Chapter 2

2.1 Synthesis of different polymeric architectures for controlled drug release

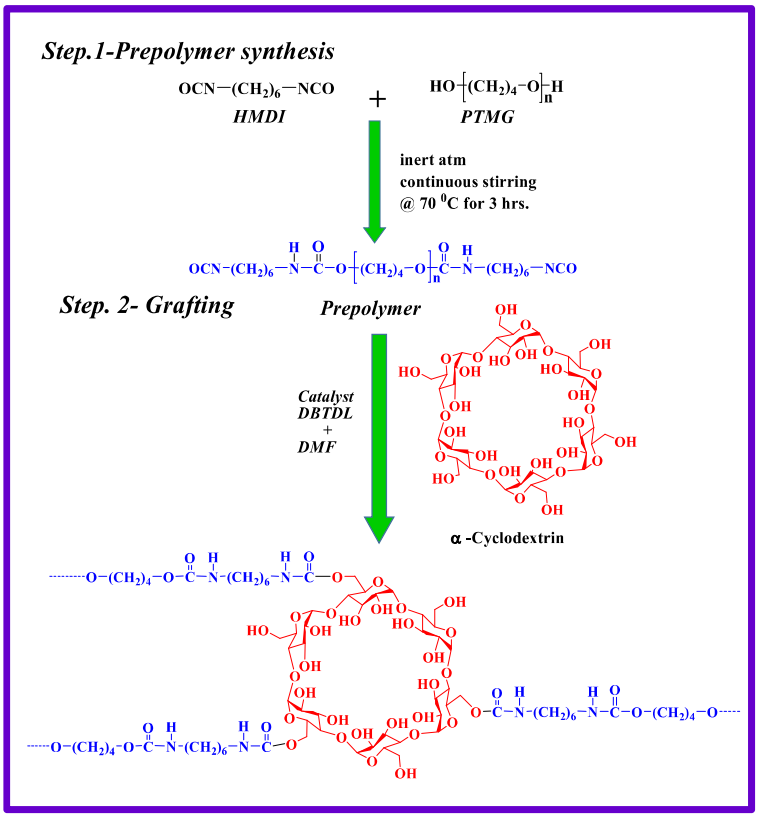
2.1.1 Materials

Poly(tetramethylene glycol) (PTMG) (terathane, Sigma–Aldrich; number average molecular weight, M_n = 2900 and 1400 $g.mol^{-1}$) was used as received. 1,6-hexamethylene diisocyanate (HMDI), dextrin, catalyst, dibutyltin dilaurate (DBTDL) and solvents, dimethyl formamide (DMF), HPLC grade water and Acetonitrile were purchased from Merck, Germany. α-cyclodextrin (CD) purity ≥98%, melanin, anti-cancerous drug (dexamethasone) bioreagent 97% (sigma), Rhodamine B were purchased from Sigma Aldrich USA. Paclitaxel was purchased from Fluorochem, Henceforth, α-cyclodextrin will be termed as CD in the entire thesis, polyurethane as PU/PP and dextrin as D.

2.1.2 Synthesis of polyurethane graft CD copolymers

Synthesis of polyurethane was carried out in two steps. First step involves making prepolymer using PTMG(2900) and HMDI in mole 1:1.05 mole ratio at 70 ^oC for 3 hrs to yield isocyanate terminated prepolymer in three neck round bottom flask equipped with mechanical stirrer and nitrogen purging inlet in a silicon oil bath. Second step involves the chain extension using different concentrations 10-100mg of CD added to prepolymer in presence of DMF (15-20 ml depending upon the viscosity) solvent and DBTDL catalyst (0.1 ml of 1 wt.% toluene solution) few drops to complete the polymerization reaction with rapid stirring at 70 ^oC for 24 h. The polymer was then precipitated by pouring the reaction mixture into deionized water as a non solvent. Grafted copolymers were separated through

