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# SYNTHESIS AND EVALUATION OF THERMO-RESPONSIVE CHITOSAN-g-POLY N-ISOPROPYLACRYLAMIDE AS A CARRIER FOR CAPECITABINE

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## ABSTRACT
In the present study temperature responsive co-polymer chitosan-g-poly (N-isopropylacrylamide) (CS-g-PNIPAAm) was synthesized by surfactant free dispersion copolymerization method with varying molecular weights of chitosan and characterized for structural analysis by FTIR and lower critical solution temperature (LCST) determination by DSC. The synthesized co-polymers were efficiently loaded with Capecitabine and nanoparticles were evaluated for their morphology (SEM, TEM), particle size, zeta potential, loading efficiency, drug content and *in vitro* drug release study at pH 6.8 and 38$^{o}$ C temperature as well as at physiological pH and temperature conditions. FT-IR and DSC study showed that there was no interaction between drug and polymers. The drug loaded nanoparticles showed the smooth and spherical morphology with loading efficiency and drug content of about 68.18% and 44.16%, respectively. The *in vitro* drug release was significantly higher at tumor extracellular pH and temperature when compared to physiological pH and temperature. Stability studies carried out for formulation F3 showed that the nanoparticles are more stable at 4-8±2$^{0}$C. In conclusion, the synthesized co-polymer may have greater potential to be used as a thermo responsive carrier to achieve targeted drug delivery system for Capecitabine with low toxic side effects.

**KEYWORDS:** Capecitabine, chitosan-g-poly(N-isopropylacrylamide), self-assembly method, thermo responsive delivery.

## INTRODUCTION
In the recent decades, intelligent materials that respond to specific stimuli, such as temperature, pH, or enzymatic activity have been developed. When used in anti-cancer drug delivery applications, the responsive nature of these materials can provide more preferential delivery of drugs to tumor sites.[1] Temperature and pH are usual unstable parameters in any biological system, which has instigated the use of temperature and pH dual responsive polymers in stimuli-responsive drug delivery. In solid tumors, reported extracellular pH is 6-7 which is lower than that of surrounding tissues and blood (7.4) and the temperature in tumor tissues is always greater than that of normal body temperature.[2,3] Based on these findings, different pH and temperature responsive co-polymers were synthesized and used as carriers for stimuli-responsive delivery of anti-cancer drugs.[4,5] For example, pH-sensitive polymers have been designed for drug release in acidic tumor microenvironments and temperature-sensitive polymers have been synthesized as drug carriers and used in conjugation with hyperthermia treatment for localized drug delivery at tumor sites. This effect has been extensively studied using the temperature-sensitive material poly (N-isopropyl acrylamide) (PNIPAAm).[1]

Thermoresponsive drug delivery based on thermoresponsive polymer PNIPAAm is due to its unique phase transition at a lower critical solution temperature (LCST) of around 32 ºC in water which is nearer to the human body temperature, biocompatible and non-toxic. Therefore, in this study, PNIPAAm is chosen as a thermoresponsive polymer to synthesize chitosan-g-poly(N-isopropylacrylamide) (CS-g-PNIPAAm) copolymer. Chitosan is natural polysaccharide, biodegradable and biocompatible polymer thus a good candidate for drug carrier to deliver drugs. Chitosan has a various biological activities including immune enhancing effects, antitumoral, antifungal, and antimicrobial activities. The unique characteristics of chitosan nanoparticles could provide a higher affinity for negatively charged biological membranes and site-specific targeting *in vivo*. Chitosan nanoparticles could elicit dose-dependent inhibitory effects on the proliferation of various tumour cell lines, while low toxicity against normal human liver cells.[6]

PNIPAAm is water soluble below its LCST through predominant hydrogen bonding interactions with the surrounding water molecules. When heating to above the LCST, the hydrogen bonding with water is disrupted leading to aggregation and collapse of the polymer chains. The LCST of PNIPAAm can be manipulated by incorporating hydrophilic/hydrophobic components to shift the hydrophilic / hydrophobic balances.[9,10] CS-g-PNIPAAm co-polymer was extensively studied as a carrier for anticancer drugs to achieve thermo and pH responsive delivery of drugs to the intended tumour sites with minimal or no adverse effects in cancer therapy.[7,8]

