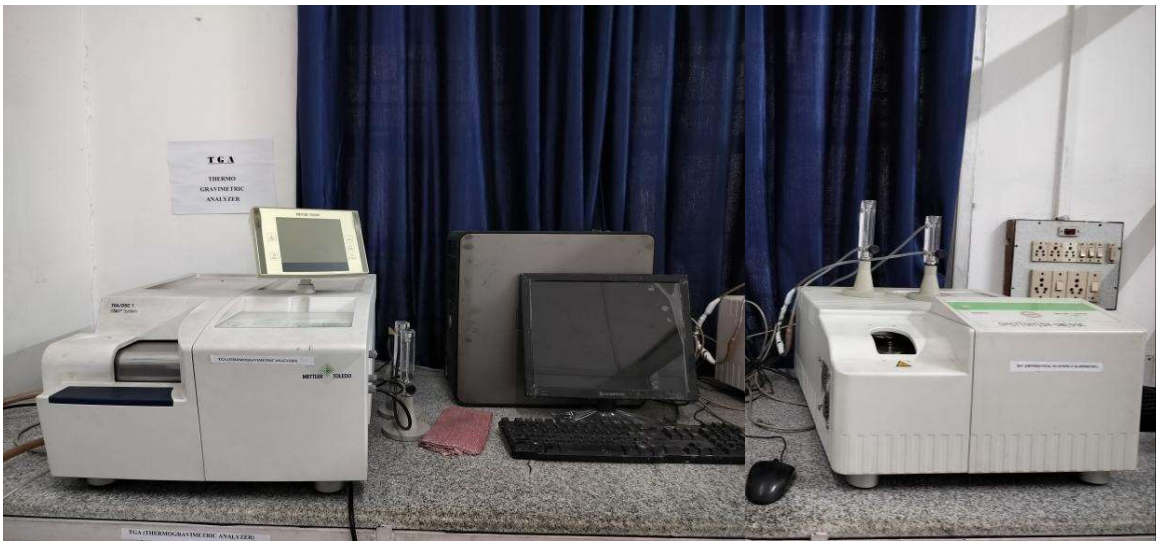


Figure 2.2.6.1: TGA-DSC Machine

2.2.7 Mechanical responses

2.2.7.1 UTM

The mechanical characteristics, including tensile strength, elongation at break, tensile modulus, and toughness, of each PU sample were assessed using universal testing machine (Tinius Olsen H50KL) with a constant strain rate of 5 mm/min. Specimens with dimensions of (50 × 10 × 10) mm^3 were fabricated using the solvent-casting method. To ensure accuracy, the measurements were replicated three times to minimize potential errors.

2.2.7.2 DMA

Dynamic mechanical analyzer (DMA): Anton Paar MCR702e model was utilized to measure the mechanical properties and relaxation behavior of the samples. The assessments were conducted in tension mode (0.001-0.01%) within a temperature range spanning 25 to -150 ℃. The measurements were performed with a scanning rate of 2 °/min at a constant frequency of 1 Hz. The sample thickness was kept at 5 mm.

Figure 2.2.7.2: Dynamic Mechanical Analyzer Machine

2.2.8 Morphological investigation

2.2.8.1 SEM

A Scanning Electron Microscope (SEM) uses a focused electron beam to capture images of a sample, (with a schematic diagram of the SEM shown in [121]**Figure. 2.2.8.1)**. In this microscope, a cathode within the electron gun emits a narrow electron beam at both low and high energy levels, which enhances the spatial resolution while minimizing the risk of sample charging or damage. The vacuum in the microscope generates the electrons, which pass

through electromagnetic lenses that focus the beam onto the specimen. The interaction of the electron beam with the sample generates various types of electrons. A detector captures the secondary electrons, and by comparing the intensities of secondary and primary electrons, a detailed image of the sample surface is created. The electron beam scans the sample in a

