Chapter@2
Experimental

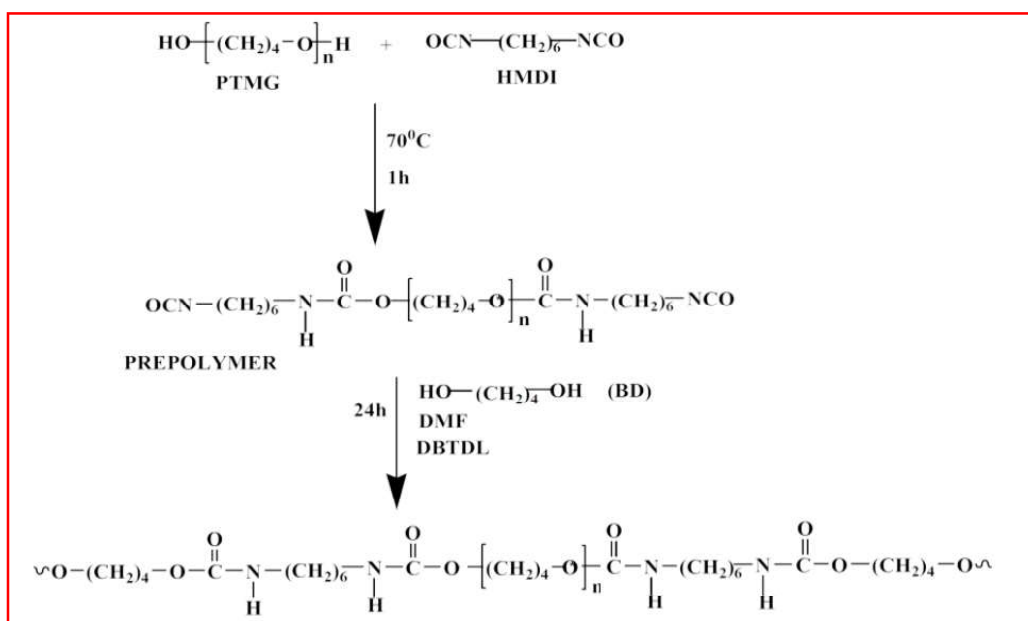**2.1 Synthesis:**

2.1.1 Materials:

Poly(tetramethylene glycol) (PTMG) (Sigma-Aldrich, number average molecular weight, M_n = 2900 g/mol), 1, 6-hexamethylene diisocyanate (HMDI), 1,4-butanediol (BD) (Merck, Germany) and Ethylene glycol (EG) (Loba Chemie) were used as received. Graphene nanoparticles were purchased from Redex Nano Lab (Noida, India). The catalyst dibutyltindilaurate (DBTDL) and solvent, dimethyl formamide (DMF) were purchased from Himedia and Merck, respectively. Graphite flake, Potassium permagnate ($KMnO_4$), Sulphuric acid (H_2SO_4), Ammonium Sulphate [$(NH_4)_2SO_4$] and Ortho-phosphoric acid (H_3PO_4). Ethylene diamine, hexamethylene diamine and dodecane diamine (Merck, Germany).Tetracycline hydrochlorides an antibiotics and Dexamethasone an anticancerous was purchased from Sigma-Aldrich.

2.1.2 Synthesis of polyurethane:

Pure polyurethane with butane diol as a chain extender (BD-PU) was synthesized in two steps: prepolymer preparation using PTMG and HMDI followed by the addition of chain extender BD. DMF was used as solvent and catalyst (DBTDL: 0.1 ml of 1 wt. % toluene solution) was used to enhance the reaction rate and complete the polymerization process with continues stirring at 70^0C for 24h. Hard segment content of polymer was maintained at 30% by using a predetermined amount of polyol, diisocyanate and chain extender as mentioned with molar ratio of PTMG: HMDI: BD was kept 1: 5: 4, respectively. The polymer flakes were obtained by pouring the solution in de-ionized water and dried at 70^0C for 48 h in oven followed by the vacuum

dried at 55^{0}C for 24h. Schematic representation of formation of pure polyurethane is given below. Hard segment content (HSC) of polyurethane is calculated by following equation.