For instant, Archana SP et al. prepared a high Oxaliplatin Loaded CS-g-PNIPAAm Co-polymeric nanoparticles for thermo and pH responsive delivery. CS-g-PNIPAAm co-polymer was synthesized by surfactant free dispersion copolymerization method. It was observed that, self-assembly method gives a high amount of Oxaliplatin loaded nanoparticles with loading efficiency of 83±4.4 % and drug content of about 47.9±5.4 % than that of direct loading method which shows only 55.87±5.6 loading efficiency and 4.3±1.2 drug content.[11] Wang Y et al., synthesized thermo responsive nanogel using chitosan (CTS) and NIPAAm. Paclitaxel (PTX) was loaded in CTS-poly(NIPAAm-co-AAm5.5) nanogels and the loading efficiency was 9.06 ± 0.195%. Results supports the potential of CTS-poly(NIPAAm-co-AAm 5.5) nanogels for the combined thermal and chemotherapy.[12] Duan C et al. synthesized CS-g-PNIPAm co-polymer and utilized to prepare pH-responsive nanogels for the targeted oridonin delivery to tumor with encapsulation efficiency of 86.3% and the loading efficiency of 5.34% only.[13] Recently, Huang C et al. developed pH and thermo-sensitive chitosan-PNIPAAm core–shell co-polymeric nanoparticles of doxycycline hyclate with encapsulation efficiency and loading content of 60.3 and 3.02 %, respectively.[14] In all these studies CS-g-PNIPAAm co-polymer was utilized as a carrier for drug and it has shown effective response to release drug at the predicted sites but unfortunately a very low percentage of drug loading was observed with this co-polymer, which is a major issue to be considered for economical development of such delivery systems. Thus, in the present study, efforts were made to develop a thermo responsive CS-g-PNIPAAm co-polymeric nanoparticulate delivery system for Capecitabine and intended to improve its percent drug loading efficiency (LE) and drug content (DC) with controlled drug release by using different molecular weight of chitosan. First, the CS-g-PNIPAAm co-polymer was synthesized by surfactant free dispersion copolymerization method, characterized and applied as carrier for Capecitabine. Drug loaded nanoparticles were evaluated for morphology, particle size, zeta potential, loading efficiency, drug content, *in-vitro* drug release study and stability study.

## MATERIALS AND METHOD

### Materials

Chitosan (degree of deacetylation>90%) was HiMedia Laboratories Pvt. Ltd. Mumbai, India. Capecitabinepure drug was obtained as gift sample from Shilpa antibiotic Pvt. Ltd, Raichur, India. Ammonium persulphate (APS), N,N-methylenebisacrylamide (MBA), N-isopropylacrylamide (NIPAAm) and glacial acetic acid were purchased from Acros (Geel, Belgium). Dialysis membrane with molecular weight cutoff of 12000-14000 Da was purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India (LA 401-HiMedia). India. All the other chemicals used were of analytical grade.

### Synthesis of chitosan-g-PNIPAAm co-polymers

Chitosan-g-PNIPAAm (CS-g-PNIPAAm) co-polymer was prepared by a surfactant free dispersion co-polymerization method.[11] Chitosan (325mg) was dissolved in 1% glacial acetic acid with continuous stirring. Copolymerization was carried out under inert nitrogen atmosphere. NIPAAm (1 gm) and MBA (20 mg) were added to the chitosan solution with vigorous stirring and when the temperature reached to $50^0$C and APS (100 mg) was added to initiate the polymerization. The reaction medium turned turbid within 15 min after that, the reaction was allowed to proceed for 4h at 50-$55^0$C. The reaction solution was purified by dialysis method using dialysis bag (molecular weight cut off 1200-1400) and dialyzed against water for a week at room temperature by daily replacing the water. After dialysis, the solution containing PNIPAAm and CS-g-PNIPAAm was obtained. The homopolymer PNIPAAm was removed by the acetone extraction at $20^o$C for 48 h to get the pure CS-g-PNIPAAm.

### Characterization of chitosan-g-PNIPAAm

The synthesized co-polymer was characterized by using Fourier-transform infrared (FTIR). The diffused cell technique was used to obtain FTIR spectra (Shimadzu) of co-polymer. The co-polymer sample was mixed properly and ground to fine power with KBr. Then, the obtained fine powder was pressed to pellets and the spectrum was recorded over the wavelength region from 4000 to 400 $cm^{-1}$.

### Determination of lower critical solution temperature (LCST)

The co-polymer was immersed in phosphate buffer of pH 6.5 & 6.8 at room temperature and allowed to swell for 24h. The LCST was determined by using DSC-60 Differential Scanning Calorimeter (Make - Shimadzu). The thermal analysis was carried out between temperature range 25 to $70^0$C under dry nitrogen atmosphere with a flow rate of 30mL/ min and heating rate of $2^0$C/min. Temperature at the onset point of DSC thermograms were taken as the LCST of the polymer.

