MATERIAL AND METHODS/ Chapter -3

Materials: Chitosan (CS) with molecular weight of 200kD and tripolyphosphate of sodium salt (TPP) purchased from Sigma Aldrich, Mumbai, India. The degree of deacetylation of chitosan was kept at minimum with 85% (the data provided by the company). amphotericin b, vancomycin, ciprofloxacin, chloramphenicol and ketoconazole (antimicrobial agents with poor bioavailability) were obtained as a gift from Hi-Media laboratory, Mumbai, India. All other reagents used were of analytical grade.

Equipments: Cooling centrifuge (REMI), Freeze Dryer/lyophilizer (Lab Tech), Magnetic stirrer, Sonicator, Zeta-sizer (Malvern), Scanning electron microscope, Fourier transform infra-red (FTIR), X-Ray crystallography, UV-spectrophotometer, weighing machine, pH meter.

3.1. Preparation of chitosan nanoparticles by Ionotropic gelation method and best standard of process and formulation variables

Chitosan nanoparticles were prepared on the basis of the ionotropic gelation method given by Calvo et al., 1997. Chitosan was dissolved in acetic aqueous solution at various concentrations (1, 2, 3, 4 mg/mL) to prepare four different formulations of chitosan nanoparticles viz CS1, CS2, CS3, CS4. The concentration of acetic acid in aqueous solution was 1.5 time higher than that of chitosan (Xu et al, 2003). Meanwhile drug (amphotericin b, ketoconazole, ciprofloxacin, vancomycin and chloramphenicol) was also added to different chitosan solutions for active loading with 1mg/ml concentration. The TPP solution (0.85%) was prepared by double-distilled water. Chitosan nanoparticles were spontaneously fabricated with the drop wise addition of 5 ml of the TPP solution to 25 ml of CS solution containing drug under magnetic stirring (500rpm) at room temperature i.e. 37ºC.

The opalescent suspension was formed under the same above mentioned conditions. The chitosan nanoparticles were separated by centrifugation at 15000 rpm and 14°C for 30 minutes, freeze-dried and stored at 5 ± 3°C. The weights of freeze-dried nanoparticles were also measured.

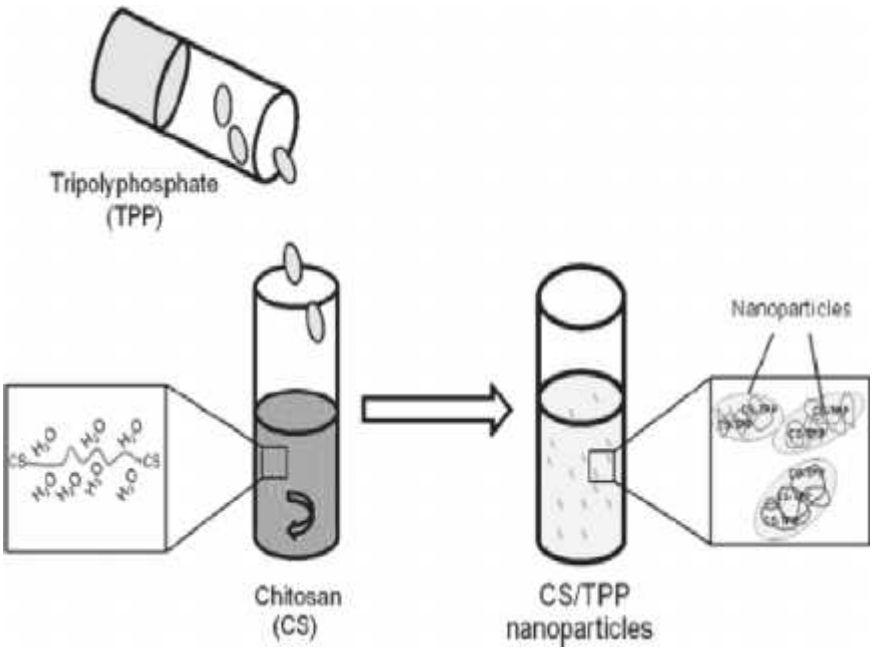

Figure.13: Schematic representation of Ionotropic gelation method for the preparation of chitosan nanoparticles at 37ºC.

3.1.1. Formulation variables

Different formulation variables were optimized to prepare nanoparticle viz. Polymer chitosan concentrations (1, 2, 3, 4 mg/ml) and cross-linking agent (TPP) concentrations (0.25%, 0.50%, 0.75%, and 1%). The effect of these variables on the nanoparticles size, shape, size distribution, drug entrapment efficiency were studied.

3.1.2. Process variables

Process variables that could affect the preparation and properties of final preparations were optimized i.e. magnetic stirring speed (250, 500, 750, 1000 rpm) and magnetic stirring time (30, 60, 90, 120mins). Effect of these variables on nanoparticles size, shape, size distribution and drug entrapment efficiency were studied.