$$\text{Hard Segment Content (HSC)} = \frac{(Wt.of\ diisocyante + Wt.of\ chain\ extender)}{(Wt.of\ poly-ol + Wt.of\ diisocyanate + Wt.of\ chain\ extender)}$$

Scheme 2.1: Reaction scheme for polyurethane synthesis

2.1.3 Synthesis of polyurethane / graphene nanocomposites:

Synthesis of polyurethane / graphene nanocomposites were done by the dispersing the required amount of graphene in poly-ol medium in early stage of polymerization. Incorporation of graphene in early stage leads to better dispersion as observed in TEM image improved the properties of polymer. Different nanocomposites of polyurethane were obtained by pouring the solution mixture into aqueous solution and dried at 70^{0}C for 48 h in oven followed by the vacuum dried at 55^{0}C for 24h.
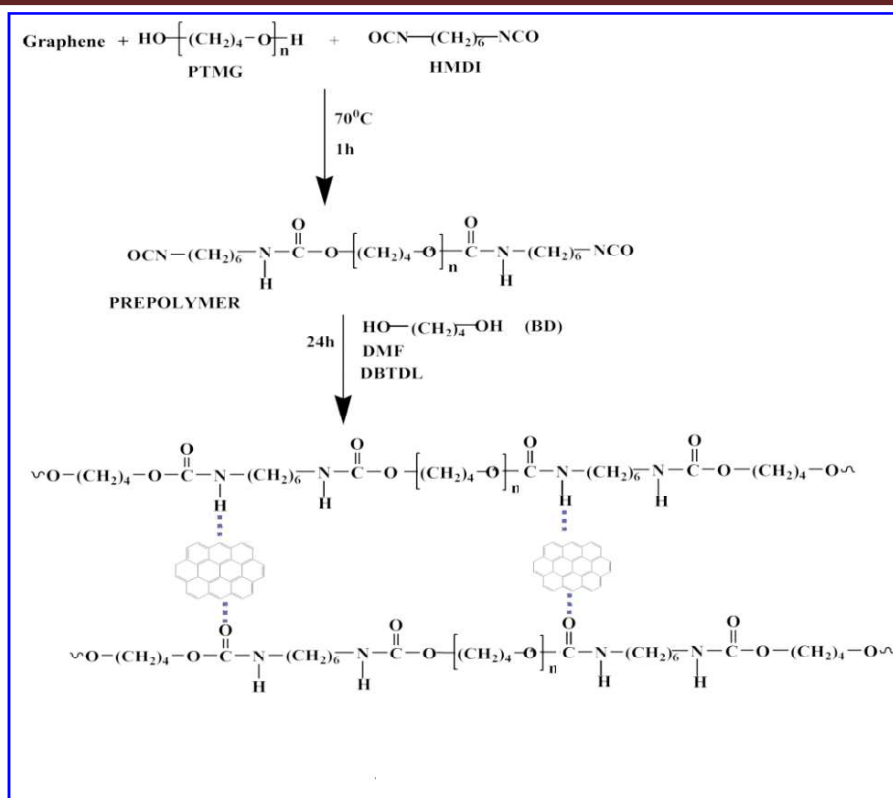
Scheme 2.2: Reaction scheme for polyurethane / graphene nanocomposites synthesis.

2.1.4 Synthesis of graphene oxide:

Graphene oxide was synthesized through modified Hammer's method [Marcano et al. (2010)]. For this, graphite flake (3.0g, 1wt. equiv.) was added to 9:1 ratio of concentrated H_2SO_4 and H_3PO_4 (360:40 ml) followed by slow addition of $KMnO_4$ (18.0g, 6wt. equiv.). After this reaction temperature was increased up to 50^0C with continuous stirring for 12 h. Addition of ice water and 30% H_2O_2 was performed after cooling the reaction mixture and centrifuged at 4000 rpm. Repeated washing of the solid product was done using the mixture of distilled water, 30% HCl and ethanol. Finally, the sample was washed with distilled water until the media reached neutral pH. The solid obtained was dried at 70^0C for 48 h followed vacuum dried at 55^0C for 24h.