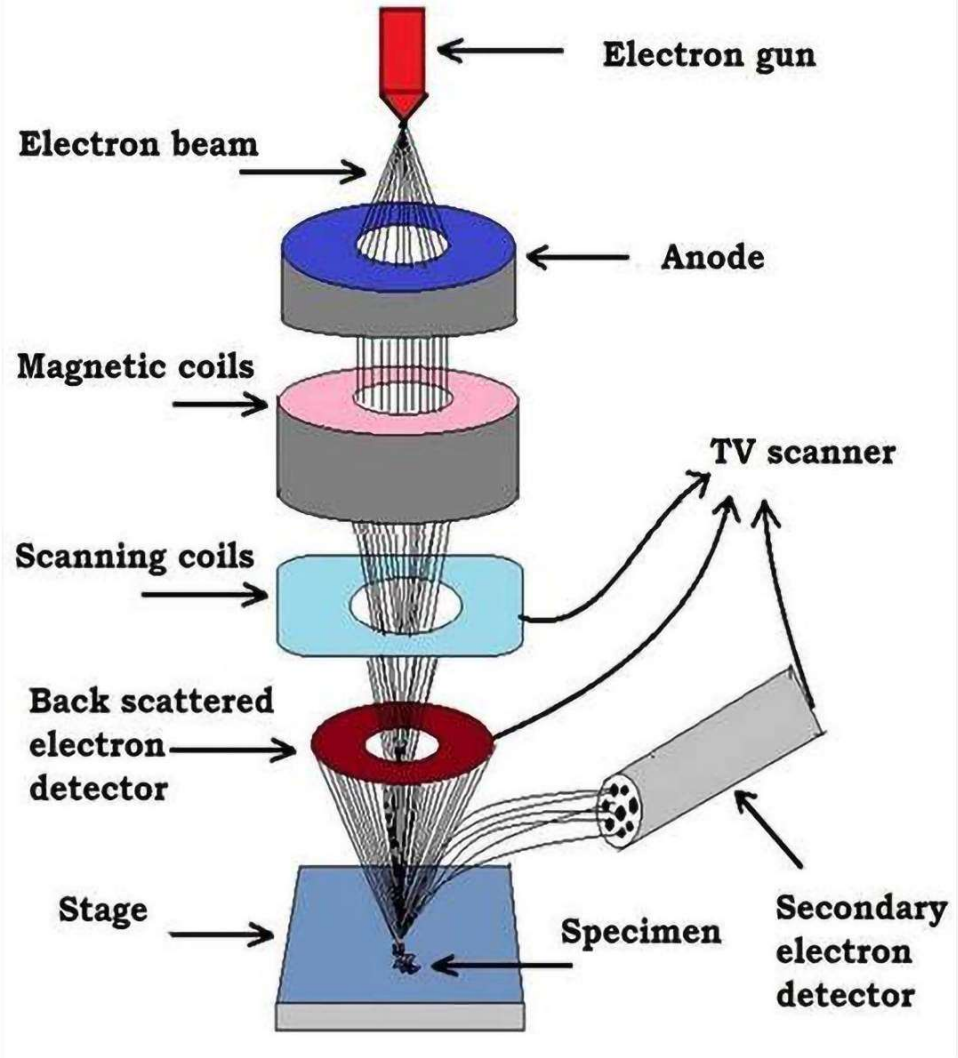


Figure 2.2.8.1: Schematic representation of SEM

faster pattern, and by correlating the beam's position with the detected signals, an image is formed. In this study, images of the material were obtained using a Zeiss SUPRA 40 SEM.

Prior to imaging, a Pd–Au coating was applied to the sample for 120 seconds using a sputtering apparatus. Additionally, the bulk morphology of all samples was analyzed following solvent evaporation.

2.2.8.1 BT-SEM

Bench Top Scanning Electron Microscope (BT-SEM). Morphological investigation of all the samples was done using JCM-6000 Plus BenchTop Sem Neoscope (JEOL Asia PTE Ltd). Prior to imaging, the samples were coated with a Pd-Au layer for 120 seconds using a sputtering apparatus. All the samples, prepared through solvent evaporation, were observed at a magnification of 1000x (10kV) using a polarizer attached.

2.2.8.2 TEM

Transmission Electron Microscopy. Morphological investigation has been done using HRTEM (Technai G220 TWIN, FEI Company of USA) operating at an accelerating voltage of 200 kV. Polymers were drop-cast on the carbon-coated copper grid. Before measurement, the Solvent was slowly evaporated by blow drying at 70°C before the measurement.