**Preparation of Capecitabine-loaded CS-g-PNIPAAm nanoparticles**

**Self assembly method**

Three formulations (F1-F3) of Capecitabine-loaded nanoparticles were prepared by using self-assembly method.[11] (Figure 1) with varying drug: polymer ratios as summarized in Table 2. The aqueous solutions of specific amount of drug and co-polymers (C-1, C-2 and C-3) were prepared separately and mixed together. The volume of the mixture was made up to 35 mL with distilled water. The dispersion was sonicated in ice bath using a prob-type sonicator for 15min. Dry nanoparticles were obtained by centrifugation and freeze drying.

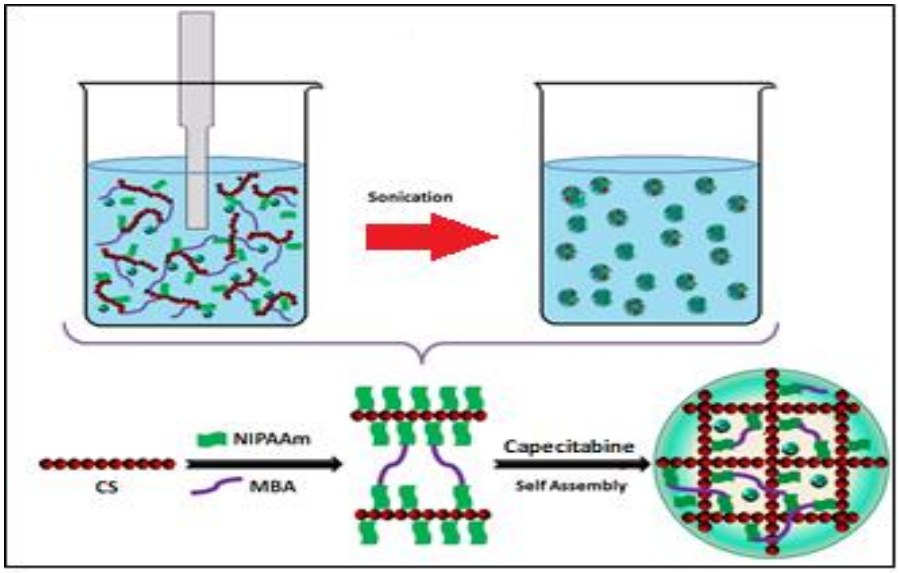


**Fig. 1: Representation of Self -assembly method.**

**Determination of particle size and zeta potential**

Particle size of all the formulations was determined by DLS using particle size analyzer (Nanotrac R- 150 USA). Samples were prepared by diluting the small quantity of dry nanoparticles in double distilled water with constant stirring for 1 h and filtered through 0.45µm millipore filter to get the desired nanoparticle dispersion. The mean diameter (±SD) was obtained from 6 determinations. The zeta potential was determined by Malvern Zetasizer and the average values of triplicates were taken.

**Determination of loading efficiency from self assembly method**

Ultracentrifugation method was used to determine the percent drug loading efficiency of all formulations. The encapsulation efficiency of nanoparticles was determined by the separation of drug- loaded nanoparticles from the aqueous medium containing non-associated Capecitabine by ultracentrifugation method.[15] The nanoparticle suspension was centrifuged at 30,000 rpm at 4˚C for 2 hours. The supernatant was collected and drug content was analyzed by using UV.

Capecitabine was detected by using UV spectrophotometer at $\lambda_{max}$ of 303nm. The standard curve for the quantification of Capecitabine was linear over the range of 10-30 µg/ml with a correlation coefficient of 0.999. Drug loading efficiency was calculated by using the following equation:

$$\text{Loading efficiency (\%)} = \frac{weight\ of\ total\ drug - weight\ \ of\ free\ drug\ found}{weight\ of\ total\ drug} \times 100$$

**Determination of drug content**

The drug content was determined by hydrolyzing the exactly weighed amount of drug loaded nanoparticles (100mg) in 1mol/L phosphate buffer at 60$^{o}$C for 1 h till the clear solution was obtained. Free drug was separated from the nanoparticle by centrifugation at 12000 rpm for 30 min and the supernatant was taken, appropriate dilutions was made and further analyzed for drug content by UV spectrophotometer. The data were expressed as the mean value of three independent experiments.[16]

**Transmission Electron Microscopy (TEM)**

External Morphology of prepared nanoparticles was determined using transmission electron microscopy. The nanoparticle suspension was prepared and placed a drop onto copper grid. Digital Micrograph and Soft Imaging Viewer software were used to perform the image capture and analysis, including particle sizing.[17]

**Scanning Electron microscopy (SEM)**

For scanning electron microscopy images, samples of

nanoparticles were dusted onto a double-sided tape on an aluminum stub. The stubs containing the **sample** were coated with gold using a cool sputter coater. Photomicrographs were taken at the accelerated voltage of 20 kV and chamber pressure of 0.6 mmHg.