3.2. Drug Loading/Incorporation into nanoparticles of chitosan

The drug was incorporated into chitosan nanoparticle either by entrapment during the preparation of the particles or by adsorption onto the surface of the particles. In the second method the nanoparticles were incubated in a drug solution, which resulted in the drug being entrapped

directly on the surface of the nanoparticle. In this research drug loading was done actively i.e. while preparation of CS nanoparticles. The concentration of chitosan, polyanion (TPP), ratio of polymer as well as polyanion used during preparation and drug concentration (amphotericin b, ketoconazole, ciprofloxacin, vancomycin and chloramphenicol) taken described in table no. 5-9:

Table.5: Concentrations of chitosan, polyanion TPP and Amphotericin B

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
CSI	1mg/ml	1mg/ml	5:1	0.85%
CSII	1mg/ml	2mg/ml	5:1	0.85%
CSIII	1mg/ml	3mg/ml	5:1	0.85%
CSIV	1mg/ml	4mg/ml	5:1	0.85%

Table.6: Concentrations of chitosan, polyanion TPP and Ketoconazole

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
CSI	1mg/ml	1mg/ml	5:1	0.85%
CSII	1mg/ml	2mg/ml	5:1	0.85%
CSIII	1mg/ml	3mg/ml	5:1	0.85%

Table.7: Concentrations of chitosan, polyanion TPP and Ciprofloxacin

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
CSI	1mg/ml	1mg/ml	5:1	0.85%
CSII	1mg/ml	2mg/ml	5:1	0.85%
CSIII	1mg/ml	3mg/ml	5:1	0.85%
CSIV	1mg/ml	4mg/ml	5:1	0.85%

Table.8: Concentrations of chitosan, polyanion TPP and Vancomycin

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
CSI	1mg/ml	1mg/ml	5:1	0.85%
CSII	1mg/ml	2mg/ml	5:1	0.85%
CSIII	1mg/ml	3mg/ml	5:1	0.85%

Table.9: Concentrations of chitosan, polyanion TPP and Chloramphenicol

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
CSI	1mg/ml	1mg/ml	5:1	0.85%
CSII	1mg/ml	2mg/ml	5:1	0.85%
CSIII	1mg/ml	3mg/ml	5:1	0.85%
CSIV	1mg/ml	4mg/ml	5:1	0.85%

3.3. Characterization of poorly soluble drug loaded chitosan nanoparticles

X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Zeta Potential measurements were employed to characterize the synthesized nanoparticles. Crystal structures of synthesized NPs were analyzed by XRD method. The chemical groups and chemical interactions involved in synthesized NPs were identified with FTIR plots. The sizes and shapes of NPs were measured by TEM/SEM images.

3.3.1. X-Ray Diffraction (XRD)

X-ray diffraction is used for the phase identification of crystal materials and provides information about average dimensions of nanocrystals. XRD (Rigaku Ultima-IV X-Ray Diffractometer) was performed to understand crystal structure of the synthesized drugs loaded chitosan nanoparticles.

3.3.2. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared Spectroscopy used to understand the chemical composition of the chitosan, chitosan nanoparticles and poorly soluble drugs loaded chitosan nanoparticles. 0.2 g of drug loaded chitosan nanoparticles was compressed with 0.18 g KBr for the pellet formation. Pellets were then analyzed in FTIR instrument within range of 400-4000cm^{-1} (Thermo Scientific, Nicolet 6700).

3.3.3. Freeze Drying

Freeze-drying is a dehydration process, working by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to the gas phase. After the drug loading studies, if the loading capacity of nanoparticles

found inefficient, increasing the porosity of nanocarriers by freeze drying would be an effective solution. The freeze drying process was applied on CSNPs in order to observe the formation of a porous, sponge like structure so that the drug loading capacity of nano-carrier is increased.

3.3.4. Scanning Electron Microscopy (SEM)

Scanning electron microscopes scan the surface of sample line by line with a fine probe of electrons and give information about sample's surface topography and composition. SEM images of drug loaded chitosan nanoparticles were taken in order to visualize the surface properties and size dispersion of nanoparticles.

3.3.5. Zeta (ζ) Potential

The zeta potential values of the chitosan NPs (CSNP) for the determination of stability of nanoparticles were measured with Zetasizer (Malvern Instruments Co., UK), in aqueous NaCl solutions at varying pH of 3.0–11.0 (adjusted by 0.1N NaOH or HCl).