2.2.8.3 AFM

Scanning Probe Microscopy (SPM) is an imaging technique that uses a small-dimension probe to scan specimens. This method allows for the assessment of surface topography and physical properties by analyzing the motion of the probe. SPM is commonly employed to examine surface characteristics through the probe's interaction with the surface. It includes techniques such as Atomic Force Microscopy (AFM) and Scanning Tunneling Microscopy (STM). In the present study, AFM was used for morphological analysis. The samples were scanned in semi-contact mode across the surface using an NT-MDT instrument (Russia). The

AFM utilized a silicon nitride tip mounted on a 100 mm long single-beam cantilever, with a resonant frequency of 240-255 kHz and a spring constant of 11.5 N/m.

Figure 2.2.7.4: AFM machine

2.2.9 Molecular Weight determination by GPC

The molecular weights of the polymers were determined using GPC with DMF as the eluent, and the values were calculated using Empower 3 software.

2.2.10 Particle Size Determination

Particle size was measured using a Nanoparticle Analyzer (sz-100, Horiba Scientific). The samples were crushed in liquid nitrogen to obtain fine powder for better dispersion. The particle sizes of PU-T and PU-M could not be determined due to their rubbery texture,

which prevented them from forming fine particles.

2.2.11 Swelling Study and Degradation Study

Swelling property has been checked using 0.2 mm thickness of polymer film in PBS (10 ml solution) medium without any enzyme. The weight of the polymer has been calculated by carefully drying the outer surface's excess water. The formula below is used to calculate the values,

$$\% \text{ of swelling} = \frac{(Final\ weight - Initial\ weight)}{Initial\ weight} \times 100$$

The images of films were taken to see if any degradation takes place for up to 5 consecutive days.

2.2.12 Contact angle measurement

To determine the hydrophobicity of the synthesized prepolymer samples, the contact angle was measured using a Kruss GmbH-DSA25S (Germany) drop-shape analyzer. Measurements were performed in triplicate to avoid errors.

2.2.13 General method for determination of Critical micelle concentration by UV-VIS spectroscopy

The determination of critical micelle concentration (CMC) was conducted at room temperature. A 15 mL aliquot of samples from the 1 mg/3 mL stock solution (in DMF) was introduced to a quartz cuvette, and the UV-Vis spectrum was recorded. Sequential additions of incremental amounts (0.1 or 1000 µg) of solutions were made using a pipette to the contents in the cuvette, and a series of spectra were recorded. Adequate mixing of the components was ensured after each incremental addition of the sample stock solution. The absorption spectra in the range of 200–800 nm were recorded after allowing the solution to

reach a steady state, typically taking about 5 minutes. The inflection point of the absorbance (optical density) versus log concentration was considered as the CMC of the solutions. Typically, approximately more than 10 spectra were accumulated for each sample's CMC determination. From the intersection of the two tangent straight lines, the critical micelle concentration (CMC) value was determined.

2.2.14 Fluorescence spectroscopy

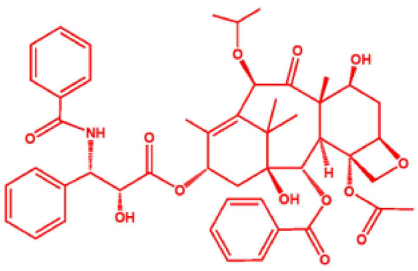
Fluorescence measurements were done on a Cary-Eclipse fluorescence spectrophotometer (Agilent Technologies) at an excitation wavelength of 280 nm (λ_{max} of TRP is 280nm). A stock solution of 1mg/3ml concentration in DMF was made, and from there, dilution was done; these various solutions of different concentrations, ranging from (1-1000) µg/ml, were used for the study at room temperature.

2.2.15 Fluorescence imaging of samples

Images were captured by making a very thin layer (100 µg/ml in DMF and dried at room temperature) of sample on a glass slide under a fluorescent microscope (Nikon, USA) equipped with NIS software for visualization and analysis.