***In-vitro* drug release study**

Drug release profile of formulations F1-F3 was performed at different pH and temperature conditions. The drug release profile of Capecitabine nanoparticles was determined by using the dialysis bag. Briefly, 100 mg of formulations was transferred to a dialysis bag with molecular weight cut off 12,000–14,000 Dalton, pore size 2.4 nm and sealed. The sealed bag is tied and dipped in the beaker containing 100 ml phosphate buffer of pH 6.8 at 38°C temperature on magnetic stirrer up to 12hrs at a constant stirring speed of 100 rpm and temperature of 38±0.5°C. Aliquots of 5 ml of the sample were withdrawn at predetermined time intervals and the same was replaced with fresh buffer. The withdrawn aliquots was analyzed by UV spectrophotometry. The concentration of drug released was calculated by using standard calibration curve.

Release study of drug-loaded nanoparticles was also performed at physiological pH and temperature conditions (37$^{o}$C & pH 7.4). All release measurements were triplicated for each sample and average values were plotted.[18]

**Stability study**

The stability study were conducted according to ICH guidelines.[19] The stability of optimized formulation F3 was determined by keeping the formulation at 25 ± 2°C/60 % RH and 4°C ± 2°C/60 % RH. The sample were tested after 15$^{th}$ and 30$^{th}$ day for % loading efficiency and drug content.

## RESULT AND DISCUSSION

**Synthesis of CS-g-PNIPAAm co-polymer**

The co-polymers were successfully synthesized by using NIPAAm and chitosan monomers via surfactant free dispersion copolymerization method with MBA as a cross linking agent and APS as a radical initiator. Elevated temperature is required for the decomposition of APS to produce sulfate anion radicals and to phase separate the growing PNIPAAm chains to produce colloidal particles. The radicals of APS then interact with the hydroxyl and amino groups of chitosan to form alkoxy radicals which initiate the graft copolymerization of NIPAAm onto chitosan with crosslinking agent MBA. When APS is used as an initiator in the synthesis of co-polymer, three complex particles were produced such as, PNIPAAm, CS-g-PNIPAAm (negatively charged) and CS–$NH_3^+$ (positively charged). These positively charged particles form polyelectrolyte complexes establish electrostatic interactions with excess of chitosan which causes the prevention of the coagulation of particles.

**Structure analysis of CS-g-PNIPAAm**

IR spectra of chitosan (A), co-polymer (B) were compared in Figure 2. For chitosan, CH stretching of heterocyclic ring appears at 2983cm$^{-1}$. Chitosan, peaks of the OH stretch appears in the region 3539 cm$^{-1}$ to 3686 cm$^{-1}$. For CS-g-PNIPAAm, the several peaks observed in pure chitosan in the region 3543 cm$^{-1}$ to 3689 cm$^{-1}$ are diminished in the IR spectrum of synthesized copolymer, which indicates the initiation of linkage between chitosan and NIPAAm at NH and OH groups of chitosan. The graft polymerization of chitosan and NIPAAm was confirmed by FTIR spectroscopy. From these observations it was confirmed that graft polymerization was successful.

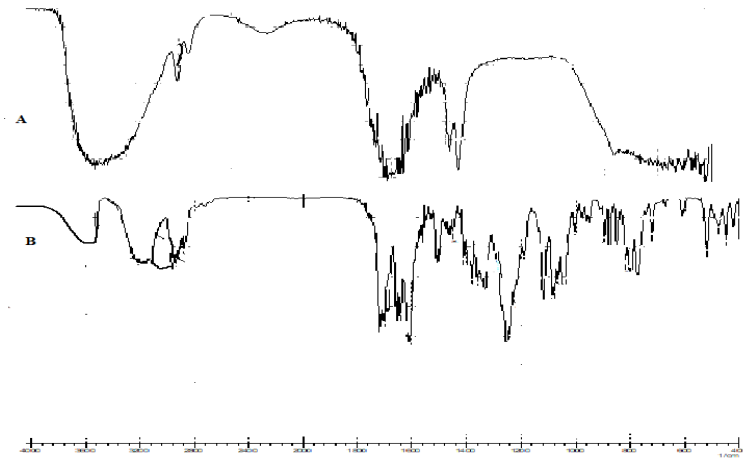


**Fig. 2: FTIR spectrum of chitosan (A), CS-g-PNIPAAm copolymer (B).**

**LCST of co-polymer**

DSC thermograms of synthesized CS-g-PNIPAAm co-polymeric nanoparticles are shown in Figure 3(A) at pH 6.5 and Figure 3(B) at pH 6.8 and temperature at the onset point of DSC thermogram was taken as the LCST of the sample. The LCST of CS-g-PNIPAAm co-polymer in phosphate buffer of pH 6.5 was lower than the LCST co-polymer in phosphate buffer of pH 6.8

