# Chapter 4: Development of Chitosan based non viral Vectors

**4.1 Introduction**

Chitosan is widely used as non-viral vector system for safe and efficacious nucleic acid and siRNA delivery. It is a safe, biocompatible, biodegradable, and a natural polycation obtained by deacetylation of chitin and also having minimum immunogenicity. These characteristics make chitosan a better candidate for development of the non-viral vector based gene delivery systems(1, 2). Chitosan, owing to positive charged at below 6.6 pKa, favors the interaction with the DNA and siRNA yielding to development of the polyplexes of different particle size, shape and other characteristics. Solubility, ionization constant, degree of deacetylation and molecular weight are the most important factors that influence the characteristics of chitosan mostly(3).

Moreover, poor solubility of the chitosan at physiologic pH results into colloidally unstable complex. The poor solubility of polymer results from the deprotonation of the amino groups of D-glucosamine. Moreover, pKa of chitosan is around 6.6, indicates that it is in an unionized form at the physiologic pH. Several studies demonstrated that optimal DNA or siRNA delivery obtained between the pH 6.5-7.0 and transfection efficiency may rapidly decrease at the higher pH owing to the premature release of DNA or siRNA from the polymer complex. Chitosan with high molecular weight (100-400 kDa) forms very stable complexes but then have poor colloidal stability because of low solubility of the polymer and they also adds viscosity at the suitable concentrations for gene/nucleic acid delivery

The perfect polymer-based siRNA delivery gratify the conflict necessities for chitosan - siRNA binding and offer a balance between intracellular release and protection (4). Recent finding demonstrated that the interactions amongst the chitosan and siRNA were pH dependent and also the adhesive interactions shown decreased at the increasing pH from the 4.1 to 6.1, 7.4, and 9.5 confirmed by atomic force microscopy(5).

To overcome these limits of the chitosan, various modifications have been tried to increase the colloidal stability such as PEGylation, which may induce positive charge at physiologic pH via quaternization by trimethylation of amino group moieties(6, 7). Trimethylation of the native chitosan improves the solubility of polymer at the physiological pH and assist intracellular release of the DNA or siRNA at particular target and could be potential non-viral based vectors for the gene delivery. Supplementary, modification or conjugation of amines of the chitosan remains promising strategy to enhance the transfection efficiency

along with intracellular release of the vectors. To increase the buffering capacity of chitosan in the range of endosomal pH and therefore facilitating endosomal escape of the polymer complexes by means of the osmotic burst mechanism. In addition, heterocyclic amine compuonds with the different pKa values like Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acid were conjugated through their –COOH (Carboxyl) groups onto remaining amines of trimethylated chitosan by amide linkage by known EDC/NHS Chemistry.

### 4.2 Material and Methods

Medium molecular weight chitosan, Piperazine 2-carboxylic acid(PC), EDC,NHS and sodium hydroxide were procured from Sigma aldrich, Bangalore, India, Methyl iodide, Pyridyl 3-Acetic acid(PA), Ethanol, Diethyl ether, N-methyl Pyrollidinone (NMP) were purchased from Spectrochem; Urocanic acid (UA) was purchased from Himedia(Mumbai).

### 4.3 Preparation of Trimethylated Chitosan (TMC)

Trimethylated Chitosan was synthesized according to previous protocol performed with slight modifications (8). Briefly, appropriate quantity of chitosan weighed and dissolved into 20 ml NMP into a 250 mL round bottom flask. And content was stirred using magnetic stirrer at the temperature 60°- 65°C. 7 ml of 5% w/v NaOH solution was then added into above stirred content. Then 6 ml of the Methyl Iodide (MI) was added and stirred for 1 hr. After stirring, trimethylated chitosan was precipitated by mixture of ethanol and diethyl ether (1:1) and adding sodium hydroxide solution till pH reached at 9 and. The precipitated trimethylated chitosan was centrifuged for 10 min at 10,000 rpm and the supernatant was then decanted and sediment settled at the bottom of the centrifuge tube was collected and washed twice with mixture of ethanol and diethyl ether. Finally, residue was dried.

Furthermore, degree of the methylation was increased by the using synthesized trimethylated chitosan in second step and repeating the all the steps and increasing the reaction time. Synthesized Polymers were lyophilized and characterized for degree of quaternization, NMR, FTIR, Solubility improvement at physiological pH and *in vitro* cytotoxicity assay. Structures with different degree of quaternization of trimethylated

chitosans were confirmed by $_1$H-NMR and FTIR spectra illustrated in the result and discussion section.

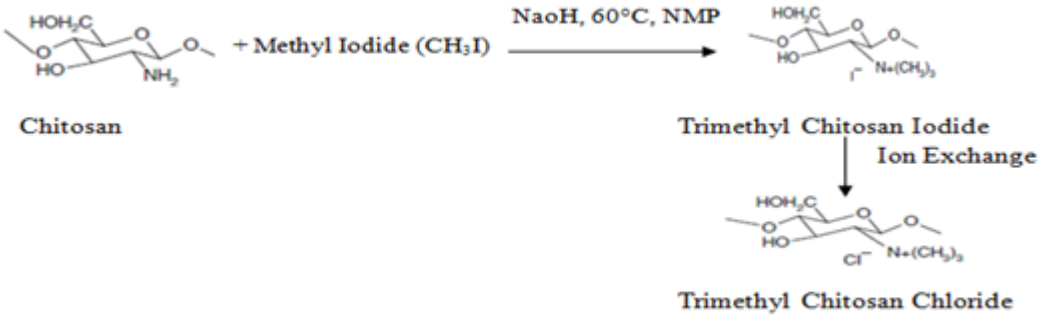



**Figure 4.1: Synthesis steps of N,N,N-Trimethylated chitosan**

### 4.4 Determination of degree of Quaternization

NMR spectroscopy was utilized to determine the degree of substitution or quaternization. Proton NMR ($^1$H-NMR) spectra were measured with 400 MHz spectrometer (Bruker) by dissolving the trimethylated chitosan samples in the $D_2O$. Degree of quaternization was estimated based on the following equation:

$$DQ\% = \left[\frac{[(CH_3)_3]}{[H]} \times \frac{1}{9}\right] \times 100$$

where DQ% is the Degree of quaternization as a percentage, [(CH3)3]-integral of the trimethyl amino group (quaternary amino) peak at 3.1–3.4 ppm, and[H]- integral of the $^1$H peaks between 4.7 and 5.7 ppm on the $^1$H-NMR spectrum. $^1$H corresponds to the hydrogen bonded to $C^1$ of the glycoside ring.

### 4.5 Solubility improvement determination

Solubility improvement after trimethylation of chitosan was studied by turbidimetry analysis. Briefly, trimethylated chitosans of different degree of methylation were firstly dissolved in 0.1% v\v acetic acid and pH was then adjusted with 1N sodium hydroxide and after that the transmittance was measured at 600 nm as the function of pH. pH 50 i.e. pH at

which transmittance value are higher than 50% and cloud point pH i.e. pH at which transmittance value is higher than 98% were measured.

**4.6 *In vitro* cytotoxicity assay (MTT Assay)**

All Synthesized trimethylated chitosans were accessed for *in vitro* cell cytotoxicity by MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-di-phenyl tetrazolium bromide) assay. As MTT assay is very rapid and versatile technique to access the cell viability *in vitro*(9).Other general method for the determination of cell viability like dye exclusion (trypan blue) can also be used. Before the cellular internalization, destabilization of the cellular membrane or any DNA interaction after cell internalization could be the basis for the cytotoxicity of the polymer.

MTT assay was performed in different concentrations of synthesized trimethylated chitosan polymers. The A549 alveolar epithelium cells were seeded at density of $5\times10^3$ cells/well in a 96 well microtiter plate (corning, New York) using DMEM supplemented with 10% Fetal Bovine Serum (FBS). The cell culture was grown for 24 hrs in $CO_2$incubator maintained at 5%concentration and humidified with saturated Copper sulphate solution. After 24 hrs, the cells were exposed at different polymer concentrations and evaluated for six hrs. After exposure time, cell the media was exchanged with the complete medium containing 1% antibiotic solution and 10% Fetal Bovine Serum (FBS). After 24 h, cells were then washed with phosphate buffer saline and 20 µl of the MTT dye (5mg/mL) solution was added to each of the well plate. The MTT dye was allowed to react for the 4 h under incubator condition, after that cell medium in each plate was then replaced with 100 µl of DMSO (Himedia, Mumbai) and microtiter plate was shaken gently to completely dissolve the formazan crystals. The color of the completely dissolved formazan was then accessed using the microtiter plate reader (Biorad, California). Cells treated with DMEM media and 0.1% Triton X were used as negative and positive control respectively. The absorbance values of cells treated with DMEM media were taken as 100% cell viability and all other treatments were expressed relative to it.

**4.7 Conjugation of Heterocyclic moieties on TMC**

The synthesized TMC were conjugated with different heterocyclic compounds such asUrocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acid at different molar ratios by covalent conjugation using carbodiimide chemistry, i.e. EDC-NHS crosslinking assay. Briefly, appropriate quantity of TMC and heterocyclic compounds were dissolved in the 20 mM MES buffer. The pH was adjusted to the 5.5-6.0. Moreover, Carboxylic groups of the heterocyclic compounds were activated by EDC-NHS dissolved in MES buffer in these 20 times molar excesses of EDC was then added to the solution followed by addition of NHS. After that, the activated solution was then immediately added to the TMC solution. The conjugation reaction was carried out for 24h at the room temperature. The reaction was quenched by adding 0.1 N sodium hydroxide drop wise to raise pH of the media to 9-10. The resulting solution was dialyzed by dialysis membrane (MW Cut off-12,000kDa) for 48 hr against distilled water. After dialysis, solution was then centrifuged at 10,000 rpm for 10 min. Obtained precipitates was again centrifuged and washed by addition of water. Resulting solution was then freeze dried (Lyophilizer- VirTis Advantage Plus) to get dried product. Further, synthesized Polymers were characterized for $^{1}$H-NMR, FTIR, TNBS assay to determine conjugation of heterocyclic compounds on TMC etc.

Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acidwere used in different concentrations to attain different degree of conjugation of the amines on the trimethylated chitosan (TMC). Different quantity of the heterocyclic compounds used were denoted by A,B and C on descending order of concentrations used while Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acid were denoted as UA,PC and PA , respectively. Consequently this nomenclature mentioned in the following table 4.1 has been used onward.