2.1.5 Synthesis of amine modified graphene oxide:

Amine functionalization of graphene oxide was done through ammonia solution as describes by Lai L. et al. (2011). For such modification 500 mg of the graphene oxide was taken in ethylene glycol followed by the ultrasonication for 30 min. After this addition of the ammonia solution was done and kept the reaction mixture for 12h at 180^0C with continuous stirring. Color of the reaction mixture was changed from yellowish to dark brown. Brownish solid material was obtained after the filtration and repeated washing of the material was performed using distilled water. Solid material was dried at 70^0C for 48h in normal oven followed by vacuum dried at 55^0C for 24h.

2.1.6 Synthesis of diamine modified graphene oxide:

Diamine surface functionalization of graphene oxide was done as reported by Kim et al. (2013). For this each diamine (ethylene diamine, hexamethylene diamine and dodecane diamine) solution (3g in 175ml of ethanol) was slowly added in graphene oxide suspension (1g in 165ml of water) separately with continuous stirring at room temperature for 24h. The resulting materials was centrifuged and washed with the ethanol: water mixture (1:1ratio). Obtained material was dried at 70^0C for 48 h followed by the vacuum dried at 55^0C for 24h.

2.1.7 Synthesis of amine sulfonated graphene oxide:

Synthesis of sulfonated graphene oxide was done as using ammonium sulphate as reported by reported by He et al. (2014). Ammonium sulphate and graphene oxide was dispersed in distilled water in mass ratio of 1:5 followed by the ultrasonication of

45min. The mixture was completely dried and heated at 245^0C for 1h. It is believed that at 245^0C $(NH4)_2SO_4$ was decomposed and gives SO_3 moiety and this form SO_3 react with carbon through its surface hydrogen atom to formed $-SO_3H$ moiety to graft graphene sheet. Amine functionalization in sulfonated graphene was done as describes earlier. (Lin J. et al. (2011)

