Chapter 4

Materials and Method
Chitosan is a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine produced by the deacetylation of chitin, a natural polymer of major importance. Chitin is the second most abundant biopolymer after cellulose. The main sources of chitin are marine crustaceans, shrimps, crabs and lobster etc. Chitosan has unique properties among biopolymers due to the presence of primary amino groups which can easily bind with the metal ions. Chitosan is more versatile in comparison to chitin due to the presence of amino groups at the C-2 position. Chitosan have chemical and biological properties which includes biocompatibility, biodegradable and non-toxic [196].

4.1 MATERIALS

A powdery material chitosan with different degrees of deacetylation 75% and ≥ 80% crab shell chitosan (CSC), medium molecular weight chitosan (MMWC), low molecular weight chitosan (LMWC), middle viscous chitosan (MVC) and low viscous chitosan (LVC) powder were from Sigma-Aldrich chemicals. Potassium dichromate (GR) from E Merck and cadmium chloride monohydrate (AR) and lead nitrate (AR) were purchased from CHD Laboratory Reagent, New Delhi. Crystal violet (CV) dye (AR) Loba Chemie laboratory, acetic acid (glacial 99-100%) was procured from E Merk, specialities pvt. Ltd. Worli, Mumbai. Sodium tripolyphosphate anhydrous (STP) and KBr (AR) of CHD Laboratory Reagents, New Delhi, Deuterium oxide (D₂O) 99.9 atom % D were purchased from Sigma-Aldrich Chemicals. All other chemicals viz HCl, HNO₃ and NaOH were of analytical grade. Double distilled water used to prepare all solutions.

Cross-linking agents as epichlorohydrin (ECH) (GR) a Loba Chemine product, glutaraldehyde (GLA) 25% in water was from Spectrochem pvt. Ltd. Mumbai, India and ethylene glycol diglycidyl ether (EGDE) of Sigma-Aldrich were used to cross-link the chitosan beads to form cross-linked chitosan derivatives.

Distillation unit for double distilled water, magnetic stirrer with hot plate, Ubbelohde viscometer and non-woven polypropylene bags, whatman filter paper No.1 etc. were utilised at various stages of the experimental work.
4.2 METHOD

4.2.1 Preparation of Chitosan Beads

Chitosan solution was prepared by dissolving 2g of chitosan powder of ≥75% deacetylated in 90 ml of 2% acetic acid and 20ml double distilled water in a 100ml standard flask. Solution was kept for stirring for 24 hours and after that it was transferred in a burette. 50 ml of 1% STP solution was taken in a patry dish and brought under the burette. Slowly and carefully released the knob of the burette so that chitosan solution comes out in the form of tiny drops. The chitosan solution forms uniform spherical beads as it comes in contact with STP solution in patry dish. Beads were left in STP solution for an overnight. The wet chitosan beads were extensively rinsed with double distilled water and finally air dried to remove the water from the pore structure, hereafter called chitosan beads.

Different types of chitosan beads including medium molecular weight chitosan (MMWC) bead, low molecular weight chitosan (LMWC) bead, middle viscous chitosan (MVC) bead and low viscous chitosan (LVC) beads were prepared in the same manner as mentioned above.

4.2.2 Preparation of Cross-linked Chitosan Beads

Three different cross-linking agents i.e. ECH, GLA and EGDE used to cross-link chitosan beads of different types CSC, MMWC, LMWC and MVC at a ratio of 1:1, chitosan: cross-linking agent, as given below.

4.2.2 (I) Chitosan-ECH beads

Chitosan-epichlorohydrin beads were obtained by a procedure described by Wei, Y. C [197]. A solution of 0.01N ECH was prepared in 0.06 N NaOH in 250 ml standard flask. Freshly prepared wet chitosan beads were added to the epichlorohydrin solution to obtain a ratio of 1:1 with chitosan beads. The mixture was heated to a temperature between 40 to 50°C for two hours and stirred continuously. The chitosan beads were filtered and washed repeatedly with double distilled water to remove the traces of
unreacted epichlorohydrin and finally air-dried. The newly formed beads are called chitosan- ECH beads.