2.3 *In-vitro* Drug Release

Standard curve was drawn, and the values were taken at 227 nm in different concentration ranges (1-100 µg/ml). PBS buffer (release medium) at pH~7.4.was being used for *in-vitro* drug release model. Prepolymer and its graft copolymers were loaded with drug utilizing solution route, by dissolving the polymer and drug (5 wt.% % with respect to



Scheme 2.3: Structure of paclitaxel

polymer weight) in DMF. The mixture was stirred overnight to ensure maximum dissolution of the drug. Later on, the solution was kept aside to evaporate the solvent naturally. These drug-loaded grafted polymers were put into 50 ml of release medium (PBS, pH ~7.4) in an incubator shaker with moderate shaking at 37°C. Aliquot amount was taken at different time intervals from the release medium and replaced with the same quantity of fresh PBS at each time to maintain the volume. UV-VIS spectrum was recorded at 227 nm (as the drug in buffer solution exhibits a peak at this position) using UV-VIS spectrophotometer, Jasco 650, and by this, the concentration of anti-cancerous drug in the PU was measured.

2.4 Molecular Docking

Molecular docking analysis was performed to investigate possible interactions between PTX and the polymers. The PDB file format was used to access the Protein Data Bank, which included paclitaxel's structures (PDB ID: TA1). ChemDraw 20.1.1 was used to draw the structure of polymers, and Chem3D 20.1.1 was used to reduce the energy of the structure. The receptor and ligand molecules were prepared using the Biovia Discovery Studio 2024 Client in order to facilitate docking. During the preparation procedure, polar hydrogen atoms and Kollman charges are added after the water molecules are removed. The molecular docking technique was done by using the grid box containing the active sites and using the blind docking protocol. The docking was then performed using a Lamarckian genetic technique with the AutoDockTools-1.5.7 software and AutoDock Vina. The docking analysis and 3D representations were carried out using the Discovery Studio 2024 client.

2.5 Cell Culture

2.5.1 Cell Adhesion and imaging on Grey Filter

The cell adhesion properties of all the samples were studied on SiHA (human cervical squamous carcinoma cell line) and 3T3 (Swiss albino mouse embryo tissue, normal cell line) cells, each at a confluency of 2×10^3 cells/well in a 96-well plate. Sample solutions and cell mixtures were added simultaneously to grow cells and then incubated in a 5% CO_2 atmosphere for 24 hours. Next, the wells were washed twice with PBS to remove unattached cells. Cellular fixation was performed with 4% paraformaldehyde solution for 20 min, followed by another PBS wash. With a volume of 20% methanol, cell permeabilization was done for around 20 min. Staining was done using an aqueous solution of 0.2% crystal violet for around 20 min. Excess stain was removed by gentle washing twice with PBS, and residual crystal violet was eluted with 10% acetic acid. At a wavelength of 570 nm, optical density (OD) was recorded using a microplate reader. The OD values were directly proportional to the number of cells attached to the material. Cell adhesion images were taken with the help of a phase contrast microscope (Leica, Germany) after fixing the cells with 4% paraformaldehyde solution and gentle washing with PBS buffer. The following formula was used to calculate the percentage of cell adhesion:

$$\% \text{ cell adhesion} = \frac{OD \text{ of sample}}{OD\ of\ control} \times 100 \ldots\ldots\ldots \text{(I)}$$

Samples were used in dispersion state during this study.

2.5.2 MTT assay

Cell viability of the samples was analyzed using MTT assay with two cell lines, 3T3 and SiHA. Sample suspensions were prepared and placed into 96-well plate, followed by cell seeding in complete DMEM media, containing 10% fetal bovine serum, supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin. Each well received 1×10^4 cells[122,123] and

was subsequently incubated for 1, 2, and 3 days in a 5% CO_2 atmosphere at 37 ℃. All MTT assay measurements were performed in triplicate. Subculture was done once the cells were around 70% confluent, to avoid overgrowth and ensure health, logarithmic phase of growth. Here, the cells have maximum metabolic activity, and this is important for reproducible results in downstream processes like the MTT assay. For MTT cytotoxicity analysis, cells were plated at optimal densities so that the cells grow uniformly and maintain the same level of metabolic activity throughout the course of the assay. Confluency and passage number were closely monitored and maintained the same throughout experiments to avoid variability. The complete media containing nonviable cells was replenished with 100 µl of fresh media in each well. 0.1 mg/ml MTT solution in complete media was added to each well and incubated for 4 hours at 37 ℃ to produce DMSO soluble formazan. Absorbance was recorded at 570 nm using a microplate reader (BioTek, SYNERGY H1). The percentage of cell viability was calculated using the formula provided below:

$$\% \text{ cell viability} = (\text{ODt}/\text{OD c}) \times 100 \ldots\ldots\ldots \quad (\mathbf{II})$$

where optical density is termed as OD, c represents control, and test samples represent t.