2.1.8 Synthesis of physically mixed and chemically grafted nanocomposites:

Amine modified graphene oxide was used in synthesis of physically blend and chemically grafted nanocomposite of polyurethane. In physically mixed nanocomposite amine modified graphene was dispersed at the last stage of polymerization polyurethane matrix whereas in chemically grafted nanohybrids these modified graphene act as a chain extender. Chemically tagged graphene nanohybrid was synthesized by the addition of amine functionalized graphene oxide in prepolymer solution and continued the chain extended reaction for 24hrs, similar to butanediol (BD) chain extended system. Physically mixed and chemically tagged graphene nanocomposites will be termed as PUG-P and PUG-C, respectively. Molecular weight of pure polyurethane and its nanocomposites are found to be ~17000 with PDI~ 1.6, as measured using gel permeation chromatography (GPC) with DMF as eluent at 70^0C with 1mL / min flow rate.
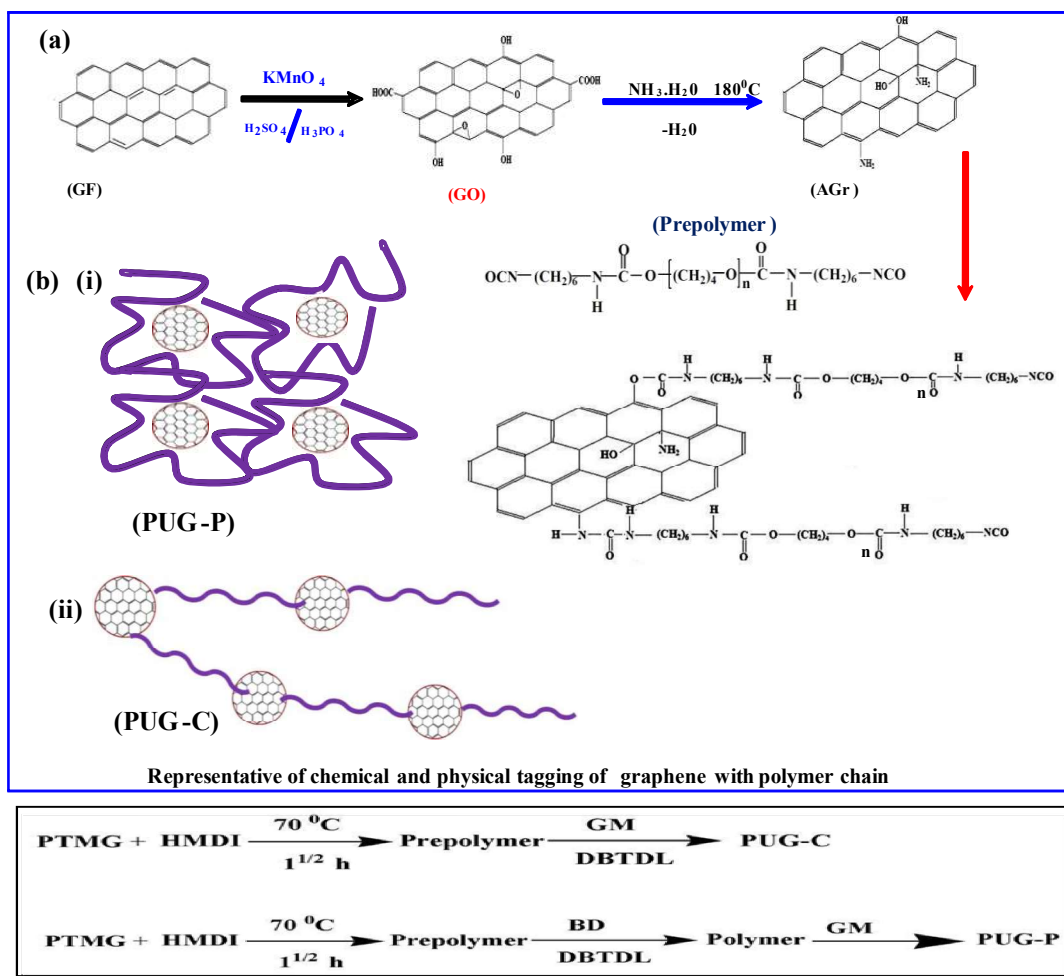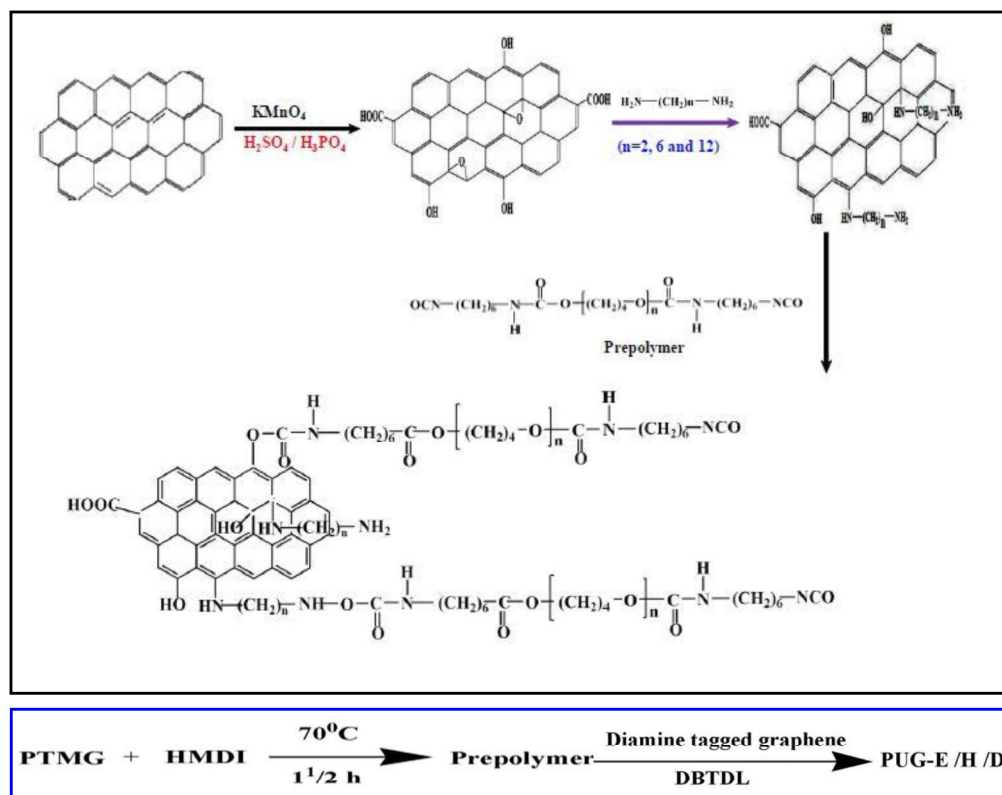
Scheme 2.3: Reaction scheme for synthesis of chemically tagged nanocomposites. **(b) (i&ii)**- schematic representation of physically mixed (PUG-P) and chemically grafted (PUG-C) nanocomposites.