4.2.2 (II) Chitosan-GLA Beads

Chitosan-glutaraldehyde beads were prepared using a similar procedure to that described by Guibal et.al [198]. Freshly prepared wet chitosan beads were kept in 0.05 M glutaraldehyde (GLA) solution for 24 hours to obtain a ratio of 1:1. After 24 hours the cross-linked chitosan beads were thoroughly washed with double distilled water, filtered, and air-dried. The newly formed beads here after called chitosan-GLA beads.

4.2.2 (III) Chitosan-EGDE Beads

Chitosan-ethylene glycol diglycidyl ether beads were obtained by a procedure described by Zeng and Ruckenstein [199]. Freshly prepared wet chitosan beads were kept in 5% of EGDE solution and stirred for 3 hours at a temperature between 40 to 50°C. Filter the beads, washed thoroughly with double distilled water to remove unreacted EGDE solution and then air-dried. The newly formed beads are called chitosan-EGDE beads.

Cross-linking of chitosan beads with 10% and 20% EGDE solution forming 10% and 20% cross-linked chitosan-EGDE bead was done in the same procedure and confirmed by XRD system, Bruker of model D 8 Discover.

4.2.3 (I) Cross-linking of Crab Shell Chitosan (CSC) Beads

a) ECH-CSC  
b) GLA-CSC  
c) EGDE-CSC

4.2.3 (II) Cross-linking of Medium Molecular Weight (MMWC) Beads

a) ECH-MMWC  
b) GLA-MMWC  
c) EGDE-MMWC
4.2.3 (III) Cross-linking of Low Molecular Weight (LMWC) Beads

a) ECH-LMWC
b) GLA-LMWC
c) EGDE-LMWC

4.2.3 (IV) Cross-linking of Middle Viscous Chitosan (MVC) Beads

b) GLA-MVC
c) EGDE-MVC

4.2.4 Dissolution and Swelling Test of Chitosan and Cross-linked Chitosan Beads

Chitosan and cross-linked chitosan beads were tested with regard to their solubility in each of 0.10N CH₃COOH, distilled water and 0.10M NaOH solution. 0.1g of chitosan or cross-linked beads were taken in a polypropylene bag and dipped in each of the acetic acid solution, distilled water and sodium hydroxide solution for 24 hours in a closed environment. The percentage swelling of these beads was calculated from the following:

\[
\text{Percentage swelling} = \frac{(W_s - W_o) - W}{W} \times 100
\]

where \(W_s\) is the weight of swollen bag, \(W_o\) weight of empty bag before swelling and \(W\) is the weight of dry beads. The observed swelling behaviour of cross-linked chitosan beads is lesser than the pure bead.

The high hydrophilicity of chitosan beads is due to a primary amine group makes chitosan easily soluble in dilute acid solution to yield hydrogel in water. It was observed, during the course of research work, the cross-linked chitosans were found to be insoluble in acidic and alkaline media as well as in distilled water.

4.2.5 Viscosity Measurements

In order to evaluate the molecular weight of polymeric chains, various methods can be used. Widespread are viscosity and gel permeation chromatographic techniques. Viscometry is commonly selected as it is one of the simplest and most rapid methods.
for determining these molecular weights. The correlation between viscosity and molecular weight is given by the Mark-Houwink equation.

\[ [\eta] = K M^\alpha \]

\([\eta]\) is intrinsic viscosity, \(M\) is molecular weight and \(K\) and the exponent \(\alpha\) both are constants for a given well-defined polysaccharide-solvent system [200].

**4.2.6 Infrared Spectroscopy**
IR characterization of chitosan biopolymers were performed with Perkin-Elmer FTIR spectrophotometer, Spectrum RX-I instrument with a frequency range of 4000-400 cm\(^{-1}\). IR is primarily used to identify bond types, structures and functional groups in organic and inorganic compounds. IR sensitive vibrations are associated with changes in dipole moments.