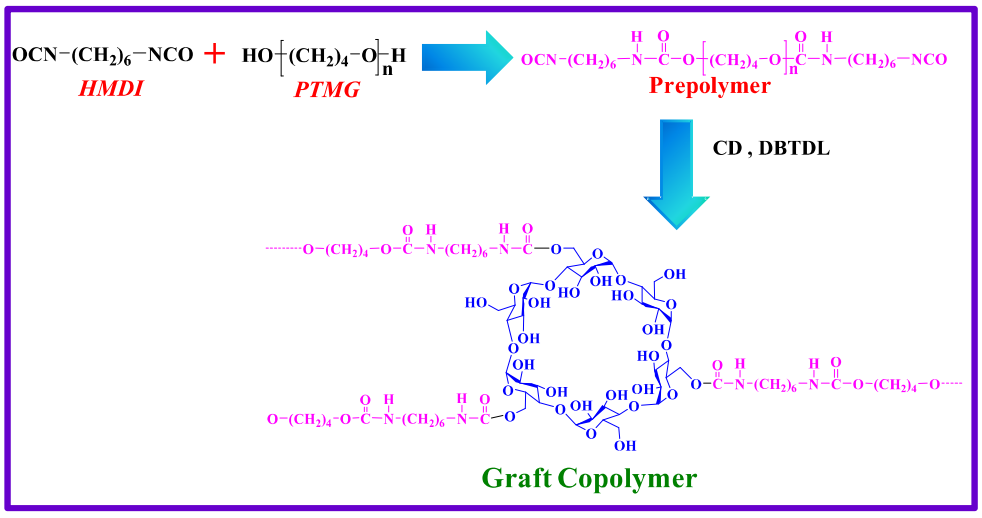
filtration and successively washed with water to remove the unreacted CD and diisocyanate. The final products were dried under reduced pressure at 60 °C overnight. The reaction details are given in **Scheme 2.1**. Different graft copolymers were synthesized by altering the reactant concentrations for different reaction time. The molecular weight of all the graft copolymers was determined using gel permeation chromatography (GPC) using DMF as an eluent and maintaining 1ml/min flow rate. The graft copolymers are termed as 'CDgPU-L' and 'CDgPU-H' for low and high graft density, respectively. The molecular weight of prepolymer is ~15,000 and, henceforth, termed as PU.



Scheme 2.1: *Chemical scheme showing grafting of polyurethane onto CD.*

2.1.3 Synthesis of short chain prepolymer and its grafting on CD

Polyurethane grafting on CD was carried out in two steps. In the first step prepolymer was synthesized using PTMG(1400) and HMDI at 70 ^{o}C for 3 hrs to yield isocyanate terminated prepolymer in three neck round bottom flask equipped with mechanical stirrer and nitrogen purging inlet in a silicon oil bath. Second step involves grafting using different concentrations of CD added to prepolymer in presence of DMF solvent and DBTDL catalyst (0.1 ml of 1 wt. % toluene solution) to complete the polymerization reaction with rapid stirring at 70 ^{o}C for 24 h. Precipitation of was done by pouring reaction mixture in deionized water as a non solvent. Separation of grafted and unreacted species was done through filtration and successively washed with water. The final grafted copolymers were dried under reduced pressure at 60 °C overnight. The mechanism of chemical reaction is given in **Scheme 2.2**.



Scheme 2.2: *Chemical scheme showing grafting of short polyurethane chains onto CD.*

Graft polymers with two different degree of substitution were synthesized by varying the CD weight ratios and other reaction conditions. Short chains of polyurethane are prepared by using PTMG soft segment of lower molecular weight (*i.e.* 1400) and followed by reacting it with HMDI yielding NCO terminated prepolymer. The molecular weight of both the graft copolymers was determined using gel permeation chromatography (GPC). The graft copolymers are termed as 'CgP-H', and 'CgP-L'. The term CgP stands for cyclodextrin grafted prepolymer and 'H' and 'L' after P are meant for high and low graft density of PP. The molecular weight of prepolymer is 7000 and it is termed as 'PP' in chapter 4.