2.1.9 Synthesis of diamine modified graphene oxide grafted nanocomposites:

Different diamine (ethylene diamine, hexamethylene diamine and dodecane diamine) modified graphene was used in synthesis of nanocomposites and compared it properties with pure polyurethane. Nanocomposites of polymer were synthesized by adding the different modified graphene oxide in prepolymer solution. These diamine modified graphene was chemically grafted with prepolymer chain and synthesized

nanocomposites are term as PUG-E, PUG-H and PUG-D for ethylene diamine, hexamethylene diamine and dodecane diamine tagged graphene, respectively.


Scheme 2.4: Reaction scheme for synthesis of diamine modified graphene nanocomposites

2.1.10 Synthesis of amine sulfonated graphene oxide grafted nanocomposites:

Polyurethane sulfonated graphene nanocomposites was synthesized by the addition of different weight of sulfonated graphene oxide in prepolymer solution and continued the chain extended reaction for 24hrs. (*Scheme* 2.5) represents the preparation of amine sulfonated graphene and chemically tagged these graphene as a chain extender in nanocomposites. The following are the two different reaction steps showing the synthesis of pure PU and nanocomposites.
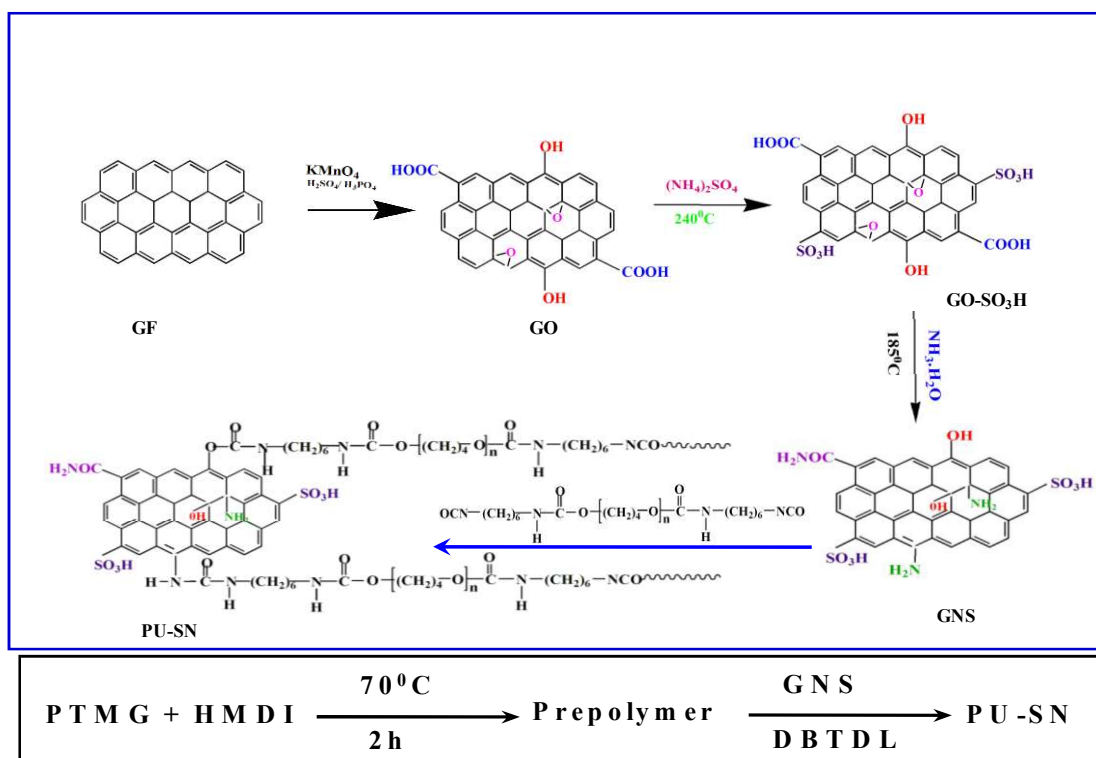
Scheme 2.5: Modification chemistry of graphene oxide and grafting of modified graphene with prepolymer chain.