For example, double and single bonds associated with carbon-hydrogen and carbon oxygen bonding (=C−H, −C−H, C−O and C=O) can be distinguished by IR absorption. When functional groups can be bonded at different locations on molecules, IR spectroscopy can frequently identify the positions at which the functional groups are attached. The reason is that vibrational frequencies differ when functional groups are attached at different sites in molecules. Each bending and stretching vibrational mode of a molecule or functional group will absorb at a particular frequency. When exposed to appropriate IR frequencies, energy will be absorbed from the incident radiation as vibrational intensities increase [201].

The FTIR spectrophotometer used to record IR-spectra. The samples were prepared in the ratio of 1:100 was made and a pressure of 5 ton was applied using a pellet holder, 1-2 mm thickness of KBr pellets is prepared and the analysis of sample was carried out.

**4.2.7 Proton Nuclear Magnetic Resonance (\(^1\)H NMR) Spectroscopy**
NMR is one of today’s most powerful tools in the study of polysaccharide composition and sequential structure. NMR is a non-destructive method resulting in retained structure and conformation of the polysaccharide.
NMR of model Bruker Spectrospin 300 MHz was used for the determination of degree of deacetylation of chitosan biopolymer. It is a powerful method used in the determination of the structure of unknown organic compounds. The $^1$H NMR spectrum of an organic compound provides information concerning, the different types of hydrogens present in the molecule, the relative position of different types of hydrogens, the electronic environment of the different types of hydrogens and the number of neighbouring hydrogen atoms [202].

Degree of deacetylation (DDA) of chitosan was determined by using $^1$HNMR spectroscopy, 5mg of chitosan sample vacuum dried at 50°C for 1 day to which 0.5 ml of D$_2$O solvent was added and finally the test tube was kept at 70°C to dissolve the polymer in solution.

4.2.8 Elemental Analysis

Vario EL III elemental analysis apparatus was used to determine the amount of C, H, and N in chitosan biopolymers. The carbon, hydrogen and nitrogen contents of the samples are determined from the quantities of CO$_2$, H$_2$O and NO$_2$ produced by the combustion of the dried solid in oxygen. In this case samples were heated to a temperature of 1000°C and approximately 2mg of chitosan was placed inside a tin boat and was dropped into the CHNS-furnace, where it was completely combusted. This instrument relies upon infrared detection to measure the weight percentage of carbon, hydrogen, and sulfur while nitrogen were measured using thermal conductivity detection.

4.2.9 X-Ray Diffraction Study

Powder diffractometers are routinely used for phase identification and quantitative phase analysis but can be configured for many applications, including variable-temperature studies, texture and stress analysis, grazing incidence diffraction, and reflectometry. The radiation used in a typical diffraction measurement contains several wavelengths, denoted K$_{\alpha 1}$, K$_{\alpha 2}$, K$_{\beta}$, which are characteristic of the material producing the X-rays [203].
Powder XRD of the material was obtained by using Discover-D X-Ray diffractometer with Cu Kα radiation (40 kV and 40 mA) at a scan rate of 1º/minute and was analysed using standard software provided with the instrument.

### 4.2.10 Thermal Gravimetric Analysis (TGA/DTA)

Thermal Gravimetric Analysis (TGA) measures the masses of samples as they are heated and cooled through standard firing programs. TGA analyses are very similar to DTA analyses. In the case of TGA analyses, the increase, decreases, or constancy of mass of samples at each temperature in the firing program indicates the presence or absence of reactions and the nature of each reaction that takes place. For example, phase changes occur without change of mass; some decomposition reactions are accompanied by weight loses; and oxidation reactions are accompanied by weight gains.

TGA analyses require small samples (several grams) of dry powders or particulate suspensions. All such samples must be thoroughly dried before performing before performing the analyses. Differential thermal analysis (DTA) a technique in which the temperature difference between a substance and a reference material is measured as a function of temperature whilst the substance and reference material are subjected to a controlled temperature programme.