2.1.4 Synthesis of different generations of CD

Prepolymer synthesis; PTMG and HMDI were taken in 1:1.05 mole ratio in three neck flask equipped with nitrogen purging inlet and mechanical stirrer with continuous stirring for 3h at 70 ^{o}C.

Modification of CD with HMDI; In the same way as prepolymer synthesis CD and HMDI in 1:3 mole ratio were taken and dissolved in DMF under inert atmosphere with stirring at 70 ^{o}C for 3 h. Now tosylation of unreacted hydroxyl groups in CD was done according to method reported.[114, 115] Tosylation was done for selective reaction at position 2, 3-OH of CD, leading to first generation.

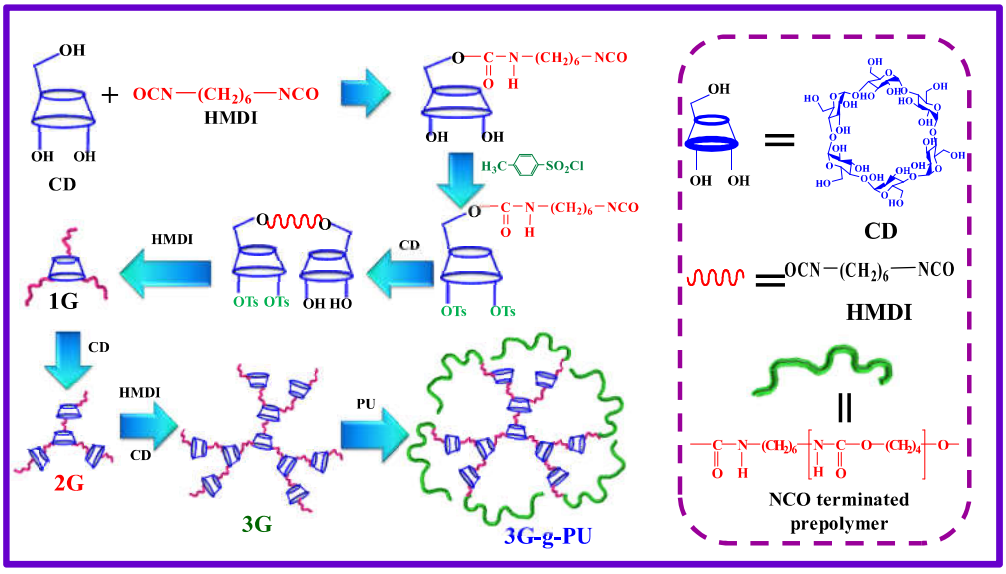
- Second generation synthesis: This tosylated NCO terminated first generation was reacted with specified amount of CD and the reaction mixture was allowed to stir for another 3 h. Hydroxyl groups of CD were reacted with NCO leading to Second generation termed as 2G:

- 2G was again tosylated in the similar method as above followed by the addition of HMDI, with stirring for 3 h. Further, a known amount of CD was added and reaction mixture was allowed to stir for another 4 h forming third generation, termed as 3G.

Now detosylation of third generation was done in acidic aq. solution. Solution was centrifuged with several times washing with water to remove any unreacted HMDI and CD. Precipitate was dried in air oven at 60 ^{o}C for 24 h.

Synthesis of grafted polyurethane; 3G was grafted with previously prepared prepolymer leading to 3G-g-PU.