In this MTT assay study, different concentrations (20, 50, 100 µg/ml) of grafted polymer (PU) and pure one were used with both cell lines to determine the maximum safe amount that can be administered without causing toxicity. The same investigation is also conducted with the drug-loaded material, where pure drug is dispersed in a (1:1) DMSO-water mixture to make the final solution less than 1% in DMEM.

2.5.3 AOPI imaging

Fluorescence microscope is being used to examine the cell proliferation factor on

polymers. Cells (both with SiHA & 3T3) were grown in 24-well plates at a confluency of 1 × 10^4, then after 24 hrs of cell seeding, sample treatment was done keeping 20 µg/ml concentration. Samples (PU and various modified PU) were sterilized before each cellular study in the Ethylene Oxide Gas sterilizer (EiligPlaz, Trueklav, vacuum sterilizer). The samples were preconditioned at 40 °C and 60% relative humidity for 6 hours, in EtO-permeable Tyvek pouches. Sterilization consisted of three vacuum pulses followed by ethylene oxide gas injection at a concentration of about 600 mg/L. The exposure was kept at 40 °C for 4 hours. Following sterilization, six air washes were performed on the chamber to expel the remaining gas. The samples were then moved to an aeration chamber and stored at 40 °C for 24 hours. For pure drug and drug-loaded samples, syringe filters were used for filtration. Each well was washed twice with fresh PBS to take out the dead cells, fixed with 4% paraformaldehyde solution for 20 min, washed with PBS again, and staining was done by using fluorescent dye acridine orange and ethidium dibromide (100 µg/ml) for 5 mins followed by washing with PBS twice and were subsequently incubated for another 15 mins in dark. Images were captured with the help of fluorescent microscope (Leica, Germany).

2.5.4 Fluorescence images of materials inside the cell

With the help of fluorescent microscope (Leica, Germany), fluorescence images of grafted copolymers are taken. Cells (SiHA) were seeded in 24-well plates at a confluency of 1 × 10^4, sample treatment was done keeping concentration at 100 µg/ml. Each well was washed twice with fresh PBS to take out the dead cells, fixed with 4% paraformaldehyde solution for 20 min, washed with PBS again, and subsequently incubated in the dark until data being recorded or images were captured at different time intervals (15min, 1hr, 3hr, 6hr, 9hr, 12hr, 18hr, 24hr).

2.5.5 Cellular uptake in healthy cells

With the help of a microplate reader fluorescence properties of PU-TRP and TRP (OD value) are calculated at different time intervals (15 min, 30 min, 1 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h), at a wavelength of 280 nm. Cells (3T3) were seeded in 96-well plates at a confluency of 2×10^3, sample treatment was done keeping a concentration of 100 µg/ml (as we calculated biocompatibility up to this concentration). Each well was washed twice with fresh PBS to take out the dead cells to assess the uptake kinetics. Then triton X (100 µl) is added and incubated for 15 minutes, collected in a 1.5 ml centrifuge tube, and centrifuged to collect the supernatant. The OD is taken with the help of the reader.

2.6 Antibacterial Assay

2.6.1 Preparation of Bacterial dilution (Micro broth dilution assay)

The two bacterial strains, *E. coli* and *S. aureus,* were cultured in 5 ml of Tryptic-soya broth (TSB) at 35 ± 1 °C for 24 h. The bacterial suspensions were prepared to a turbidity equivalent to 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml). The bacterial strains were diluted in Saline at a ratio of 1:1000 to yield approximately 1.5 x 10^5 CFU/ml.

2.6.2 Preparation of sample dilution

For the assay with *S. aureus*, cystine, the PU, and PP were prepared in the dilution range of 0.0001 mg/ml to 10 mg/ml, 5 mg/ml to 20 mg/ml, and 1 mg/ml, respectively. For the experiment with *E. coli*, the cystine, the PU, and PP dilution ranges were 0.1 mg/ml to 10 mg/ml, 5 mg/ml to 30 mg/ml, and 10 mg/ml, respectively.