Chitosan biopolymers were analysed using Shimadzu DTG 60 (TG/DTA) 20mg of the sample was used and alumina as a reference material under nitrogen atmosphere at with the flow rate of 10ºC/minute between 30-1000ºC.

### 4.2.11 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) a technique in which the difference in the energy inputs into a substance and a reference material is measured as a function of temperature whilst the substance material are subjected to a controlled temperature programme.

DSC studies were performed using a DSC Q200 TA instruments. The samples were scanned under a nitrogen atmosphere at a constant rate of 10ºC/min. The experiment consisted of 1 run, temperature between 25 to 450ºC.
4.2.12 Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) is a widely used and accepted technique capable of determining trace (µg/mL) and ultra-trace (sub µg/mL) levels of elements or metals in a wide variety of substances. Atomic absorption spectrometry uses the absorption of radiation by free gaseous atoms in order to achieve qualitative detection and quantitative determination of elements [204].

In an atomic spectrometer, atom cell dissolves the liquid sample and dissociates analyte elements into their free gaseous ground state to absorb radiation coming from light source and create a measurable signal which is proportional to concentration. The filtrate solutions obtained in the adsorption process were analyzed using flame AAS. Atomic absorption spectrophotometer (Shimadzu AA-6300 model) for Pb(II) and Atomic Absorption Spectrophotometer (model AA7000 Lab India) was used for Cd(II).

4.2.13 UV and Visible Spectroscopy

UV/VIS spectroscopy can be used to determine the concentration of a solution. UV-Visible spectrophotometer of Analytical Jena specord 250 used for the determination of crystal violet and Cr(VI).

The other instruments used for research work were pH meter (Toshniwal Instrument, New Delhi), thermostat (Model NBE, Germany) with temperature control ± 0.05°C and distillation unit to produce double distilled water of specific conductance, $0.5 \times 10^{-6}$ ohm$^{-1}$ cm$^{-1}$.

4.3 METHOD OF TAKING RUN

i) Determination of metal binding on different chitosan and cross-linked bead.

ii) Study of pH effect on adsorption

Standard solutions of different metal ions of a particular normality were prepared and pH of the experimental solution was adjusted using HCl for acidic medium and NaOH for basic medium. A calculated amount of chitosan beads of CSC, MMWC, LMWC, MVC and their cross-linked derivatives were added to the experimental solution and
kept as such for a fixed time period. Samples were taken out at a regular interval of
time to find out the concentration of unreacted metal ions by using atomic absorption
spectroscopy. The experiment was conducted at 30°C for adsorption isotherms.

Swelling behaviour of chitosan or cross-linked chitosan beads 0.1N of CH$_3$COOH and
0.1N NaOH solutions was studied by adding calculated amount, 0.1g of chitosan and
cross linked beads. The beads were allowed in the respective solution for 24 hours.
After 24 hours, percentage of swelling was calculated.

Molecular weight of chitosan powder was determined by using Ubbelohde
viscometer. A solution of (0.2%) of chitosan was prepared by using 0.1g chitosan
powder in 7ml of CH$_3$COOH of (5%) and double distilled water was added to make
the solution 50ml in a standard flask and stirring it for an overnight. 10ml of 0.1% of
chitosan solution was taken in viscometer and time of flow was recorded. The
viscometric constants were calculated by using Mark-Houwink’s.

To carry out adsorption and desorption experiment solution of cadmium of $10^{-4}$ moles
L$^{-1}$ and 0.2g of chitosan beads were placed inside the Erlenmeyer flask and pH 1 to 5
was adjusted by adding 0.1N of HNO$_3$ and pH 7 to 8 with 0.1N NaOH solution. The
chitosan mass was placed in an unwoven polypropylene bag before introducing into
the batch absorber. The solution and the chitosan were kept for 48 hours. For
desorption, the bag containing chitosan was taken out from the solution and rinsed
with reactive water set at the same pH as the solution used in the adsorption
experiment to remove metal residues that remained in the chitosan. Subsequently, the
bag with chitosan was suspended in the reactive water at the desired pH for 48 hours
the samples were analysed with atomic absorbance spectrophotometer.