2.1.11 Coupon Preparation:

Mild steel samples having percentage composition: *Carbon*, 0.23; *Manganese*, 0.11; *Silicon*, 0.02; *Phosphorus*, 0.02; *Sulfur*, 0.02; *Nickel*, 0.02; *Copper*, 0.01; *Chromium*, 0.01; iron, remainder were used as test specimens for gravimetric and electrochemical measurements. Specimens were mechanically polished with different grades of emery papers starting from coarse (320) to very fine (2000), washed with double distilled water, degreased with acetone and dried prior to each experiment. The corrosive solution (0.5 M H_2SO_4) was prepared by using Analytical Reagent (AR) grade sulfuric acid with double distilled water. For determination of corrosion resistance of nanocomposite coated mild steel in 0.5 M H_2SO_4, the electrodes were coated with PU

and its nanocomposite dissolved in DMF was used as the base solution. The coating was done by using a brush and then the sample was dried at 70 ^{0}C in vacuum.

2.2 Characterization of polyurethane and its nanocomposites:

2.2.1 X-ray Diffraction (XRD):

X-ray diffraction was measured using a Bruker AXS D8 Advance wide angle and Rigaku miniflex 600X-ray diffractometer with a graphite monochromator using Cu Ka source with a wavelength of 0.154 nm. The generator was kept at 40 kV and 20 mA. The thin films of the samples, prepared through solution casting technique, were placed on a quartz sample holder at room temperature at the scanning rate of 1^0/min.

2.2.2 Small angle neutron scattering (SANS):

Small angle neutron scattering (SANS) experiments were done on the spectrometer at the Dhruva reactor at Bhabha Atomic Research Centre, Mumbai, India. The SANS experiment was performed in the scattering vector (q) range of 0.17 nm^{-1} ≤ q ≤ 3.5 nm^{-1}. The Scattering from the samples were corrected for background contribution. Debye-Bueche and other models were fitted separately in lower range of q. The characteristic length (Λ_c) was obtained through the equation $\Lambda_c = 2\pi / q_m$, where q_m is the scattering vector q corresponding to the peak position in scattering pattern and the temperature of experiment was kept constant at 30^0C.

2.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR):

Proton NMR spectra of the sample were recorded on Bruker spectrometer using d6-DMSO as solvent. The chemical shift was recorded in parts per million relative to tetramethylsilane. It is very important technique to evaluate the electronic environment around the nucleus. Different signal in spectra indicate the chemically different proton in molecule and area of peak in spectra indicate the number of proton of each type.

2.2.4 Fourier Transform Infrared Spectroscopy (FTIR):

Fourier transform infrared (FTIR) spectrum was measured in reflectance mode at room temperature from 400 to 4000 cm^{-1} using a Nicolet 6700FTIR with a resolution of 4 cm^{-1}. FTIR spectrums indicate different functional groups in molecule and also help in determining the structure of molecule. FTIR spectrometer is superior to conventional IR spectrometer because it gives the faster and more sensitive analysis.

2.2.5 UV-Visible Spectroscopy (UV-visible):

UV-Visible Spectra indicate the conjugation in molecule through different transition like n→π* and π→π*.The UV-visible measurement was done in the range of 200 to 800 nm in reflectance mode using solid specimens (Cary Bio-100, Agilent and Jasco V-650). Position of absorption max and intensity indicates the extent of interaction.

2.2.6 Gel Permeation Chromatography (GPC):

Gel permeation chromatography (Youglin ACME-900) is used for determination of molecular weight of pure polyurethane and its various nanocomposites. Here dimethyl formamide (DMF) is used as a eluent with a flow rate of 1ml/min.

2.2.7 Morphological observation:

Surface morphology of pure polyurethane and its various nanocomposites was analyzed through Scanning electron microscope (SEM), atomic force microscope (AFM) and optical microscope. Dispersion of graphene or modified graphene in PU matrix was viewed through TEM (FEI Technai20) operating at a voltage of 200 kV. Zeiss scanning electron microscope is operated at 5kV. 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 μm long, single beam cantilever with resonant frequency in the range 240-255 kHz, and the corresponding spring constant of 11.5 N/m. Bulk Surface morphology of thin-film was studied through optical microscope (Leitz). Samples were prepared through solvent casting technique for optical measurement.