**Table 4.1: Nomenclature of heterocyclic moieties conjugated TMC**

| Sr. No. | Concentration of Heterocyclic moieties used | Type of Heterocyclic moieties | Nomenclature |
|---|---|---|---|
| 1 | 1.08 mmole (A) | Urocanic acid | TMC-UAA |
| 2 | 0.72mmole (B) | | TMC-UAB |
| 3 | 0.36 mmole (C) | | TMC-UAC |
| 4 | 0.739 mmole(A) | Piperazine 2-Carboxylic acid | TMC-PCA |
| 5 | 0.49 mmole(B) | | TMC-PCB |
| 6 | 0.246 mmole(C) | | TMC-PCC |
| 7 | 0.864 mmole(A) | 3-Pyridyl acetic acid | TMC-PAA |
| 8 | 0.576 mmole(B) | | TMC-PAB |
| 9 | 0.288 mmole(C) | | TMC-PAC |

### 4.8 Nuclear Magnetic Resonance spectroscopy (NMR) Analysis

Proton NMR spectroscopy was carried out to determine the characteristics peaks of the functional group to confirm the synthesized polymers. $^{1}$H-NMR spectra of TMC conjugated with Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acidindividually were recorded on Bruker 400 MHz spectrometers at frequency of 400 MHz. Native chitosan and modified polymers were dissolved in the D2O in 5 mm NMR tubes and $^{1}$H-NMR spectroscopy were performed.

### 4.9 Fourier Transformed Infrared Spectroscopy (FTIR) Analysis

FTIR spectra were recorded for all the synthesized polymers by potassium bromide (kBr) pellet technique. Appropriate quantity of the polymers was mixed with the KBr and compressed to obtain a pellet and analysis was performed by FTIR Spectrophotometer (Bruker and Shimadzu). Baseline correction was done and samples were then scanned against the blank KBr pellet background in the range of 4000-600 $cm^{-1}$ wave number with 1.0 $cm^{-1}$ resolution.

**4.10: 2, 4, 6- Trinitrobenzene 1-Sulphonic acid (TNBS) assay**

After, structure confirmation by NMR and FTIR, a method based on the UV-visible spectroscopy was used to quantify the substitution of the amine groups of the trimethylated chitosan. TNBS assay is popular among other methods and specific to primary amine groups. Since, the degree of substitution plays a significant role in quantification, TNBS assay is used to control the substitution.

Free primary amines of the modified trimethylated chitosans were estimated by TNBS assay. TNBS reacts with primary amines of the TMC making it possible to determine the percent of substituted primary amines in modified TMC with compared to unmodified TMC. Briefly, polymers were dissolved in the reaction buffer,0.1 M Sodium bicarbonate buffer.0.25 ml of 0.01% w/v TNBS was then added into 0.5 ml solution of each sample and mixed well and after that incubated at 37 °C for 2 h. Then, 0.25 ml of 10% SDS and 0.125 ml of 1 N HCL were then added to each sample. Absorbance of each of the sample was measured at 335 nm (10, 11). Experiment was performed in triplicate and percent of primary amines substitution was calculated accordingly.

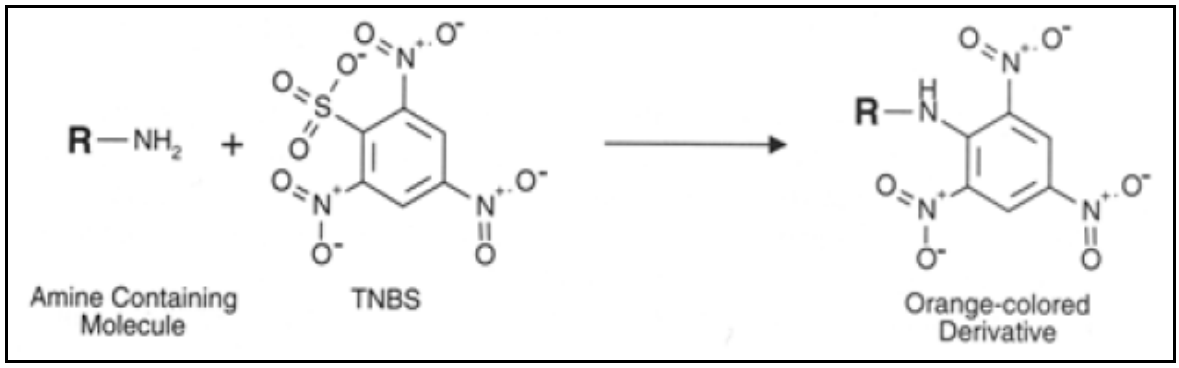



**Figure 4.2: Reaction of TNBS with a primary amine-containing molecule to produce a chromogenic derivative.**

**4.11 Buffering Capacity/Acid Base titration**

Endosomal escape of the formulation is necessite for the successful transfection. This property directly relates to buffering capacity of the vectors in the endosomal pH range. Hence, proton sponge study was performed. Study was performed by titrating synthesized polymer solution from acidic pH to basic as chitosan was insoluble at basic pH

and it took more time to dissolve during titration(12).Briefly, 10 mg of the polymer was dissolved in the sodium chloride solution(150 mM) by hydrochloric acid by adjusting initial pH 3.5 and then this polymer solution was then titrated with 0.1 N NaOH solution. pH of the polymer solutions were recorded by pH meter (Lab India, Model: Pico$^{+}$). The amount of NaOH consumed was calculated and compared.

**4.12 Hemolysis Study**

Erythrocytes (RBCs) were collected from fresh heparinized rat blood through centrifugation at 1000 g for 10 minutes at 4$^{o}$C for the hemolysis study. Obtained pellet was then washed with PBS Ph 7.4 several times until supernatant became clear. RBCs were reconstituted in PBS pH 7.4 to obtained 2% v/v RBCs suspension. The 500 µl of cell suspension was added into different concentrations of the modified polymers prepared in eppendrof tubes. Then mixture was mixed by vortex mixer thoroughly and incubated at 37$^{o}$C for 1 hr under the constant shaking in Shaker incubator. After that, RBC suspension was centrifuged at 3000-3500 rpm for 10 min. The supernatant was estimated for hemoglobin content release by UV-Visible spectrophotometer at 540 nm (13-18). Triton X (0.1%) was used as positive control, the % hemolysis was estimated using the following equation.

$$\text{Haemolysis} = \frac{A_s - A_n}{A_p - A_n} \times 100$$

Where, As- absorbance of supernatant, An- absorbance of negative control treated cell supernatant, Ap- absorbance of positive control treated cell supernatant. All assays were performed in triplicate and data were reported as mean ± SD.

**4.13 Erythrocytes aggregation**

Due to the cationic charge, Cationic polymers are known to induce aggregation of the erythrocytes in vivo and hence to evaluate biocompatibility of newly synthesized polymers, erythrocyte aggregation study was performed using rat blood cells. The erythrocyte suspension was prepared by the same procedure as that described above section for Hemolysis study. 500 µl of 2% erythrocyte suspension containing synthesized polymers at different concentration were prepared in the PBS pH 7.4 mixed thoroughly by vortexing

and incubated for 2 h at 37°C in incubator. The erythrocyte aggregation was observed using inverted microscope (Nikon Eclipse TS100) in a phase contrast mode.

### 4.14 *In vitro* cytotoxicity assay (MTT Assay)

MTT assay was performed by same protocol described in *in vitro* cytotoxicity assay for Trimethylated chitosans of different degree of quaternization. MTT assay was performed in different concentrations of conjugated trimethylated chitosan polymers.

### 4.15 Result and Discussion

**Nuclear Magnetic Resonance spectroscopy (NMR) Analysis**

Successful quaternization of the medium molecular weight chitosan with different degree was confirmed by the $^{1}$H-NMR spectra shown in the figure 4.3, figure 4.4, figure 4.5 and figure 4.6. Characteristic peaks of the quartenized amino groups appeared at $\delta$ 2.8 ppm and 3.4 ppm for all the quartenized chitosans. In case of low degree quartenized chitosan peak intensity was small while higher degree quartenized chitosan demonstrated intense characteristic peaks. Peak between $\delta$ 1.5 ppm to $\delta$ 2.0 ppm attributed to hydrogen of the Acetyl group of the chitosan while peak between 4.5 ppm and 5.5 ppm demonstrates the hydrogen bonded to the anomeric carbon 1 of the chitosan molecule. These$^{1}$H- NMR findings suggest the successful synthesis of the quartenized chitosans.