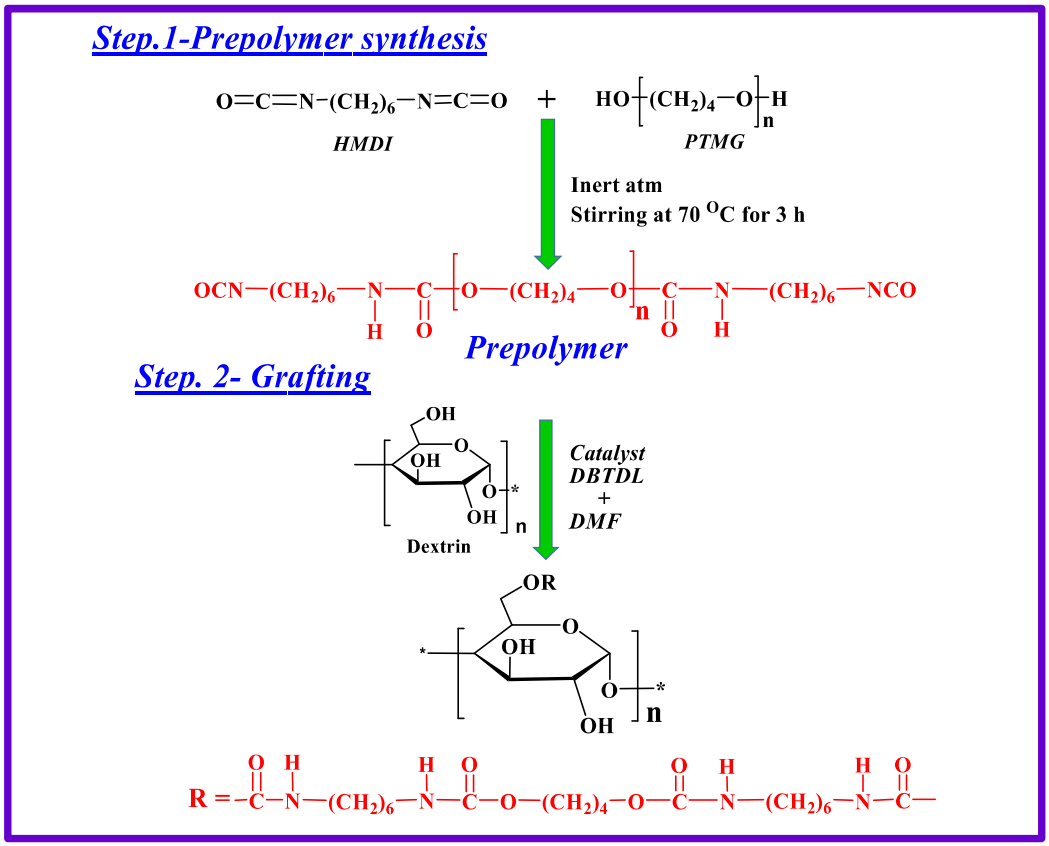
Preparation of 3G-g-PU methyl cellulose hydrogel; A known amount of 3G-g-PU was dissolved in DMF (5 % (w/w) drug was added with respect to 3G-g-PU) and it was added to methyl cellulose hydrogel of 10 % (w/w). It was stirred for 2 h to ensure complete mixing.



Scheme 2.3: *Scheme showing formation of different generations of CD using small spacer HMDI and its subsequent grafting with polyurethane.*

2.1.5 Synthesis of polyurethane grafted linear (dextrin) brush Copolymers

Polyurethane synthesis was carried out in two consecutive steps first step prepolymer formation using soft segment PTMG and HMDI for three hr at 70 ^{o}C yielding isocyanate terminated prepolymer while the second step involves the chain extension by adding various weight ratios of dextrin in the presence of catalyst DBTDL (0.1 ml of weight % toluene) and DMF as a solvent to carry out the polymerisation process with vigorous stirring.



Scheme 2.4: *Chemical scheme showing grafting of polyurethane onto linear biopolymer dextrin.*

The reaction was performed in three neck flask well equipped with mechanical stirrer and nitrogen purging inlet to maintain inert atmosphere in silicon oil bath keeping temperature 70 ^{o}C for 24 h. Reaction mixture was poured in deionised water which is a non solvent for precipitation of the copolymers, washing was done repeatedly to separate out unreacted dextrin and HMDI. Polymers were dried in air oven at 60 ^{o}C for 24 h and for next 24 h in vacuum oven. Reaction scheme details for copolymers synthesis is given in **Scheme 2.4**. The molecular weight determination of synthesised copolymers was done through GPC technique.