2.6.3 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of all the samples was measured using the broth microdilution assay following the Clinical and Laboratory Standards Institute (CLSI)

guidelines. The dispersion of all the samples was prepared by serial dilution with MHB in Microcentrifuge tubes. The adjusted bacterial suspension (20 µL of 1.5×10^5 CFU/ml) was applied into each well containing 80 µL of sample to make up 100 µL of total volume. The bacterial growth was monitored at one-hour intervals using a BioTek SYNERGY H1 microplate reader at 600 nm with continuous shaking while incubating at 35 ± 1 °C for 24 h. The lowest concentration of the samples, indicating inhibition in bacterial growth, was taken as MIC of that sample.

2.6.4 Determination of bacterial ROS

The generation of ROS (reactive oxygen species) from all the specimens was measured using 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) purchased from Thermofisher Scientific, India. 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, a derivative of 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). It is among the most commonly utilized detection agents for instantly measuring a cell's redox state. DCFH-DA, a cell-permeable, nonfluorescent precursor of DCF, is utilized as an intracellular probe for oxidative stress since it is effortless to use, susceptible to adjust in the redox state of a cell, cost-effective, and useful as it follows changes in ROS over the timespan. For the study of ROS, 1.5 x 10^5 CFU/ml of bacterial suspension of *S. aureus* and *E. coli* were treated with 30 mg/ml and 60 mg/ml of PU, respectively, after 18 h of incubation at 37 ℃, later for 15 min it is centrifuged at 4 ℃ at 5000 rpm and the supernatant formed was treated with 10 μM CM-H2DCFDA for 1 h. The ROS produced was calculated using BioTek SYNERGY H1 microplate reader using fluorescence spectrometry.

2.7 Animal studies

2.7.1 Gelation study for localized treatment

Methylcellulose (MC-gel) with a weight percentage of 7 in water is made, then drug-loaded polymer (keeping 5% drug with respect to polymer, in DMSO) is taken in a 1.5 centrifuge tube, (2.5mg of drug-loaded polymer) with that 200 µl of MC-gel added, and with the help of vortex mixed well so that no particle remains and used as is for animal application. One important note is that it took 7 minutes to solidify. Images of mice before and after injection were captured with the help of a phone camera.

2.7.2 Histopathology

Mice were slaughtered, and the skin from the area where the localized gel injection was administered, along with normal skin, was collected from mice for histological examination. Afterward, they were removed for the analysis. Skins that had been dissected were kept in a 10% buffered formalin solution. Graded ethanol was used for the dehydration process, and paraffin was embedded. The tissues were cut into slices 3-5 µm thick, and hematoxylin and eosin (H&E) were used for staining. After that, these stained slices were seen under a light microscope at a magnification of 20×. TNF-alpha pictures were also taken at this time to check for necrosis.

2.7.3 *In-vivo* Drug-Release/Biodistribution Study

Albino mice weighing (20-25) g were randomly handpicked in two groups for the biodistribution study & these were treated with pure drug (5 mg/kg dosage with respect to body weight) and drug-loaded MC-polymer (encapsulated inside methylcellulose gel) (an equivalent amount). In this method, the retro-orbital in mice is penetrated with a capillary tube & blood was collected in heparinized centrifuge tubes (1.5 ml) for both the groups at a predetermined time interval (5min,10min,15min,30 min,1, 3, 6, 12,18, and 24 hr.). To secure

the necessary plasma, it was instantly centrifuged at 4000 rpm for 10 min at 4 ℃ after blood collection. Plasma drug concentration was determined using acetonitrile and water mixture (60:40 v/v) estimated with the help of reverse phase HPLC (Waters 1500, Milford, MA, USA), attached to Waters 2998 photodiode array detector, and the data were evaluated using waters Breeze software (Waters, USA) to check out drug dispersal. The mobile phase flow was detected at a wavelength of 227 nm & with a rate of 1.0 ml/min.

2.8 Statistical analysis

The absorbance values were ascertained by statistical analysis utilizing the two-way investigation (MTT assay) and one-way (cell adhesion) of variance (ANOVA) method in GraphPad Prism-9. A predetermined significance level of $p < 0.05$ was used for the Tukey test, which compares means. Significant levels were denoted by the symbols *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Following three test runs, all cell culture data were gathered.