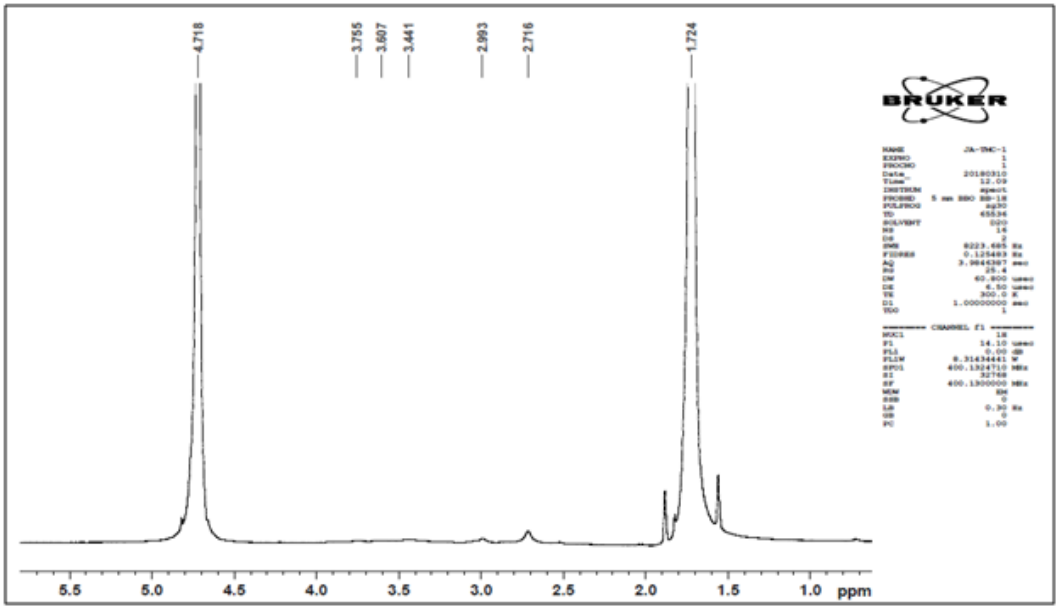



**Figure 4.3: $^{1}$H-NMR spectrum of Trimethylated Chitosan-1 (TMC-1)**

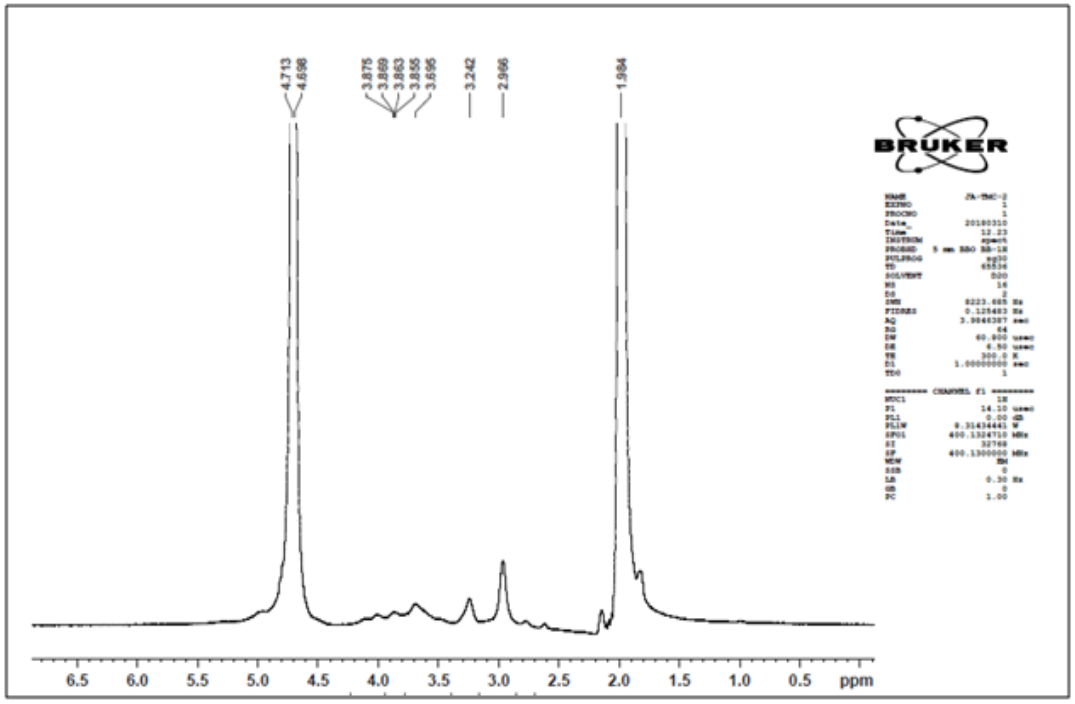



**Figure 4.4: $^{1}$H-NMR spectrum of Trimethylated Chitosan-2 (TMC-2)**

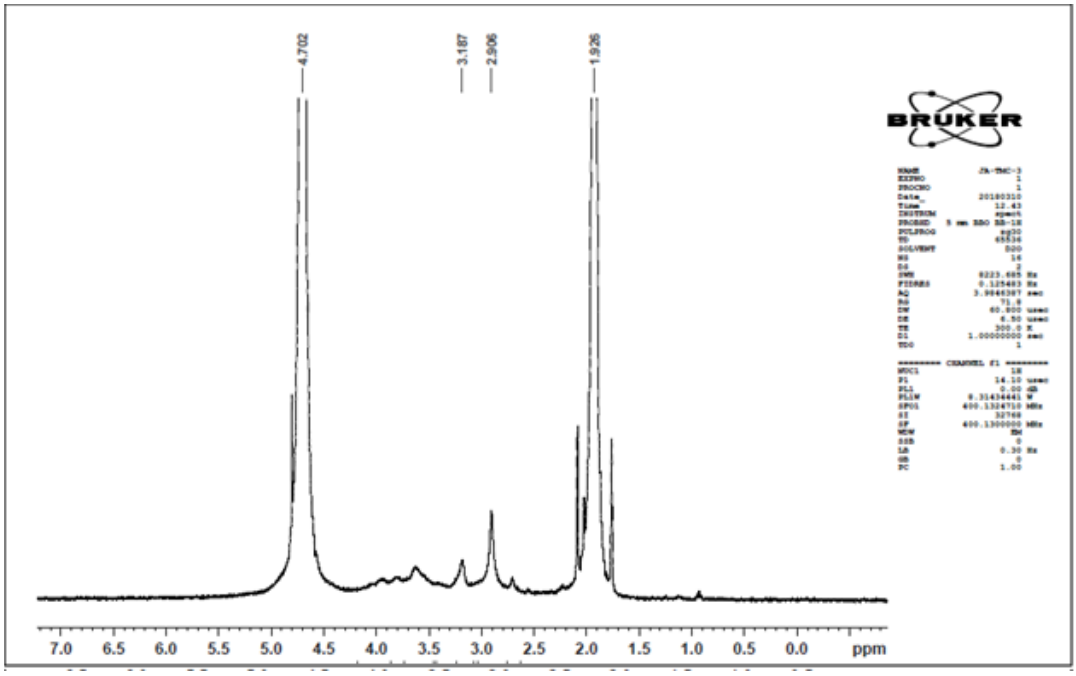



**Figure 4.5: $^{1}$H-NMR spectrum of Trimethylated Chitosan-3 (TMC-3)**

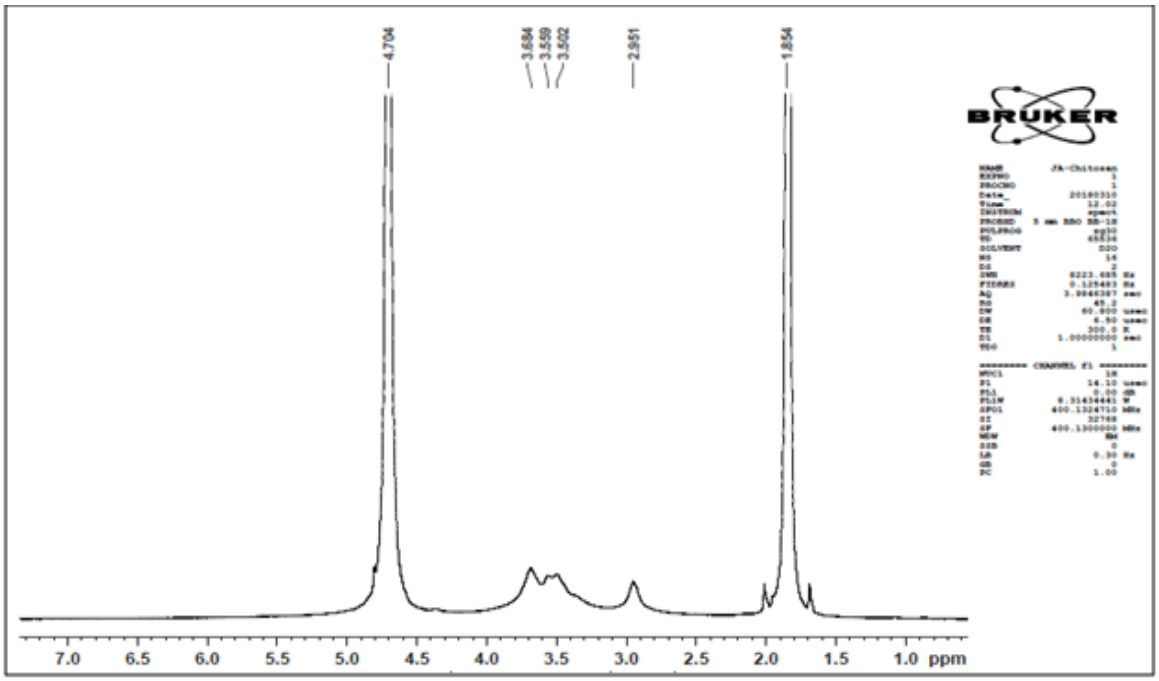



**Figure 4.6:$^{1}$H-NMR spectrum of Trimethylated Chitosan-4(TMC-4)**

**Fourier Transformed Infrared Spectroscopy (FTIR) Analysis**

The FTIR spectrum of the trimethylated chitosan provides the evidence for the occurrence of the methylation particularly in the region between 1,700–1,200 cm-1. Band centered at 1455-1475 $cm^{-1}$ in FTIR spectra of the trimethylated chitosan (Figure 4.7 and Figure 4.8) attributed to asymmetric angular deformation of the C–H bonds of methyl groups of TMCs, which is absent in the Chitosan spectrum, shown in figure 4.9. Band as a result of the angular deformation of the N-H bond of the amino groups occurred at between 1500-1620 $cm^{-1}$ however, it is weaker because of the happening of the N-methylation. Peak at 1630-1660 $cm^{-1}$ assigned to quaternary ammonium group.