2.2.8 Mechanical behavior:

In solid state mechanical behavior of pure polyurethane and its different nanocomposites was done through universal testing machine (UTM) in tensile mode. Standardized samples were prepared through microinjection using microinjector (model FD-1, Fly Tech Engineering). The samples were microinjected at a barrel

temperature of Tm + 20^{0}C and a mold temperature of 40 ^{0}C with a pressure of 100 bar. The tensile test of the samples were performed with standardized sample through Instron 3369 tensile tester at a strain rate of 5 mm/min at room temperature with the specimen dimension of 25 mm gauge length, 4.05 mm breadth, and 2.12 mm thickness. Several samples were analyzed to minimize the error.

Mechanical response of pure polyurethane and its nanocomposites in liquid state was studied through dynamic frequency sweep test, on a Reologica (model: Nova) using parallel plate geometry (25 mm) at a constant temperature of 210^{0}C, keeping the strain rate 1% to maintain linear response of the samples. During the experiment, angular frequency (u) was kept in the range from 0.4 to 300 rad/s. The storage moduli and complex viscosities of polymer and its nanocomposites were measured as a function of angular frequency.

2.2.9 Thermal studies:

Melting behavior and heat of fusion of Polyurethane and its nanocomposites were measured using differential scanning calorimeter (DSC, Mettler 832) over a temperature range of -30 to 200 ^{0}C with scan rate of 10^0 / min. The peak temperature and heat of fusions were obtained from the endotherms using a computer attached with the instrument. Degradation stability of pure polyurethane and its nanocomposites was studied through thermogravimetric analyzer (TGA, Mettler-Toledo) in the temperature range from 40 to 600 ^{0}C. All the experiment was done at a heating rate of 20 ^{0}/ min under inert atmosphere (nitrogen atmosphere).

2.2.10 Contact angle measurement:

Nature of sample play very important role in determining the biocompatible properties. Contact angels of the samples were measured through the Kruss Tensiometer K-100 for evaluation the surface hydrophilic nature. To avoid the error every sample is measured in triplicate form.

2.2.11 Enzymatic degradation:

Enzymatic degradation of PU and its nanocomposites were studies through *Lipase* and *Protease* at 37^0C in Phosphate buffer solution (pH 7.4) containing 0.2 mg / ml enzyme. Polymer and its nanocomposites dimension of 9 x 8 x 0.3 mm^3 were taken into the vial containing 5ml of phosphates buffer. These vials were incubated at 37^0C with constant shaking for different time interval. Samples were taken from the vial washed it with distilled water for 3-4 times to removed surface material from samples and dried in vacuum before analysis.

2.2.12 Gravimetric Measurement:

To evaluate the rate of corrosion of mild steel gravimetric measurement was done using the 150 ml of acid solution in 250 ml beaker and keeps it at desired temperature in thermostat. The testing specimens were weighed accurately and immense in corrosive solution with and without presence of inhibitors for 24h at 25^0C. The specimens were taken out from the corrosive solution and washed with water followed by acetone and dried in oven and weighed. The percentage inhibition efficiency (%IE) was calculated by using the following formula.

$$\%\text{IE} = \frac{W^0 - W}{W^0} \times 100$$

Where, W^0 and W are the weight loss of mild steel samples without and with the addition of the samples, respectively.

2.2.13 Drug assay and release:

Standard stock solution (1 mg/ml) of tetracycline hydrochlorids an antibiotic was prepared first. Standard curve was drawn after taking absorbance measurement using a UV-visible spectrophotometer (Shimadzu 1700) taking the absorbance at 360 nm in the concentration range of 1-100 µg/ml. In case anticancerous, dexamethasone standard curve was drawn after taking absorbance measurement using a UV-visible spectrophotometer (Jasco V-650) taking the absorbance at 242 nm in the concentration range of 5-100 µg/ml. In vitro release studies were done in PBS buffer at pH~ 7.4. Drug loaded polyurethane and its nanocomposites were prepared using solution route followed by evaporation of solvent followed by putting in 100 ml of released medium at incubator shaker at 100 rpm at 37^0C. Samples were taken from the release medium at constant time interval and same quantity was replaced with fresh buffer.