2.2 Characterizations

2.2.1 Proton NMR

One-dimensional ^{1}H NMR spectra were recorded on a Bruker ADVANCE 400 MHz Spectrometer (Germany) using DMSO-d_6 (99.98%) as a solvent for all the grafts/generations and pure polymers.

2.2.2 Fourier transform infrared spectroscopy (FTIR)

(FTIR) spectrum was measured in the reflectance/transmittance mode at room temperature in the range of 600 to 4000 cm^{-1}. An FTIR spectrum gives valuable information about functional groups and thus confirms the chemical modification.

2.2.3 UV-Vis

UV-Vis measurements were performed using a Jasco V-650 spectrophotometer, Japan, in the range of 200-800 nm. The transparent thin films of the specimens were prepared through compression molding technique while pure polymer is powder form was used as received.

2.2.4 Thermal analysis

DSC: The melting and glass transition temperature of all samples was determined after quenching the samples from room temperature to −50 °C at a rate of 30 °/min followed by heating up to 200 °C at a heating rate of 10 °/min using a Mettler 832 differential scanning calorimeter. The cooling was done from 200 to -50 °C keeping the cooling rate same as of heating at 10 °/min. The heat of fusion (ΔH) of the melting process was obtained from the area under the endotherm. The DSC was calibrated with indium before use.

TGA: The degradation temperature of the specimens was estimated using Mettler thermogravimetric analyzer (TGA, Mettler-Toledo) at a heating rate of 20 ° min^{-1} under nitrogen atmosphere in the temperature range from 40 to 600 °C.

2.2.5 Mechanical responses

Specimens were prepared via microinjection using an FD-1 microinjector (Fly Tech Engineering) for the testing of tensile strength of material. The samples were microinjected from a barrel of temperature T_m+20 ^oC and mold temperature of 30 ^oC at a pressure of 650 bar. Tensile tests were performed on the injection molded specimens of 65 mm long 4.05 mm wide and 2.12 mm thick using an Instron 3369 tensile tester at a strain rate of 5 mm /min at room temperature. Three samples of each grafted copolymer were tested to obtain good error estimates through standard deviations.

2.2.6 Swelling and contact angle studies

The swelling capacity of the graft copolymers was determined through gravimetric technique. A known weight of the dry polymer was immersed in a petridish containing 10 mL of distilled water. The polymer films were taken out from the solvent at different time interval and the excess water was removed by soaking with tissue papers. The swollen

polymers were weighed and then were placed in the solution again. The dynamic weight change of the polymers with respect to time was calculated using the formula below:

$$\%\, wiegt\ cange\ = \frac{W_f - W_i}{W_i} \cdot 100$$

where, W_f is the weight of the polymer in the swollen state and W_i is the initial weight of the polymer under dried condition after regular time intervals. Contact angle of the graft copolymers were measured with a Kruss tensiometer K-100 for evaluating hydrophobicity of the surface of the films. Measurement was done in triplicate for better error estimate.

2.2.7 Structural analysis

X-ray diffraction was done using a Rigaku mini Flex Advance wide angle X-ray diffractometer with a graphite monochromator using Cu Kα source with a wavelength of 0.154 nm. The generator was kept at 40 kV and 20 mA. The films of the samples, prepared through compression molding technique, were placed on a quartz sample holder at room temperature and were scanned at the rate of 3 ^{o}/min. Small angle neutron scattering experiments were performed on a spectrometer at the Dhruva reactor in Bhabha Atomic Research Centre, Mumbai, India. The scattering range of the wave vector was kept constant for all the polymeric systems and data was collected in the range of 0.17 $nm^{-1} \leq q \leq 3.5$ nm^{-1}. Initial lower scattering wave vector range was fitted with Debye Buche model. The characteristic length was calculated through the equation $\Lambda_c = 2\pi/q_m$, where, q_m is the scattering wave vector corresponding to the peak position in the scattering pattern.