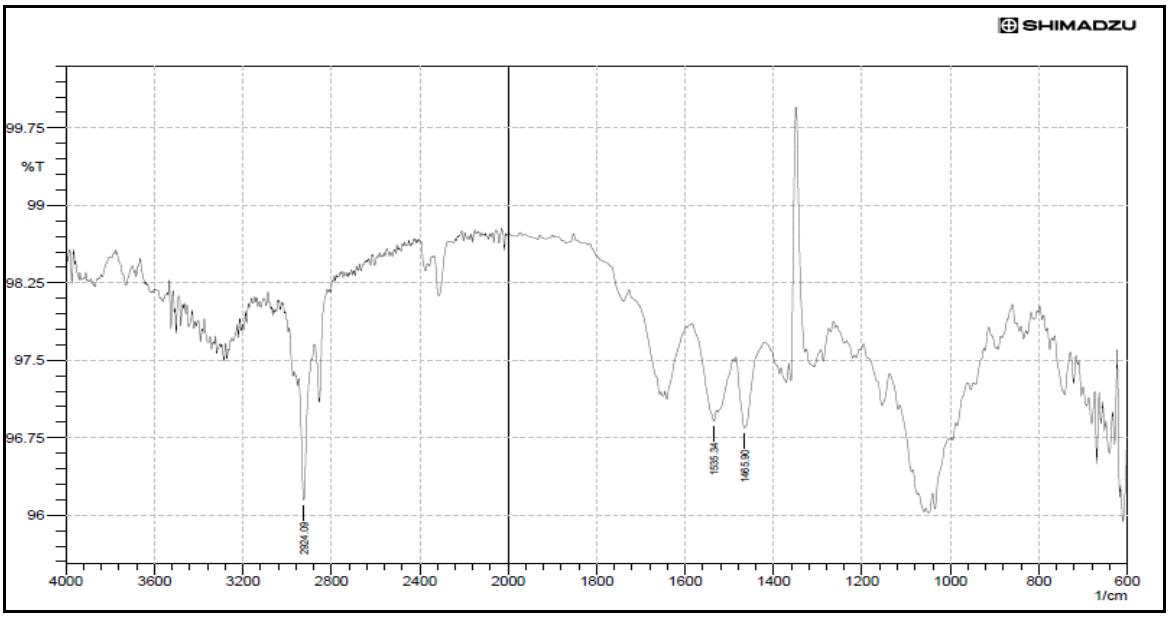



**Figure 4.7: FTIR spectrum of Trimethylated Chitosan-1**

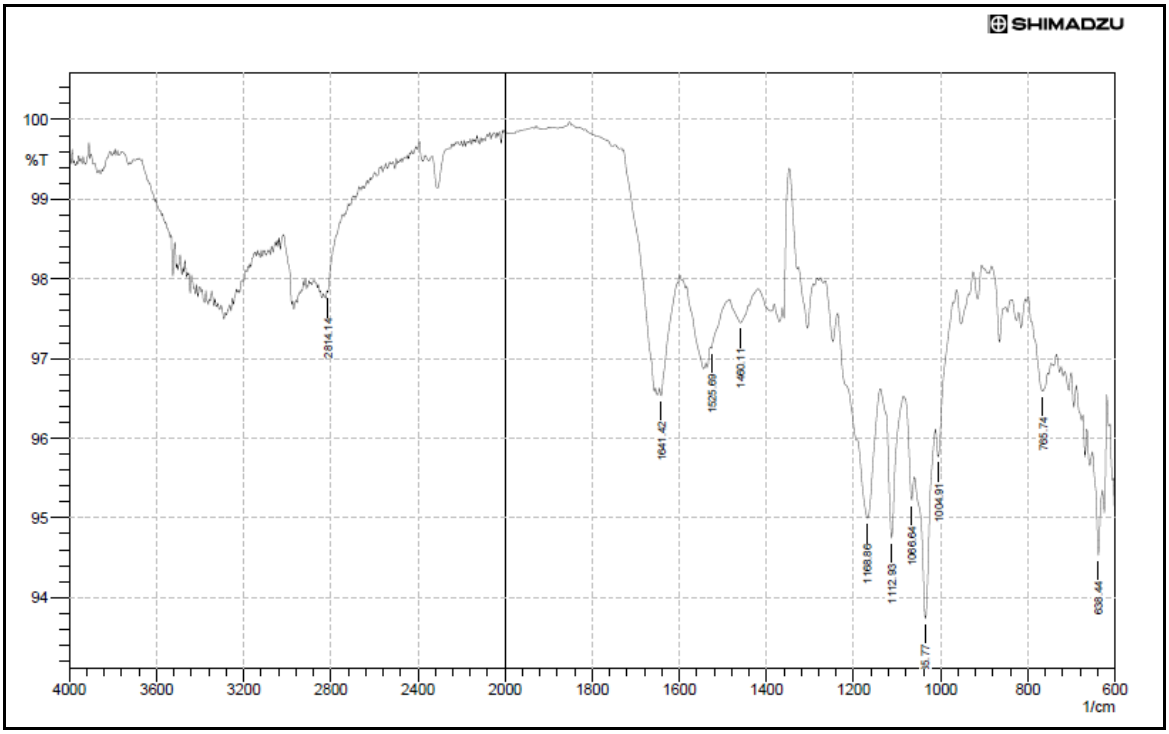



**Figure 4.8: FTIR spectrum of Trimethylated Chitosan-2**

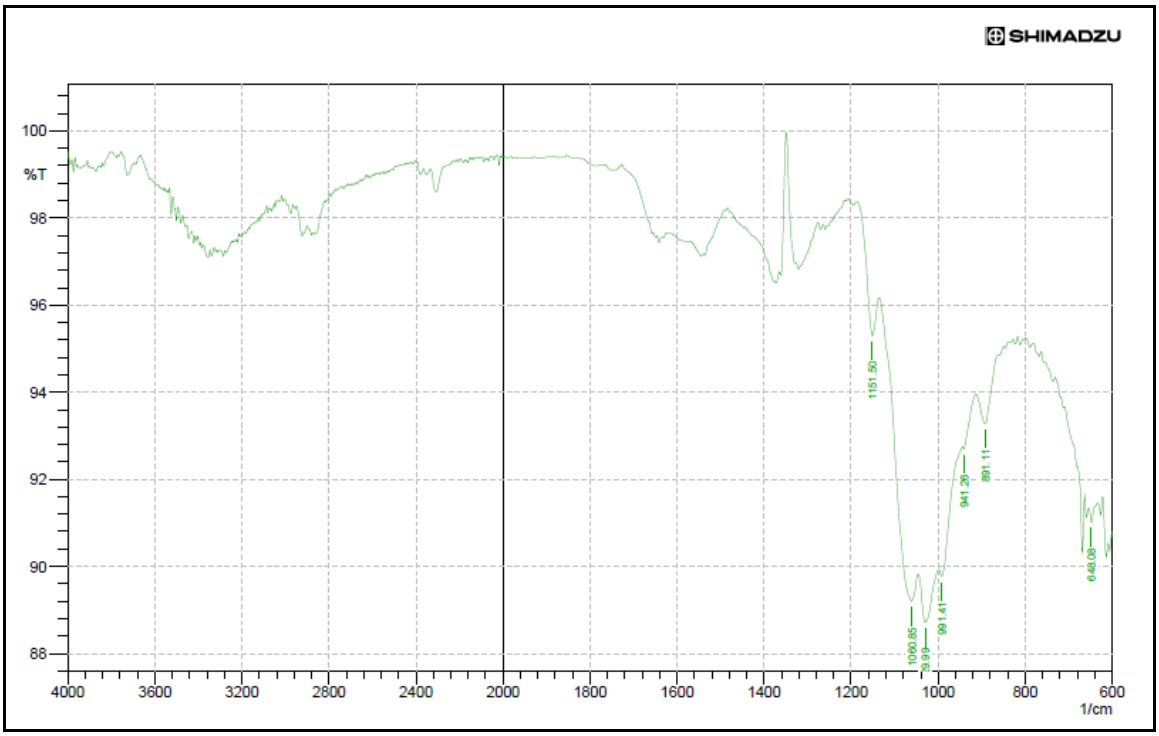



**Figure 4.9: FTIR spectrum of Chitosan**

**Degree of Quaternization**

Degree of quaternization of all the synthesized TMCs was determined by the peak integration of the $^{1}$H-NMR spectra by equation described earlier in methodology section. Degree of the quaternization was increased by increasing the reaction time. Following table 4.2 describes different degree of quaternization calculated by equation. The degree of quaternization (DOQ) was linearly increased with the reaction time for methylation. Reaction time of methylation from 60 min to 150 min yielded the DOQ 12% to 39%. DOQ is important as it influence the aqueous solubility of the native chitosan at the physiological pH. Figure 4.10 demonstrates that the reaction of trimethylation has been processed in the controllable manner as graph of degree of quaternization with reaction time shown almost linear curve.

**Table 4.2: Degree of Quaternization of trimethylated chitosan**

| Sr. No. | Synthesize trimethylated Chitosans | Reaction Time (Min) | Expected degree of quaternization (%) | Degree of quaternization (%) | Nomenclature |
|---|---|---|---|---|---|
| 1 | TMC 1 | 60 | 10 | 12.11 | TMC 12 |
| 2 | TMC 2 | 90 | 20 | 23.08 | TMC 23 |
| 3 | TMC 3 | 120 | 30 | 31.75 | TMC 31 |
| 4 | TMC 4 | 150 | 40 | 39.12 | TMC 39 |

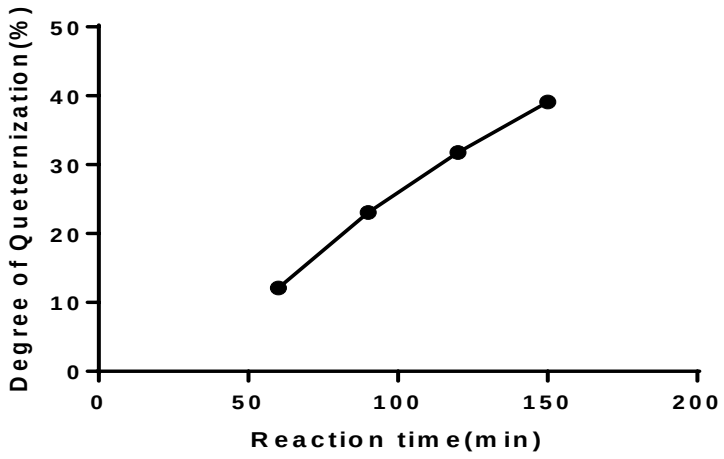



**Figure 4.10: Plot of Degree of Quaternization vs. Reaction time.**

**Solubility Improvement determination**

The Trimethylated chitosans (TMCs) were characterized for solubility profile using turbidimetry analysis to analyze the effect of quaternization on the colloidal stability of trimethylated chitosans. With change in degree of quaternization the pH dependent solubility was changed which was detected by change in transmittance value by UV-Vis spectrophotometer, A correlation was observed for transmittance and pH for all TMCs. As expected the pH50 and cloud point increased with degree of quaternization due to increase in solubility. Solubility of chitosan polymer is a function of degree of polymerization.

Nevertheless, the aim was to obtained TMCs with decent colloidal stability at physiologic pH. Soluble-insoluble transitions are initiated at the pH around the pKa of the amino groups of chitosan i.e. 6.5 – 6.8. Hence, from the values of the transmittance, it was apparent that TMCs were having better solubility property nearby physiologic pH than chitosan.