2.2.14 Biocompatibility of pure polyurethane and its nanocomposites:

2.2.14.1 Cell culture:

Different kinds of cells like Bone marrow derived mesenchymal stem cells (BMMSCs), HeLa cell and MDA-MB231cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal calf serum (10%)

(Invitrogen Carlsbad, CA), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37^0C in a humidified CO_2 incubator, maintained at 5% CO2.

2.2.14.2 Cell Viability:

Cell viability of pure polyurethane and its nanocomposites has been studied through MTT (4,5-dimethylthiazol-2-yl-2,5- diphenyltretrazolium bromide) assay. After completing the sterilization $1x10^4$ cells were seeded in each well and incubated for 1, 3 and 5 days time intervals. On completion of each treatment periods the cells were treated with MTT for another 4 h and finally the MTT containing medium was removed and 100 ml of DMSO (SRL, Mumbai, India) was added in the wells to solubilize water insoluble formazan. Absorbance was then determined in an ELISA plate reader (Oasys, Austria) at 570 nm. Calculation of the percentage cell viability has been done using the following formula.

% of cell viability = [(Optical density of sample) / (Optical density of control)] × 100 Optical density of control is that incubation of cell in medium alone, and optical density of sample is in presence of sample.

2.2.14.3 Fluorescence studies:

Cell proliferation of different kinds of cells on the surface of pure polyurethane and its different nanocomposites has been studied through fluorescence microscopic technique after 24 h of incubation. Cells were suspended in fresh medium at an approximate density of 1×10^4 cells per ml in a 96 well plate of triplicate and the

incubation was performed for 4 h. After these initial incubation, 0.2 ml extracted medium was replaced with fresh medium in each well, except the control one and was finally incubated for 24 h. Washing of cells have been done by PBS, and was stained with Fluorescent dye DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, Sigma) (0.1 mg/mL) followed by incubation in dark place for 30 min at room temperature. Image has been taken using a fluorescence microscope (Axiovert 25, Carl Zeiss, Germany).

2.2.14.4 Cell adhesion:

For this experiment, 1×10^4 cells per well of 96 well plate were seeded on pure polyurethane and its various nanocomposites surface and incubated in a CO_2 incubator for 4 h. After 4 h of incubation, the samples were washed with PBS to remove the unattached cells and then the attached cells were fixed with ice-cold 4% paraformaldehyde for 20 min. Cell permeabilization was carried out with 20% methanol for 20 min after PBS washing. The attached cells were then stained using 0.5% crystal violet aqueous solution (SRL, Mumbai, India) for 30 min. Excess stains were removed by three gentle washes in deionized water followed by elution of the residual crystal violet with 10% acetic acid for 30 min during gentle agitation on an elliptical shaker. Optical density (OD) of the eluted solution was measured in an UV-Vis spectrophotometer (double beam LI-2800, Lasany, India) at a wavelength of 570 nm, with the background absorbance value measured at 650 nm. The optical density values obtained were correlated directly with the attached cell number.

2.2.14.5 Reactive Oxygen Species (ROS):

Measurement of the reactive oxygen species indicates the biocompatible nature of materials. For this, cells were grown in 6 well plates ($2x10^6$ cells/well). After 60-70% of confluence; cells were treated with pure polyurethane and its different nanocomposites and kept it for 24 h. Cells were then incubated for 30 min at 37^0C with 10 μM carboxy H2-DCFDA for intracellular ROS. After that, cells were washed twice with PBS and analyzed on a Leica TCS-SPE (Leica Microsystem Nusloch Germany) microscope.

2.2.14.6 Mitotracker Analysis:

Mitotracker analysis provides the levels of mitochondria membrane potential (MMP) were examined using mitotracker fluorescent dye; Life technologies (M7512). For this experiment cells were seeded into 35mm dishes containing a glass cover slip after treatment cells were washed twice with pre-warmed PBS. Furthermore, cells were incubated with mitotracker red (100nM) for 15 min at 37^0C and then washed twice with PBS. The cells were then fixed in 4% paraformaldehyde for 20 min, permeabilized in triton X-100 (0.1%) for 20 min and stained with DAPI (0.1μg/ml) with mounting media and analyzed on a Leica TCS-SPE (Leica Microsystem Nusloch Germany) microscope.

Results and Discussions