2.2.8 Morphological investigation

AFM was done using a NT-MDT multimode AFM, Russia, controlled by a Solver scanning probe microscope controller. Semi contact mode was used with the tip mounted on 100 mm long, single beam cantilever with resonant frequency in the range 240 - 255

kHz and the corresponding spring constant of 11.5 N/m. Surface morphology of thin-film in optical range was studied through optical microscope (Leitz). Thin samples were prepared through solvent casting technique for the optical measurements.

2.3 *In-Vitro* Drug release

In vitro drug release study was carried out in PBS buffer at pH 7.4. Standard stock solution (1 mg/ml) of dexamethasone and paclitaxel were prepared initially and standard curve was drawn after taking absorbance using a UV-visible spectrophotometer taking the value at 242 and 227 nm in the concentration range of 1-100 μg/ml. The drug loaded specimens were immersed in 50 ml PBS in conical flask and kept in incubator shaker at 50 rpm and 37 ^{o}C. Aliquots from release medium were collected at constant time interval and same amount of fresh medium was added. The absorbance of the collected aliquots was taken at 242 and 227nm and amount of drug corresponding to that absorbance was calculated from the standard curve. 5 wt % of drug was encapsulated in films and hydrogels respectively.

2.4 Biocompatibility

2.4.1 Cell Culture

HeLa (human cervical cancer cell line) / B16-F10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat inactivated fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. The temperature of the culture was maintained at 37 °C in a CO_2 incubator with 5% CO_2 supply. The cell viability percentage was evaluated by using MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Films of each test sample after sterilization with 70% alcohol followed by UV exposure were added in each well. Cells were then seeded in 96 well plate at the confluency of 1×10^4 cells per well in 0.1 ml of DMEM containing 10% fetal bovine serum,

supplemented with 50 μL penicillin and 50 σL streptomycin and were incubated at 37 ^{o}C in 5% CO_2 and all the treatment were done in triplicate for MTT assay. The media containing dead cells was replaced by 100 μl of fresh media in each well. MTT solution of 0.5 mg/ml in DMEM was added in each well and was incubated for 3 hours at 37 ^{o}C to produce water insoluble formazan. Finally, DMSO was added to each well to solubilize this formazan and absorbance was taken in microplate reader using a spectrophotometer (Amersham) at 570 nm wavelength. The percentage cell viability was calculated using the formula

$$\%\ Cell\ viability = \frac{\mathrm{OD_c}}{\mathrm{OD_T}} \times 100$$

where, OD is optical density, C stands for control and T represents test specimen.

2.4.2 Cell adhesion

For examining the cell adhesion properties of prepared copolymers, cells at the confluency of 1 $\times 10^4$ per well were seeded on the sample surface in 96 well plate and were incubated in CO_2 incubator for 6 hrs. After incubation, the adhered cells were washed with PBS solution twice to remove the unattached cells from the specimen, fixed with 4% paraformaldehyde solution for 20 min, washed with PBS again and then cell permeabilization was carried out with 20% methanol for 20 min. Staining of the attached cells were done by using 0.2% crystal violet aqueous solution for 20 min. Excess stains were removed by gentle washes in PBS twice followed by elution of the residual crystal violet with 10% acetic acid. Optical density (OD) of the solution was measured using a microplate reader at a wavelength of 570 nm. The optical density values thus obtained were correlated directly with the number of attached cells in the sample. Cell adhesion images were also observed using a phase contrast microscope (Leica, Germany) after fixing the cells with 4% paraformaldehyde solution followed by washing with PBS. Percentage cell

adhesion was calculated using the following formula: $\%\ Cell\ adhesion = \frac{\text{OD of sample}}{\text{OD of control}} \times 100$.