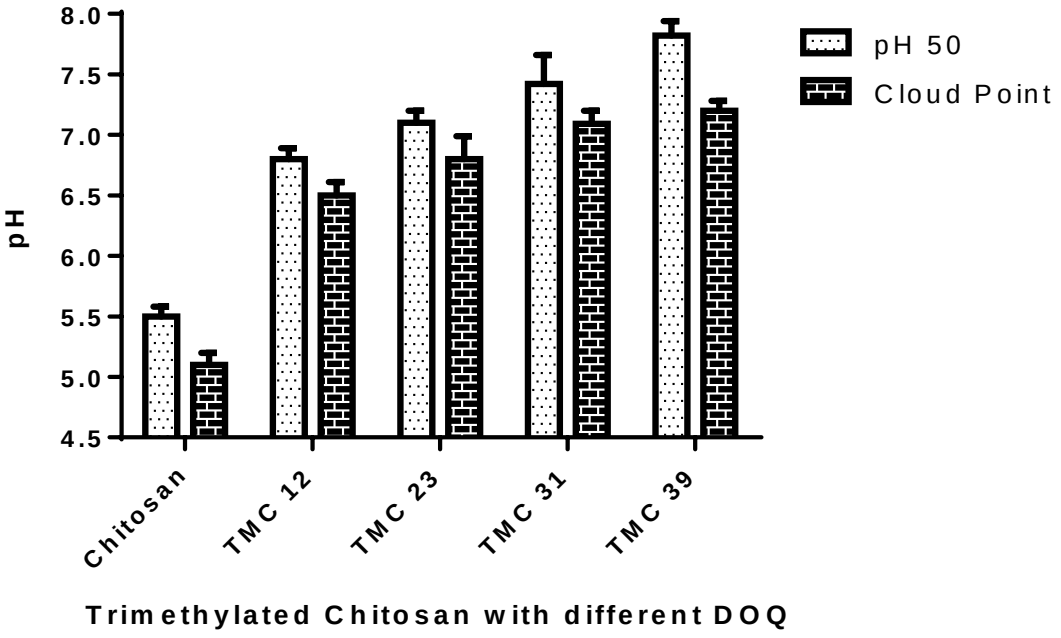



**Figure 4.11: Solubility profile of Trimethylated Chitosans with different DOQ**

Figure 4.11. demonstrates the solubility profile of trimethylated chitosan with different degree of quaternization. From the transmittance results, it was evident that trimethylated chitosans have a good solubility at physiological pH. The Cloud point of TMC was 6.5 and with increase in quaternization, cloud point was increased upto 7.2. While, pH50 values were from 6.8 to 7.82. For chitosan, values of cloud point and pH 50 were 5.1 and 5.5. Above results explains the improvement in solubility of the trimethylated chitosan at physiological pH.

***In vitro* Cytotoxicity study**

Figure 4.12 demonstrates the viability of A549 cells after 24h incubation with different concentrations of the synthesized trimethylated chitosans. The concentration ranged from 1, 10, 100, 1000 µg/mL. TMC 12, TMC 23 and TMC 31 showed negligible toxicity i.e. > 90% after 24h at concentration of 1, 10, 100, 1000 µg/mL in A549 cells. In case of TMC 39, the cell viability at concentrations of 100 and 1000 µg/mL was ~85% and 78 % after 24h, respectively. Which means that TMC 39 is toxic to the cells due to the higher cationic charged of the quartenized amines than other trimethylated chitosans. Hence, TMC 31 was found appropriate for desired vector development due to superior solubility, colloidal stability and greater cell viability and thus chosen for further experiments. The TMCs tend to take optimal physicochemical characteristics to formulation through effects on size and zeta potential.

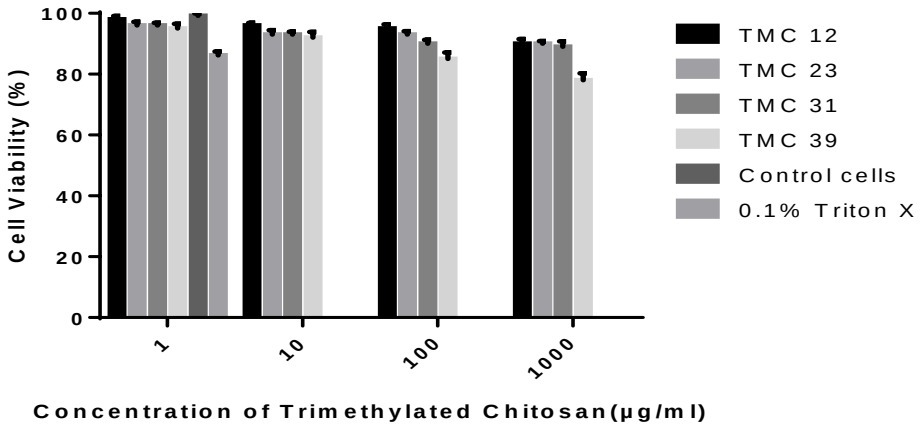



**Figure 4.12 *in vitro* cytotoxicity MTT assay of trimethylated Chitosans**

**Conjugation of Heterocyclic compounds**

The synthesized TMC 31 was conjugated with different heterocyclic compounds such asUrocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acidwith an objective to improve the buffer capacity of the TMC based vectors of siRNA in the endosomal range and so to assist in intracellular release of siRNA and transfection of the cells. These heterocyclic moieties which having pka in the endosomal range (Imidazole ring-pKa-6.04-

6.5, piperazine-pKa-5.27, pyridine-pKa-5.23) were conjugated to amines of the trimethylated chitosan through amide linkage at different conjugation levels.

**Nuclear Magnetic Resonance spectroscopy (NMR) Analysis**

Successful conjugation of the heterocyclic amine onto the trimethylated chitosan was confirmed by the $^{1}$H-NMR spectra shown in the figure 4.13, figure 4.14, and figure 4.15.TMC-UA showed characteristic peak of hydrogen of imidazole ring at around $\delta$ 8.5 ppm while TMC-PC and TMC-PA demonstrated the characteristics peak of piperazine and pyridine between$\delta$ 3.5 ppm to 4.0 ppm and $\delta$ 7.5 ppm to $\delta$ 8.5 ppm, respectively. While Peak between $\delta$ 1.5 ppm to $\delta$ 2.0 ppm attributed to hydrogen of the acetyl group of the chitosan while peak between $\delta$ 4.5 ppm and 5.5 ppm demonstrates the hydrogen bonded to the anomeric carbon 1 of the chitosan molecule. These$^{1}$H- NMR findings suggest the successful conjugation of heterocyclic compounds on the trimethylated chitosan.

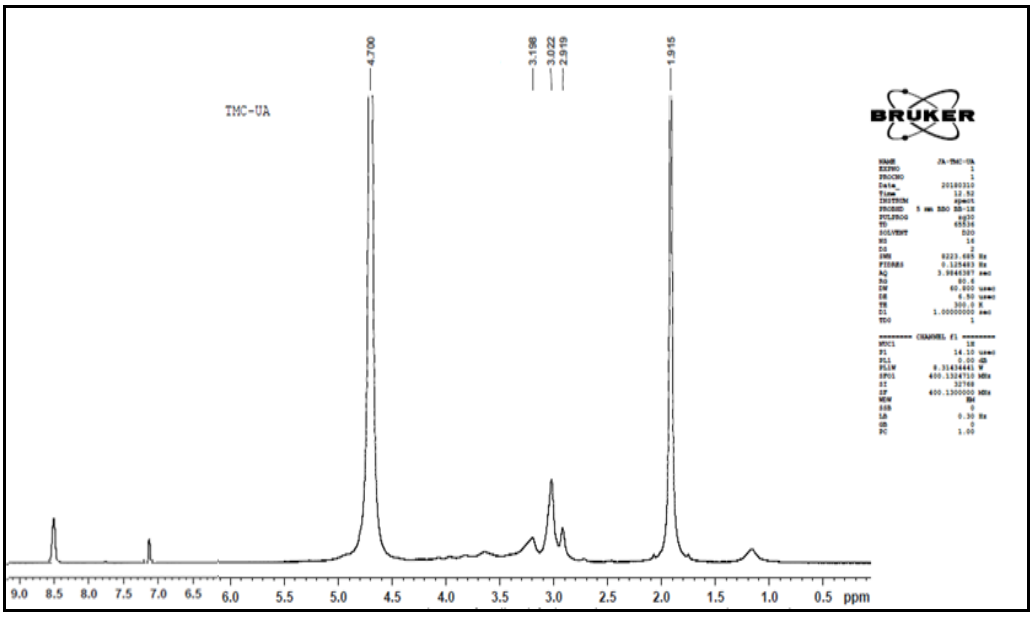



**Figure 4.13:$^{1}$H-NMR spectrum of the Urocanic acid conjugated TMC 31**

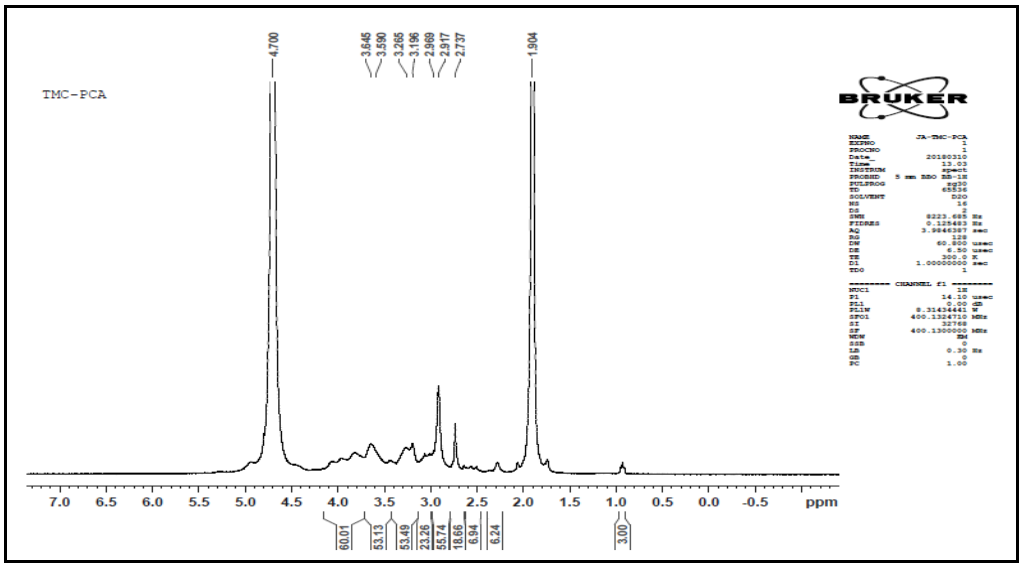



**Figure 4.14:[1]H-NMR spectrum of the Piperazine 2-Carboxylic acid conjugated TMC 31**

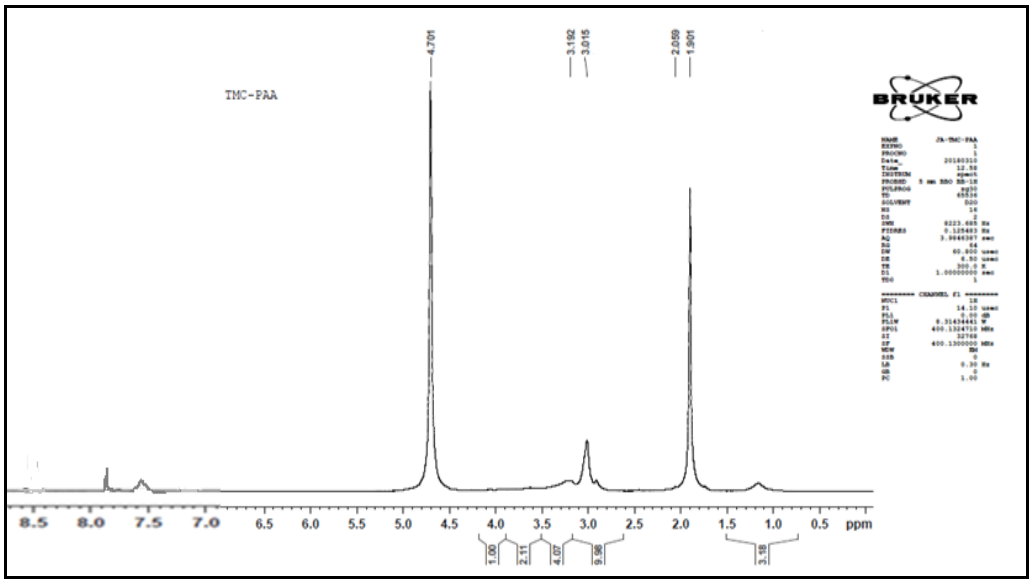



**Figure 4.15:[1]H-NMR spectrum of the 3-Pyridyl acetic acidconjugated TMC 31**

**Fourier Transformed Infrared Spectroscopy (FTIR) Analysis**

Figure 4.16, figure 4.17 and figure 4.18 shows FTIR spectra of the TMC conjugated withUrocanic acid(TMC-UA), Piperazine 2-Carboxylic acid(TMC-PC), 3-Pyridyl acetic acid(TMC-PA) respectivly.The FTIR spectra of the heterocyclic amine conjugated TMC provides the indication for the occurrence of the amide bond formation particularly in the

region between 1600-1650 cm-1 Particularly, TMC-UA shows amide band at 1633.16 cm-1, while TMC-PC and TMC-PA shows band at 1634.47 cm-1 and 1637.88 cm-1 respectively.