which is shown Table 1. The LCST results revealed that as the molecular weight of chitosan used for CS-g-PNIPAAm co-polymer increases, the LCST of co-polymer also increases.

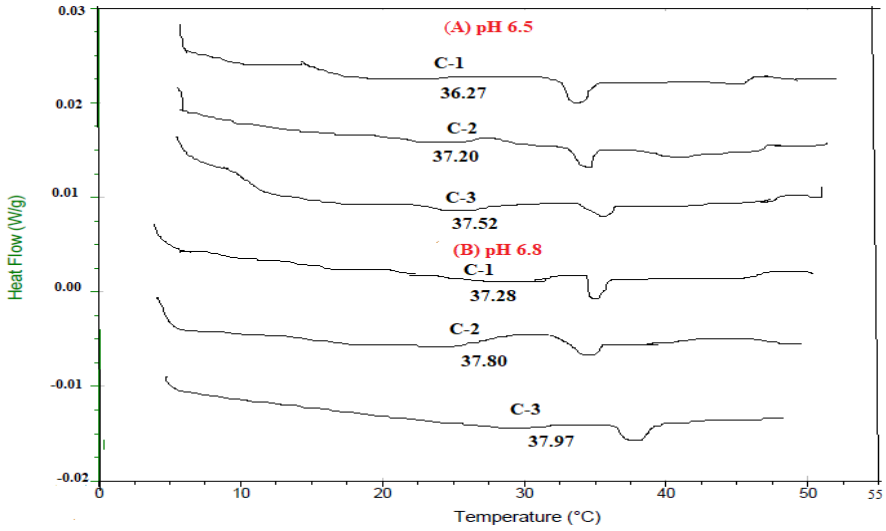


**Fig. 3: DSC thermograms of synthesised Cs-g-PNIPAAm Copolymer in different pH 6.5 (A) and pH 6.8 (B).**

**Table 1: Synthesis of CS-g-PNIPAAm copolymer with varying molecular weights of chitosan and low critical solution temperature in different pH.**

| Copolymers | Molecular Weights of Chitosan | Phosphate buffer pH 6.5 | Phosphate buffer pH 6.8 |
|---|---|---|---|
| C-1 | 161.16 | 36.27 | 37.28 |
| C-2 | 163.1 | 37.20 | 37.80 |
| C-3 | 324.15 | 37.52 | 37.97 |

**Effect of different molecular weights of chitosan content in co-polymer on drug loading efficiency**

To study the effect of chitosan molecular weights in co-polymer on drug loading efficiency, three formulations (C-1, C-2 and C-3) of Capecitabine loaded co-polymeric nanoparticles with varying chitosan molecular weights were prepared by self assembly method. Chitosan molecular weight, % drug loading efficiency (% LE), % drug content (% DC), particle size and zeta potential of all the formulations are shown in Table 2.The incorporation of hydrophilic co-monomer chitosan increased the LCST of co-polymer which was a similar finding as that of our previous work[11]. As the molecular weights of chitosan in co-polymer increased, the % drug loading efficiency and % drug content also increases. High reaction sites are found in high molecular weight chitosan which increases the electrostatic interaction between polymer and drug that in turn leads to enhance the amount of drug loaded in to the co-polymer.

**Table 2: Preparation of drug loaded CS-g-PNIPAAm co-polymeric nanoparticle by self assembly method and their evaluation.**

| Formulations | Copolymers | Molecular Weights of Chitosan | Copolymer (gm) | Drug (mg) | Drug Loading Efficiency % | Drug Content (%) | Particle size (nm) | Zeta potential (mV) |
|---|---|---|---|---|---|---|---|---|
| F-1 | C-1 | 161.16 | 1 | 100 | 58.12±0.62 | 29.18±0.72 | 338±13 | 23±2.6 |
| F-2 | C-2 | 163.1 | 1 | 100 | 62.24±0.20 | 33.0±0.65 | 203±16 | 26±4.3 |
| F-3 | C-3 | 324.15 | 1 | 100 | 68.18±0.27 | 44.16±0.28 | 132±17 | 31±1.1 |