3.3.6. Drug Quantification Calculations

Poorly soluble drug loaded chitosan nanoparticles were prepared. The amount of drug entrapped was evaluated indirectly from the difference between the total quantity of drug added to the loading solution (initial loading) and the quantity of non-entrapped drug remaining in the supernatant. This was done by measuring the absorbance of the samples determined using the UV spectrophotometer the drug concentration in the supernatant calculated. A standard curve (concentration vs. UV absorbance) was prepared diluting a standard solution of drug to various concentrations ranging from 0.5-1.5mg/ml.

By using external standard method of calibration the amount of free drug in the supernatant of drug loaded nanoparticles was calculated. This value was then subtracted from the known initial loading to measure of drug entrapped. After nanoparticle formation, the entrapment or association efficiency and practical yield can be determined from the supernatant (Reid et al., 2013; Grenha et al., 2005; Katas et al., 2006).

3.3.7. % Entrapment Efficiency (EE)

Entrapment efficiency indicates the efficiency of the preparation method to incorporate the drug into the carrier system as it expresses the amount of the drug entrapped within the nanoparticle compared to the initial drug loading (de Villiers, 2009). 100% entrapment efficiency meant that the total initial drug loaded was integrated into the nanoparticles. The performance of the drug delivery system can be determined by the amount of drug entrapped as it impacts the rate and extent of drug release from that system (Judefeind and de Villiers., 2009). Entrapment efficiency was determined according to the following equation (Zhang., 2009):

$$EE\ \% = Actual\ drug\ loaded/theoretical\ drug\ loaded \times 100\ \%$$

Where the actual drug loaded is the total amount of drug substracted from amount of free drug and theoretical drug is total amount of drug.

3.3.8. % Yield of Chitosan Nanoparticles

The percentage yields of chitosan nanoparticles were calculated from the weight of dried nanoparticles recovered and sum of initial dry weight of starting material as:

$$PY\% = Nanoparticle\ weight / Theoretical\ mass\ (polymer+drug+TPP) \times 100$$

Nanoparticle weight = 0.0254gm, polymer = 20mg, drug = 20mg, TPP= 0.025gm

3.3.9. Assessment of Antimicrobial activity of drug loaded chitosan nanoparticle formulation CS1.

3.3.9.1 Nutrient agar (NA)

0.5% Peptone - this provides organic nitrogen, 0.3% beef extract/yeast extract - the water-soluble content of these contribute vitamins, carbohydrates, nitrogen, and salts, 1.5% agar - this gives the mixture solidity, 0.5% Sodium Chloride - this gives the mixture proportions similar to those found in the cytoplasm of most organisms, distilled water - water serves as a transport medium for the agar's various substances, pH adjusted to neutral (6.8) at 25 °C.

23 g of nutrient agar was dissolved in 1000 ml of distilled water and was mixed thoroughly. This solution was heated with frequent agitation and boiled for 1min to completely dissolve the agar powder. This media was then autoclaved for 15 min at 121ºC.

3.3.9.2 Potato dextrose agar (PDA)

Potato Infusion from 200 g.....4 g*,

Dextrose.....20 g ,

Agar .....15 g

*4.0 g of potato extract is equivalent to 200 g of infusion from potatoes.

Final pH: 5.6 ± 0.2 at 25°C.

39 g of the medium was suspended in one liter of purified water. Then heated with frequent agitation and boiled for one minute to completely dissolve the medium. This medium was then autoclaved at 121°C for 15 minutes.

NA and PDA media was prepared as described above and autoclaved. The strain selected for the study, was gram negative (*E.coli, Pseudomonas aeroginosa*) gram positive (*Bacillus cereus, Streptococcus thermophilus*) bacterial strains and fungal strain (*Aspergillus fumigatus* and *Coriolus versicolor spp.*) because these microorganisms are highly infectious and frequent. The bacterial as well as fungal strain was cultured on the NA and PDA medium respectively. NA and PDA plates were prepared by pouring 20ml of the media into a sterile petridish separately. 10µl of culture was spreaded over the media and incubated at 37°C for 2h for stabilization. Further free drug and drug loaded chitosan nanoparticles dissolved in normal saline were dropped over the plate on the marked points in equal concentration. Positive control plate was with microorganism but no drug. Plates were then kept for incubation at 37°C for 48h. Zone of inhibition were determined or measured for all samples after 48h of incubation.

3.4. Synthesis of Thiol-Containing Chitosan (TCS) and drug loaded thiolated chitosan nanoparticles.

3.4.1. Preparation of thiolated chitosan

CS (0.1650 g) was dissolved in 1% w/w acetic acid solution. Then thiolactic acid or TLA (0.1055 g) was dissolved in water and then added drop wise to the CS solution. Then, EDC (0.1552 g) was added to the above reaction mixture. The pH of the solution was maintained at 5.0. The reaction was conducted at 50°C for 4 h. Then the reaction mixture was poured into acetone solution (200 mL) for precipitation. The precipitate was washed with ethanol. The product was dried in vacuum at room temperature i.e. 37ºC (Barthelmes et al., 2011; Dünnhaupt et al., 2012; Calvo et al., 1997; Bilensoy et al., 2009).