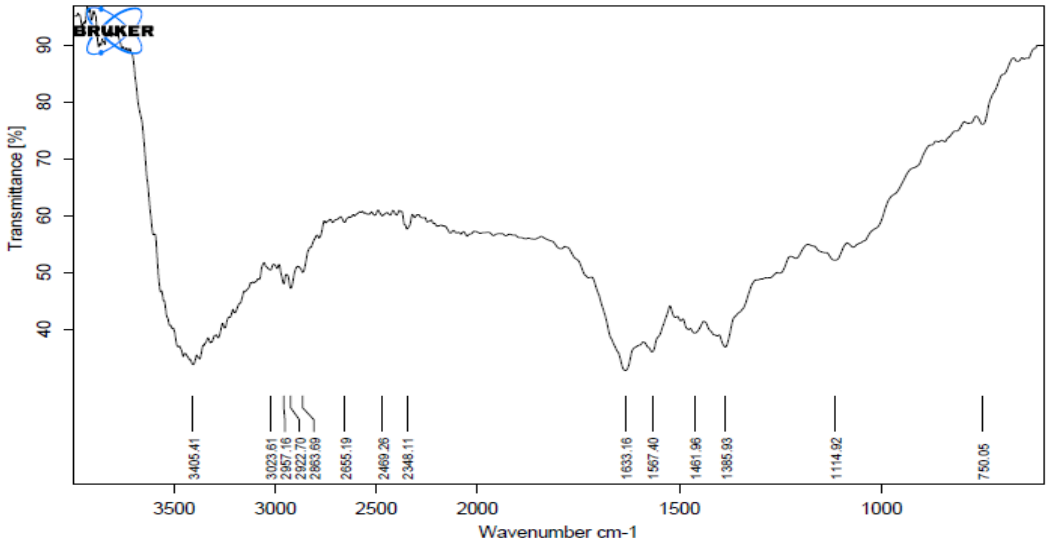



**Figure 4.16: FTIR spectrum of Urocanic acid conjugated TMC 31**

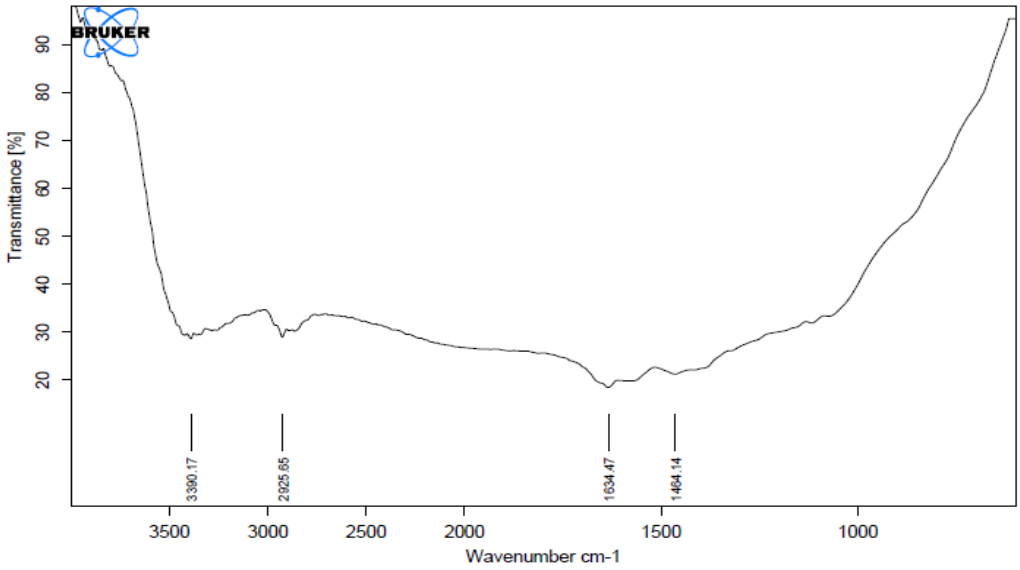



**Figure 4.17: FTIR spectrum of Piperazine 2-Carboxylic acid conjugated TMC 31**

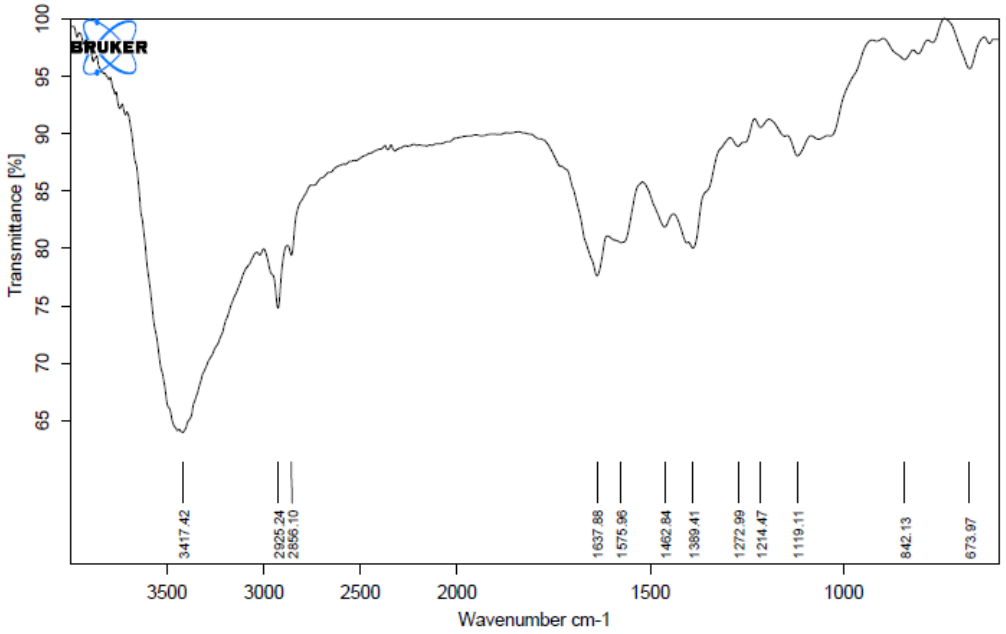



**Figure 4.18: FTIR spectrum of 3-Pyridyl acetic acidconjugated TMC 31**

**TNBS Assay**

Chitosan, natural polysaccharide is an attractive non-viral vector for the delivery of gene owing to its well-known capability to condense the siRNA or DNA through electrostatic interaction amongst the negatively charged nucleic acids and the primary amines of the chitosan polymer. Presence of the amino groups, chitosans are amenable to diverse and selective modifications or any type of conjugation on the structure. Conjugation of any group on the amino group can be assessed by the TNBS assay and degree of the conjugation or substitution can be estimated against the primary amines of the native chitosan. Degree of deacetylation of the medium molecular weight chitosan was 85 %.

**Table 4.3: Degree of conjugation of Heterocyclic moieties on TMC**

| Sr. No. | Modified Polymers | Degree of conjugation* (%) | Non-modified amines of the chitosan (%) |
|---|---|---|---|
| **1** | TMC-UAA | 23.54 ±2.42 | 29.71 |
| **2** | TMC-UAB | 18.29 ± 1.88 | 34.96 |
| **3** | TMC-UAC | 12.89 ±1.69 | 40.36 |
| **4** | TMC-PCA | 19.27 ±2.15 | 23.98 |
| **5** | TMC-PCB | 15.32 ±1.92 | 37.93 |
| **6** | TMC-PCC | 10.15 ±1.81 | 43.10 |
| **7** | TMC-PAA | 20.25 ±2.68 | 33.00 |
| **8** | TMC-PAB | 17.33 ±2.05 | 35.92 |
| **9** | TMC-PAC | 11.77 ±1.79 | 41.48 |

*Values are represented as Mean± SD, n=3

Degree of Deacetylation was 85.0 %and Degree of Quaternization was 31.75 %.

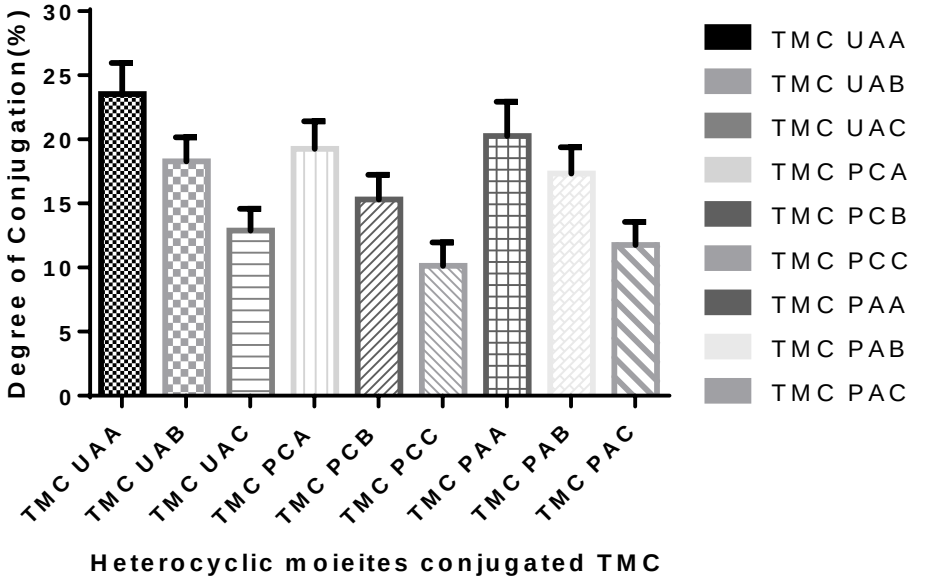



**Figure 4.19: Degree of conjugation of Heterocyclic moieties on TMC**

Above results demonstrated increasing conjugation or substitution percentage on the TMC with increasing molar ratio of the heterocyclic amines used. Heterocyclic amines conjugated at higher 20- 23%, moderate 15-18% and lower 10-12% levels.