**Particle size and zeta potential**

The mean particle size obtained for the formulations prepared by self assembly method are shown in Table 2. The particle size of F3 formulation with high molecular weight of chitosan was 132nm and that of formulation with low molecular of chitosan formulation F2 and F1 nanoparticles was 338nm to 203nm respectively. The mean particle size of prepared nanoparticles decreased with increase in molecular weights of chitosan used in co-polymer. The high molecular weight of chitosan drug loaded nanoparticles exhibit higher zeta potential than that of low molecular weight of chitosan, indicating the stability of nanoparticles. The particle size and zeta potential of drug loaded nanoparticles (F-3) prepared by using high molecular weight of chitosan in self-assembly method were found to be 132 ±17nm and 31±11mV, respectively.

**Scanning Electron microscopy (SEM)**

SEM images of the optimized formulation F3 suggested that the formulations were in nano size range (200 nm). SEM images of the optimized formulation F-3 is shown in Figure 4(A).

**Transmission Electron Microscopy (TEM)**

TEM images of the optimized formulation F3 suggested that the formulations were in nano size range 100 nm with spherical morphology. TEM images of the optimized formulation F-3 is shown in Figure 4(B).

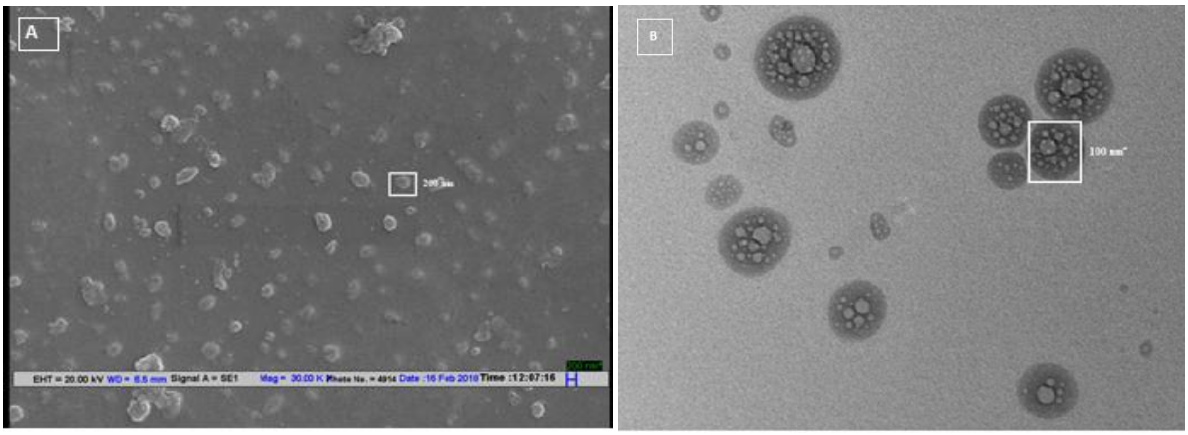


**Fig. 4: Scanning Electron Microscopy of F-3 formulation (A), Transmission Electron Microscopy of F-3 formulation (B).**

***In vitro* drug release studies**

Drug release of F1-formulation showed % cumulative drug release of about 60 % at physiological conditions (pH & temperature) and 68 % at tumor extracellular temperature and pH conditions which were depicted in Figure 5. This is because of the co-polymer used for drug loading (C1) which has the LCST of 37.28 ºC which is much closer to that of normal body temperature. Drug release profile of F2-formulation is shown in Figure 6, which showed the % drug release of about 54% at physiological conditions and 72% drug at tumor extracellular temperature and pH conditions. Here, the co-polymer used for drug loading was C2 which has the LCST of 37.80 ºC which is higher than that of body temperature but little closer to it. Drug release profile of F3- formulation is shown in Figure 7, which showed % drug release of about 47.82 % at physiological conditions and 78 % at tumor extracellular conditions. In this formulation co-polymer used for drug loading was C3 which has the LCST of 37.97 ºC which is almost 1ºC higher than that of physiological temperature. Thus the % drug release is much lower at physiological condition & much higher at extracellular conditions.

Based on the results obtained F3 formulation with higher % loading efficiency and drug content, showing enhanced % cumulative controlled drug release at tumor extracellular conditions is considered to be optimized formulation.