2.4.3 Fluorescence imaging

Cell proliferation effect on polymers was observed using fluorescence microscope. Cells were seeded in 24 well plates at a confluency of 1×10^4 on to polymer films and were incubated for 24 hrs at 37 ^oC. Specimens were washed twice with fresh PBS to remove 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 taken using fluorescent microscope (Leica, Germany).

2.5 Animal studies

2.5.1 In-vivo anti-tumor efficacy

Male mice of swiss albino strain with average body weight 20 - 25 g were purchased from the Institute of Medical Science, Banaras Hindu University, India. All the mice were housed in accordance to the ethical guidelines and were provided food and water. Anti-tumor activity of pure drug and drug loaded graft copolymers patch/hydrogels were evaluated using melanoma B16-F10 cell bearing mice and were compared with untreated mice. All the mice were injected with 1×10^6 B16-F10 subcutaneously on the right dorsal. The mice that developed palpable tumor of volume 25±5 mm^3 were randomly divided in different groups having five animals in each and treated either with pure drug dexamethasone/paclitaxel, CD-d (drug loaded CD), Dex-D(drug loaded dextrin), patch of drug loaded in graft copolymer (CDgPU-L), hydrogels of pure drug (Gel-D), Mod-Gel-D

and D-P-L-MC-D for the treatment. A drug enhancer (tritronX-100) has been applied on top of the generated tumor and the drug loaded CDgPU-L or other treatments like drug or CD-d was put over the enhancer followed by dressing with leucoplast. Control groups were left untreated (saline applied and dressed with leucoplast as usual). For quantification of anti-tumor efficacy, tumor sizes were measured in every 3rd days with Vernier-calliper and the tumor volume were calculated as $\frac{\text{length} \times \text{width}^2}{2}$. The number of mice in control group declined after 21 days due to their early death as the tumor growth was very fast and we took data up to 30 days of treatment for better comparison. The animals were observed daily and treatment was given in every 3rd day. Tumor volume, body weight, mortality rate and number of long survivals were monitored. In another tumor treatment process, drug, CD-d and drug loaded CDgPU-L material in buffer solution were injected just beneath the tumor tissue in every 3rd day and tumor size and other related data were collected as mentioned before to compare the efficacy of the drug loaded in CDgPU-L (patch) vis-à-vis conventional intravenous treatment.

2.5.2 Histopathology and biochemical assay

Mice were sacrificed and main organs *e.g.* liver, kidney and spleen were separated for histological analysis. Tumor tissues were also excised for the examination. The dissected organs were preserved in 10 % buffered formalin solution. Dehydration was done in graded ethanol and was embedded in paraffin. The tissues were sliced into sections of 3-5 μm thickness and were stained using hematoxylin and eosin (H&E).These stained sections were then examined under light microscope at a magnification of 20×.

Blood serum (50 μl / mice) was extracted from the collected blood before sacrifice at the end of treatment (30 days) for the clinical assessment of blood parameters like aspartate

aminotransferase (AST), alanine amino transferase (ALT), Alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine which were analyzed using Jasco 650 chemistry analyzer.

2.5.3 Biodistribution

For biodistribution study albino mice weighing 18-23g were randomly assigned in different groups and treated with pure drug (dexamethasone and paclitaxel) at the dose of 5mg/kg body weight and an equivalent amount in drug loaded Mod-Gel/D-PL-MC-GE;-D. For both the groups, at predetermined time interval points (0.25, 0.5,1,3,6, and 24 hr) blood was collected from tail vein into heparinized microfuge tubes, immediately after blood collection it was centrifuged at 4000 rpm for 10 min at 4 ^{o}C to obtain required plasma. Concentration of released dexamethasone/paclitaxel was estimated through HPLC technique.

Statistical Analysis: Results are presented as the mean value ± standard deviation. One way analysis of variance with t test is applied for statistical results.