**Buffer Capacity/Acid base titration**

Positively charged vectors used for the nucleic acid delivery ensures high intracellular uptake of nucleic acids (siRNA or DNA) through endocytosis. Nevertheless, key requirement for nucleic acid delivery systems is that they should escape the endosomes to circumvent exposures of therapeutic siRNA or DNA to higher pH of the late endosomes. High transfection efficiencies of the cationic polymers such as polyethylenimine, lipopolyamines, poly-L-lysine (PLL), and Chitosan have been extrapolated to their higher buffer capacity that assist in lysis of endosomes at lower pH of the endosomes by secondary and tertiary amines ionization. In the cell, endosomes have comparatively low pH (5.1 to 7.4) by ATPase proton pump. And so, called "proton sponge" mechanism can be explained by the example of PEI which once uptaken within the endosomes, takes up the protons through secondary and tertiary amines ionization which results in more proton influx to maintain pH and these influx of the protons forces the chloride ion accumulation within endosomes. Proton and chloride influx increase the osmotic pressure inside the endosome vesicles causing endosomes rupture and release of the therapeutic genes from the vectors or complex to the cell cytoplasm. We have hypothesized the heterocyclic compound conjugated TMC would increase the proton buffer capacity of the chitosan. We used acid base titration to study the buffer capacity of the polymers.

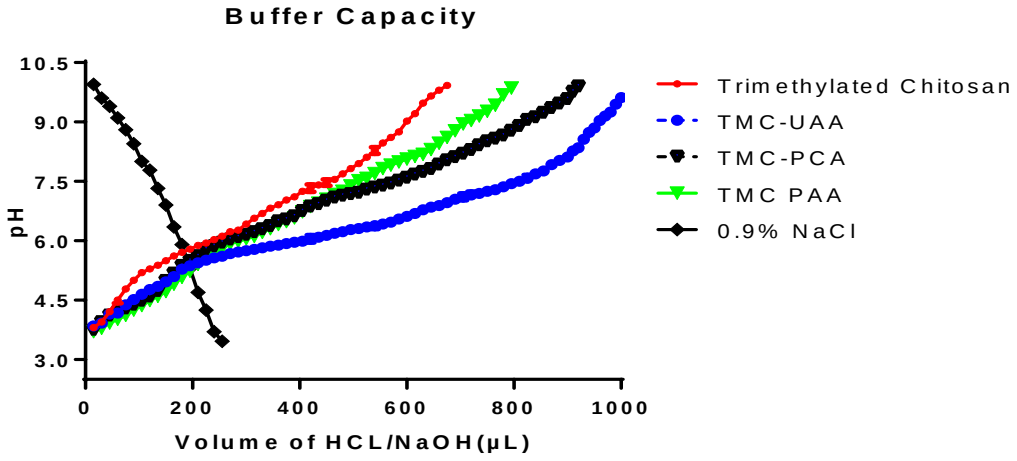



**Figure 4.20: Acid/Base titration of different modified TMCs**

Sodium chloride solution (0.9%) was titrated as negative control, when 0.1 N HCL was added, pH of sodium chloride solution rapidly dropped to 3. In contrast, the pH of polymer solutions went up gradually by continuation addition of NaoH demonstrating that

modified chitosans have a very strong buffer capacity as its pKa in the range between 6.5-6.8. Heterocyclic compounds Conjugated TMCs demonstrated improved buffer capacity as imidazole, piperazine and pyridine rings having pka in the range of endosomal pH. Among the these modified TMCs, Urocanic acid conjugated TMC (TMC-UAA) showed higher buffering capacity as it is synergist the buffer capacity of the chitosan as pKa of imidazole ring of Urocanic acid is 6.04-6.5 took part in the proton sponge effect and it will assist to release therapeutic genes into the cytoplasm by endosomal escape. TMC-PCA and TMC-PAA polymers demonstrate comparative buffer capacity as pKa of the piperazine and pyridine moiety moieties are near and hence there was no significant difference was observed in the buffer capacity. From the results, it can be say that once the polyplexes endocytosized intracellularly, good buffering capacity of the modified TMCs assists in the endosomal escape of the therapeutic siRNA into cytoplasm; which is most important parameter for efficient gene transfection by any vectors.

**Hemolysis study**

Nevertheless, positive charged vectors are a facilitator of cell membrane attachment following endosomal uptake, however too much of positive charge leads to membrane destabilization and hemolysis. During hemolysis, polymers create nano-sized pores in cellular membrane inducing solute influx into the cells which causes rupture by membrane destabilization and release of hemoglobin molecules. Along with charge, the polymer structure and conformation also play significant important role in mediating toxicity, wherein rigid and helical conformations are said to be more membrane permeabilizing.

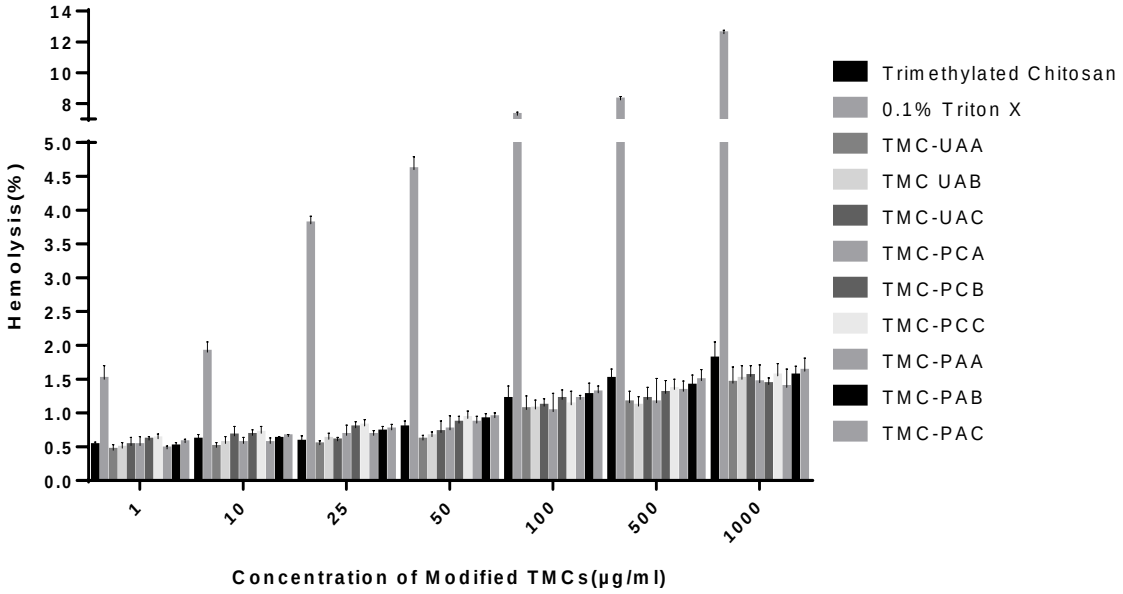
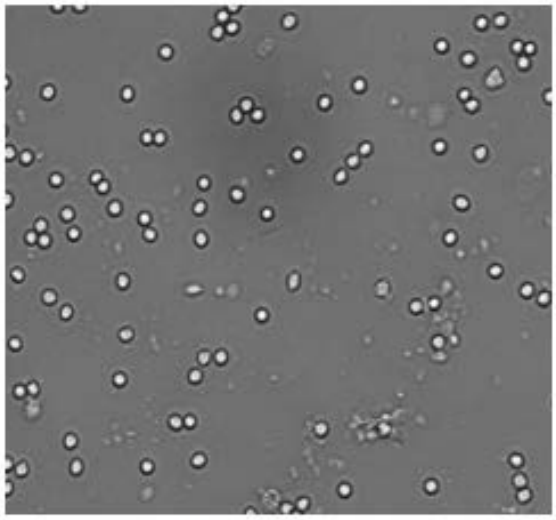



**Figure 4.21: % Hemolysis of different modified TMCs**

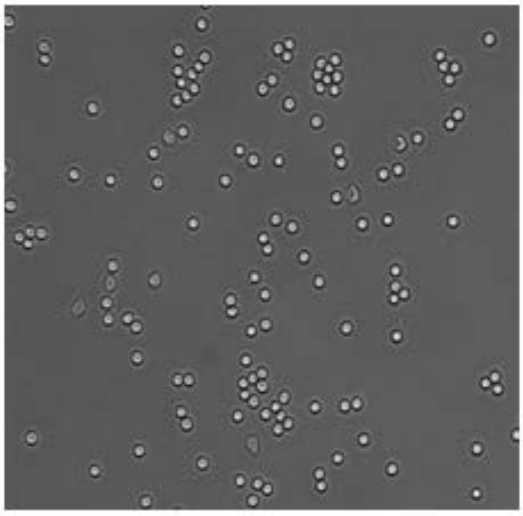
From the figure 4.21, it can be said that developed heterocyclic compound conjugated trimethylated chitosan polymers shows negligible or no hemolytic potential at different conjugated levels. However, increase in concentration of the polymers, increase in hemolytic potential was observed from 0.5 % to 1.8 % hemolytic activity. At 1000 µg/ml concentration, trimethylated chitosan showed 1.80% hemolysis while TMC-UA, TMC –PC and TMC-PA exhibited 1.42 to 1.60% hemolysis. 0.1% Triton X was used as positive control showed 12.54% of hemolysis at 1000 µg/ml concentration. It can be said that modified trimethylated chitosan polymers demonstrated superior biocompatibility.