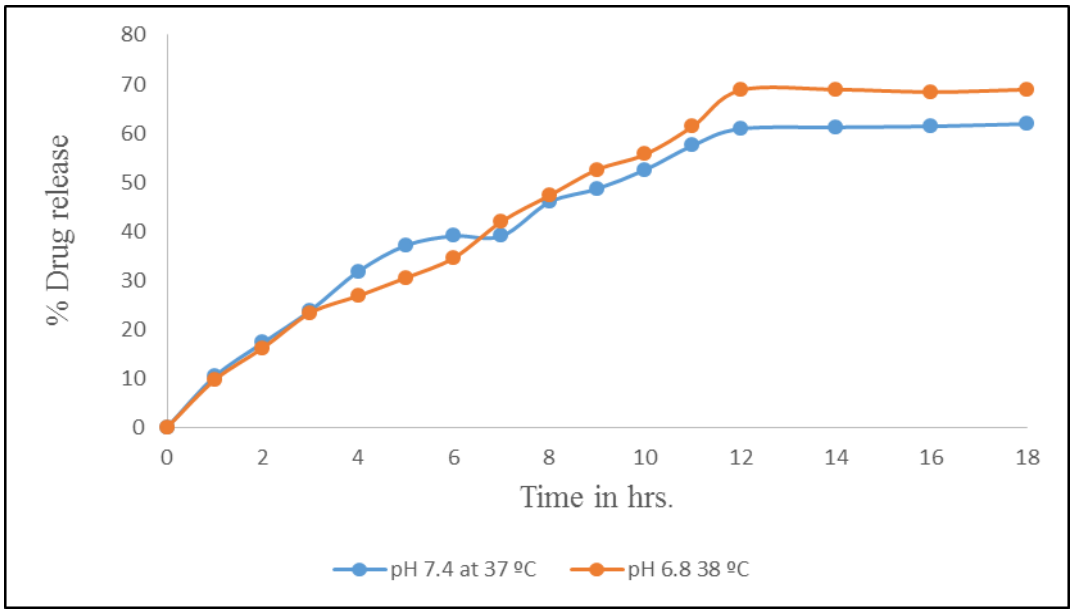


**Fig. 5: Comparative drug release profile of F-1 at physiological conditions (7.4 pH and 37º C) and at tumor extracellular conditions (6.8 pH and 38º C).**

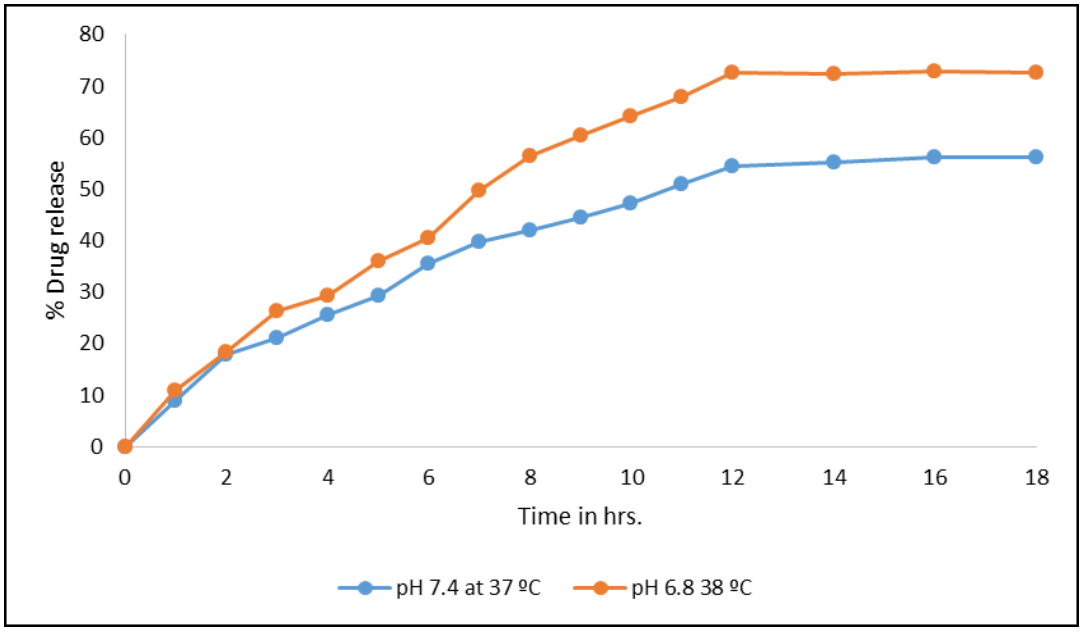


**Fig. 6: Comparative drug release profile of F-2 at physiological conditions (7.4 pH and 37º C) and at tumor extracellular conditions (6.8 pH and 38º C).**