3.4.2. Preparation of TCS nanoparticles

Thiol-containing chitosan nanoparticles were prepared by using same ionotropic gelation process with same concentration of chitosan, polyanion and drug that was used for chitosan nanoparticles preparation. The different formulations of CS1 with counter polyanion TPP as shown in table.10. TCS (1mg/ml) was dissolved in 10 ml of a 1% w/w acetic acid solution and stirred for 24 h to obtain a transparent and homogeneous solution. The TCS solution with or without drug was dropped through a syringe needle into the 0.85% TPP aqueous phase, nanoparticles were formed immediately and preparation was allowed to stand for 1 h and then centrifuged at 15000 rpm for 30 mins. The pellet was lyophilized and the dried samples of nanoparticles were collected for further study.

Table.10: Concentrations of thiolated chitosan, polyanion TPP and poorly soluble drugs.

SAMPLE	DRUG	POLYMER(CS)	RATIO(CS/TPP)	POLYANION(TPP)
T-CH-AMPHO	1mg/ml	1mg/ml	5:1	0.85%
T-CH-KETO	1mg/ml	1mg/ml	5:1	0.85%
T-CH-CIPRO	1mg/ml	1mg/ml	5:1	0.85%
T-CH-VANCO	1mg/ml	1mg/ml	5:1	0.85%
T-CH-CHLM	1mg/ml	1mg/ml	5:1	0.85%

3.5. Comparative study of *in-vitro* drug release from chitosan nanoparticle CS1 , thiolated chitosan nanoparticles and free drug

The particular amount of drug-loaded chitosan nanoparticles was suspended in separate tubes containing equal volumes of 0.2 mol/L PBS solutions (pH 7.4) and incubated by shaking at 37°C and 500 rpm. At appropriate time intervals (1, 2, 4, 6, 10, 12 h) one tube was removed and the sample was centrifuged at 15,000 rpm and 14°C for 30 minutes. The amount of drug released in the supernatant was measured. The amount of released drug from CSNPs formulation CS1, thiolated chitosan nanoparticle and free drug itself was determined by measuring the absorbance of the supernatant within time intervals at 266 nm by an UV spectrophotometer. Quantification of drug was performed by generating a calibration curve with known amounts of drug concentrations. Finally a graph was plotted between % cumulative drug release and time (Fig 14) (Wang et al., 2012). The *in-vitro* study was done for free drug, drug loaded chitosan nanoparticles CS1 and drug loaded thiolated chitosan nanoparticles.

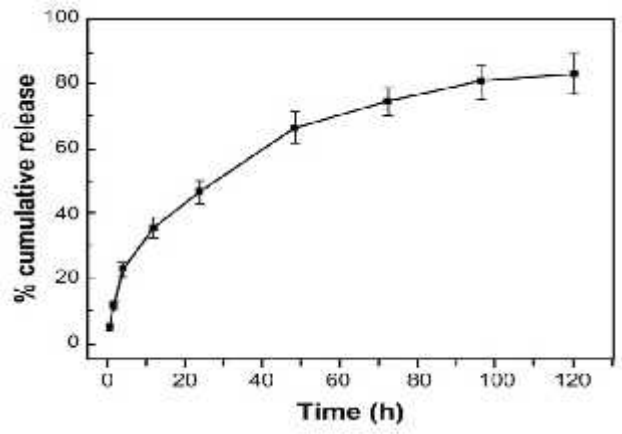

Figure.14: Graph showing percentage cumulative release of drug with respect to time

3.6. Statistical analysis

Each experiment was carried out in triplicate unless otherwise specified. The values represent the mean ± standard deviation. All data were expressed as mean standard deviation (SD) unless particularly outlined. The statistical significance of differences among more than two groups was determined by one-way ANOVA by the software SPSS 13.0. A value of $P < 0.05$ was considered to be significant.

3.7. Kinetic modeling of poorly soluble drug release from chitosan nanoparticles

Investigation of poorly soluble drugs such as Amphotericin B, Ketoconazole, Ciprofloxacin, Vancomycin and Chloramphenicol release kinetic from chitosan nanoparticle formulation CS1 performed by applying six kinetic models on cumulative release data obtained of sample which were synthesized, characterized and loaded by chitosan nanoparticle CS1 previously at temperature 37ºC.

The determination was accomplished by comparison of determination coefficient or correlation coefficients; the higher the determination coefficient the most suitable model is achieved. As it was obvious a portion of release occurred as burst release at the beginning. No burst release happened due to the low specific surface of drug loaded chitosan nanoparticles which were exposed to the media. Release kinetic data were fitted to the different models which were explained completely and in accordance with regression coefficient of determination (R^2) models.