**Erythrocyte aggregation**

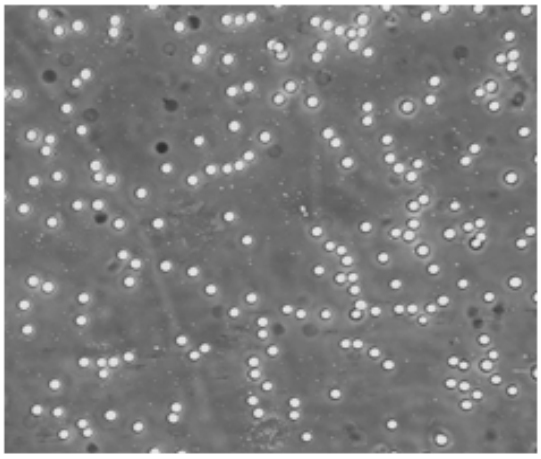
During aggregation the polymer molecules first interact with the colloid surface and then bridges inter-tangle with each other leading to flocculation and aggregation (19-21).From the images shown in figure 4.22(a to k) demonstrates that heterocyclic compound conjugated trimethylated chitosans exhibit no any types of aggregation or flocculation of the erythrocytes. These results prove the biocompatibility of the synthesized polymers to the cells.



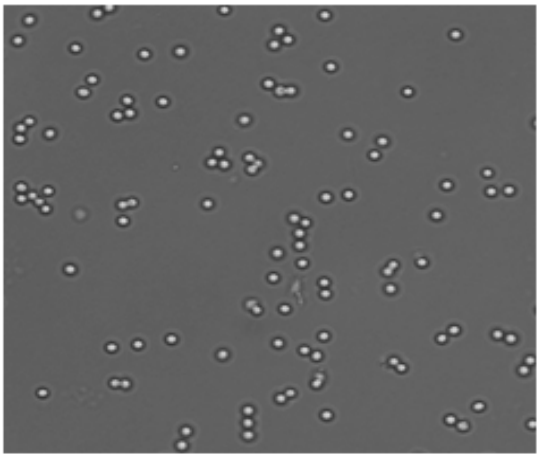
(a) TMC-UAA



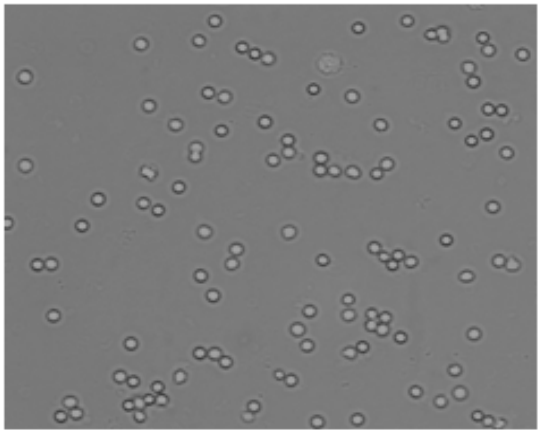
(b) TMC-UAB



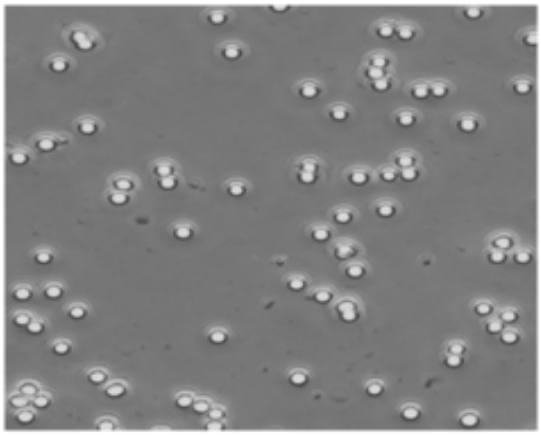
(c) TMC-UAC



(d) TMC-PCA



(e) TMC-PCB



(f) TMC-PCC

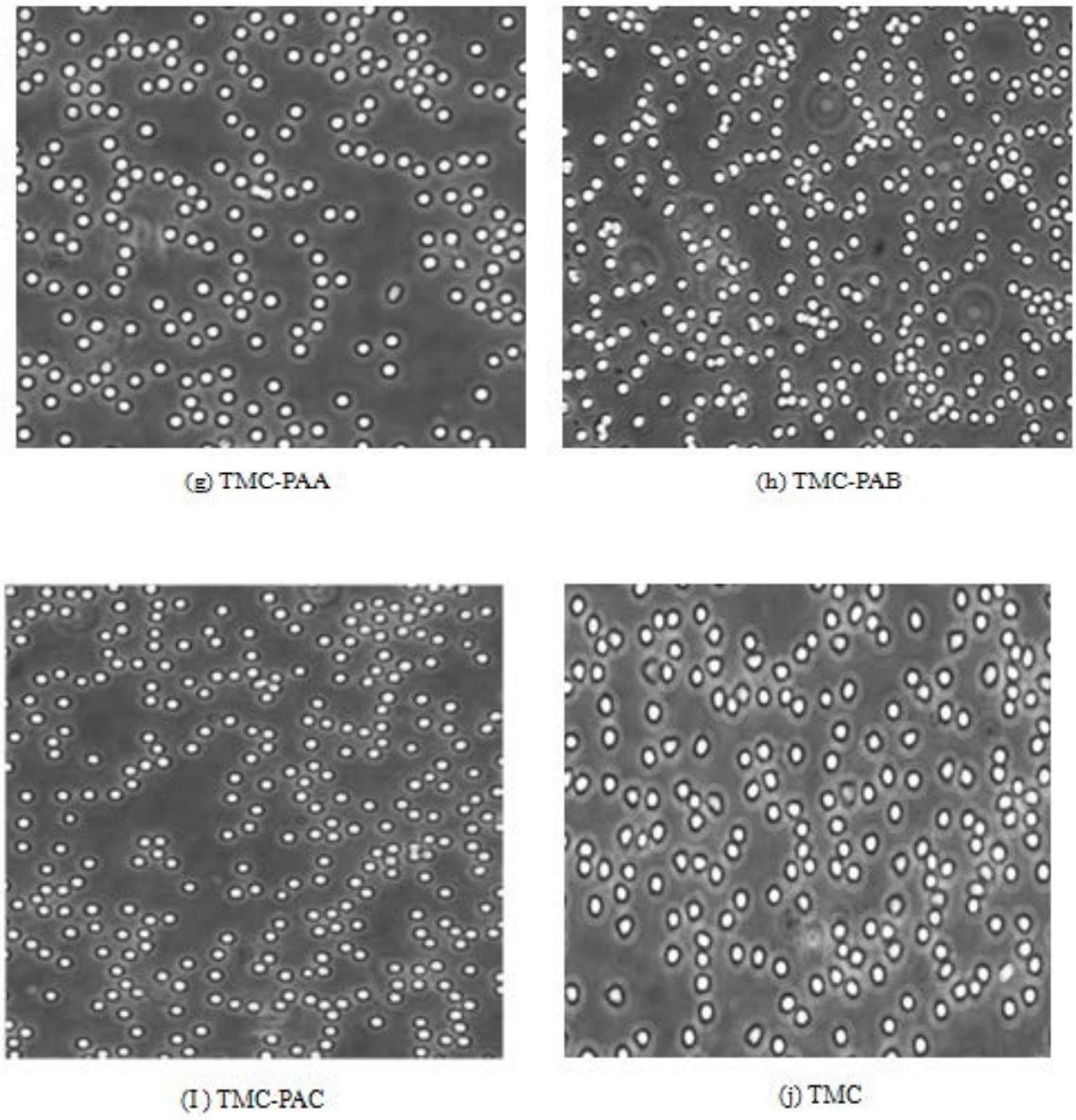



**Figure 4.22 Erythrocyte aggregations by different Modified TMCs**

***In vitro* cytotoxicity assay**

Figure 4.23 explains the viability of A549 alveolar epithelial cells after 24h incubation with different concentrations of the synthesized trimethylated chitosans conjugated with heterocyclic compounds. The concentration ranged from 1, 10, 100, 500, 1000 µg/mL. TMC- UA, TMC-PCA and TMC-PAA demonstrated cell viability of greater than 90% after 24h at each concentration in A549 cells at each conjugation levels. As the concentration of conjugated trimethylated chitosan increases from the 1µg/mL to 1000 µg/mL, there was

slight decrease in the cell viability. Cell viability was assessed by considering the cell viability of the control cells. 0.1% Triton X was used as positive (+ve) control, exhibited cell viability about 82.54%.

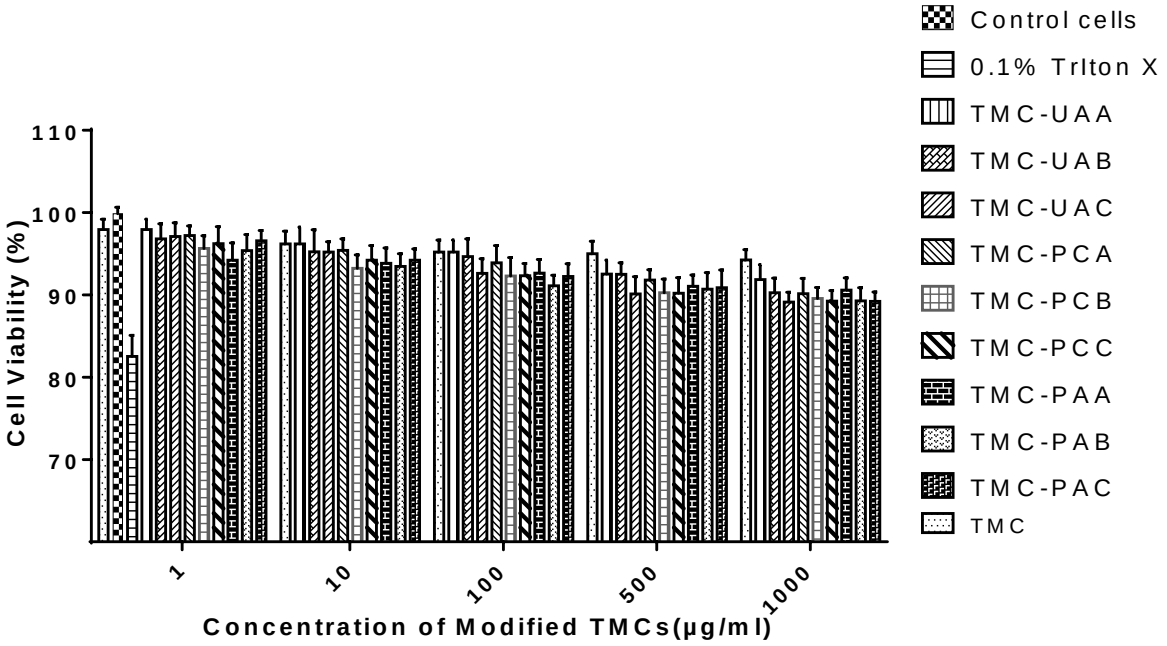



**Figure 4.23 Cell viability of modified TMCs**

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