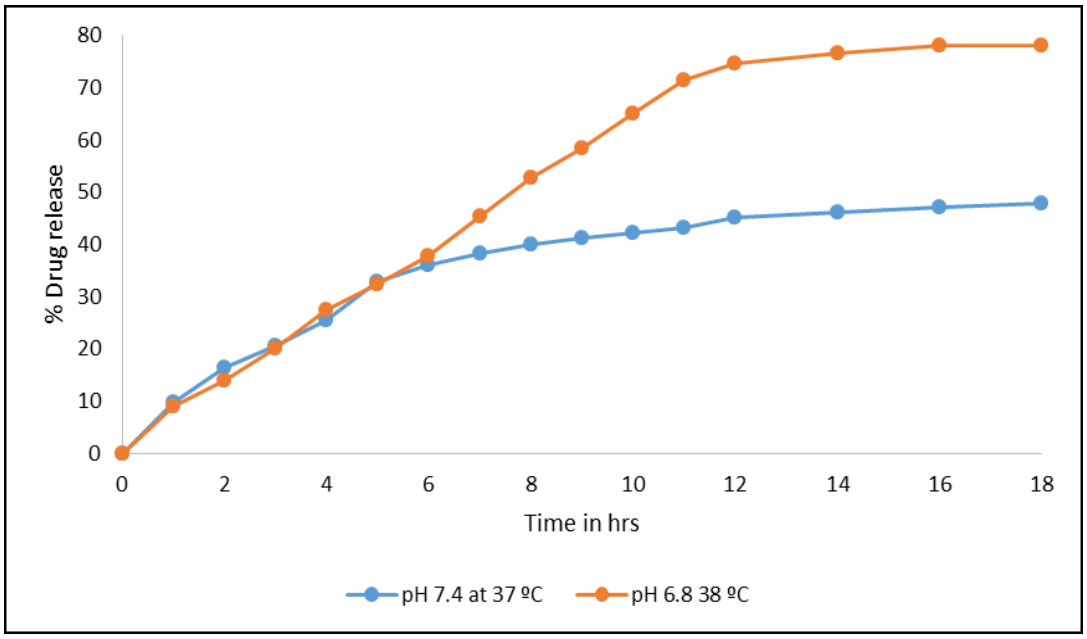


**Fig. 7: Comparative drug release profile of F-3 at physiological conditions (7.4 pH and 37º C) and at tumor extracellular conditions (6.8 pH and 38ºC).**

**Stability study**

Stability study showed no significant change observed in the % loading efficiency and drug content as conducted at an interval of 10 days for 1 month at 4°C ± 2°C/60 % RH. Thus formulation stored at 4-8±2$^0$C/60±5%RH showed better stability as compared to the formulation stored at 25±2$^0$C/60±5%RH.

**CONCLUSION**

The temperature responsive CS-g-PNIPAAm co-polymers with varying molecular weights of chitosan were successfully synthesized via surfactant free dispersion co-polymerization method. Chemical structure of the synthesized CS-g-PNIPAAm co-polymer was confirmed by using the FTIR spectrum. LCST of co-polymer was determined by DSC. The synthesized co-polymer used for optimized formulation shows the LCST about 37.97 $^o$C at pH 6.8 which was higher than that of normal body temperature and thus suitable to achieve targeted delivery of drug to tumor site.

Capecitabine loaded nanoparticles were successfully prepared by self-assembly method and were evaluated for particle size, polydispersity index, zeta potential, shape and surface morphology, percentage drug content, percentage loading efficiency, *in-vitro* release at

nanoparticles at tumor extracellular pH (6.8) and temperature (38.$^{o}$C above LCST) conditions and at physiological pH (7.4) and temperature (37$^{o}$ C) and stability studies.

In self assembly method, the co-polymer with varying chitosan molecular weights in co-polymers were used to load the drug and it was observed that as chitosan molecular weight of co-polymer increases, the drug loading and percent drug release at above LCST increases, but the rate of drug release decreases due to higher viscosity of high molecular weight chitosan. *In-vitro* drug release profile of optimized formulation has shown higher percent drug release at tumor extracellular conditions and very low percent drug release at normal physiological pH and temperature conditions. Thus, the *in-vitro* drug release study revealed the efficiency of enhanced temperature responsive drug release from nanoparticles. In conclusion, the synthesized CS-g-PNIPAAm co-polymer with higher molecular weight of chitosan can be used as a carrier for Capecitabine delivery to achieve temperature responsive sustained and targeted delivery of drug to tumor, thereby reducing the systemic adverse effects to enhance the anticancer efficacy of Capecitabine.

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