AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Development and Optimization of Capecitabine Loaded Chitosan Nanoparticles for Colon Cancer Therapy

Ankur R. Javia*[1], A.K.Seth[1]
1. Department of Pharmacy, Sumandeep Vidyapeeth University, Piparia, Vadodara, Gujarat.

ABSTRACT

The goal of this study was to develop and optimize the Capecitabine loaded chitosan nanoparticles (CS-NPs)for improved colon cancer therapy, by enhancedsurface area, sustained drug release, reduced dose and hence, most importantly, reduced toxicity. Capecitabine loaded Chitosan nanoparticles were prepared by 3^2 full factorial designs, using ionotropic gelation method by cross-linking of chitosan (CS) with sodium tripolyphosphate (TPP). CS-NPs were prepared by dissolving chitosan in 1% (w/v) acetic acid solution under magnetic stirring at room temperature. The CS solution was diluted with deionized water to produce different concentration. The capecitabine was dissolved in CS solution using sonication and aqueous TPP solution was added drop wise using syringe to the mixture with moderate stirring for 30 min. The prepared nanoparticles were characterized by FT-IR spectroscopy and DSC to confirm the cross linking reaction between CS and cross-linking agent. From the % entrapment of capecitabine, nanoparticles were optimized using regression analysis, contour plots and check point analysis.Particle size of the optimized batch (CS-NPs-8) was found to be 87 nm. The Polydispersity index of the nanoparticles was found to be 0.113. The nanoparticles formed were spherical in shape with high zeta potentials, -35mV. *In vitro* release studies in phosphate buffer saline (pH 7.4) showed an initial burst effect and followed by a slow drug release. The drug release followed first order kinetics and was found to be diffusion controlled. Optimized formulation was also showing more % inhibition than drug alone in *In-vitro* anticancer study. From the accelerated study of optimized batch, it was found to be stable.

Keywords: Capecitabine, Colon cancer, Chitosan-TPP nanoparticles, HT-29.

*Corresponding Author Email: ankur_javia@yahoo.in
Received 27July 2014, Accepted13 August2014

Please cite this article as: Javia A *et al.*, Development and Optimization of Capecitabine Loaded Chitosan Nanoparticles for Colon Cancer Therapy. American Journal of PharmTech Research 2015.

INTRODUCTION

Nanotechnology is the designing of practical frameworks at molecular or nano scale[1]. Particles are considered as nanoparticleswhen one dimension is 100 nanometers or less in size[2]. The properties of numerous common materials change when framed from nanoparticles. This is commonly in light of the fact that nanoparticles have a more prominent surface area per weight than bigger particles; this makes them be more receptive to certain molecules. It is conceivable that medications as nanoparticles may give better solubility, prompting better absorption[3]. Additionally, medications may be contained inside of a molecular transporter, either to shield them from stomach acids or to control the drug release to a particular focused area, diminishing the probability of side effects. Nanoparticular drug delivery systems are submicron-sized particles (3-200 nm), devices, or systems that can be made, utilizing an assortment of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), virus (viral nanoparticles), and even organometallic compound (nanotubes). Capecitabine (Figure 1) is an pro-drug that is changed over to fluorouracil in the body tissues taking after the oral route. It is broadly utilized as a part of the treatment of metastatic colorectal growth and breast malignancy, since it is promptly ingested from the gastrointestinal tract. The prescribed every day dosage is huge, i.e., 1500 mg/m^2 and it has a short disposal half-life of 0.5–1h[4]. The unwanted impacts with capecitabine incorporate bone-marrow dipression, cardiotoxicity, looseness of the bowels, sickness and retching, stomatitis, dermatitis, and so on. Subsequently, preparing capecitabine as a controlled release (CR) medication would give more noteworthy or more *in vitro* and *in vivo* antitumor movement, in this way decreasing its harmful effects. Specifically, particular favorable circumstances of multi-particulate formulation, for example, microspheres, beads, and so forth., over other routinedosage form like tablets and capsules have been talked about before[5]. Nevertheless, the principle target of any CR dosage form is to get formulation that would permit the drg to stay in therapeutic window as far as possible. The improvement of such pharmaceutical structures can be accomplished by utilizing particular biocompatible polymers. Among numerous such polymers, hydrogels have been broadly utilized for creating CR devices. Subsequently, it would be of enthusiasm to pick such polymers that have suitable chemical composition, physicochemical nature, biodegradability, chemical stability, mechanical properties, drug release qualities, coveted pharmaceutical dosage form and route of administration[6]. Chitosan [poly (β-(1 → 4)-2-amino-2-deoxy-D-glucose)] is a deacetylated derivative of chitin, which is a naturally occurring polysaccharide, found abundantly in marine crustaceans, insects and fungi[7]. Chitosan is a

cationic, biocompatible and biodegradable polymer having many biomedical applications. Chitosan has many advantages, particularly in developing micro/nanoparticles. These include, its ability to control the release of active agents, it avoids the use of hazardous organic solvents while fabricating particles formulating dosage form, it is soluble in aqueous acidic solution, it is a linear polyamine containing number of free amine groups that are readily available for crosslinking, its cationic nature allows for ionic crosslinking with multivalent anions, it has muco-adhesive character, which increases the residual time at the site of absorption and so on. Chitosan has been extensively studied as a carrier for many drugs[8], proteins and gels for the entrapment of cells or antigens[9] in pharmaceutical industries.

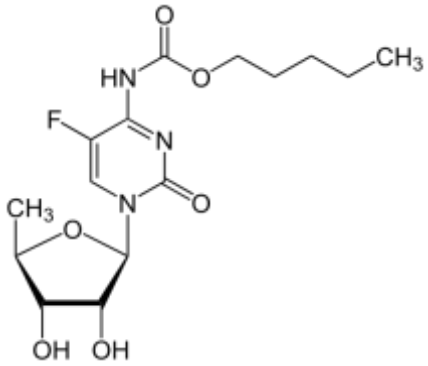

Figure 1: Chemical structure of Capecitabine

MATERIAL AND METHODS

Chitosan (CS, Deacetylation degree of 95% and molecular weight of 80 kDa), Tripolyphosphate (TPP) waspurchased from Balaji drugs (Surat). Capecitabine was obtained as a gift sample from Sun Pharma Research and Analysis Center (Vadodara). All other materials and reagents used in the study were analytical grade.

Preparation of Capecitabine Nanoparticles

Chitosan nanoparticles were prepared, by 3^2 factorial design, by ionic cross linking of chitosan solution with TPP anions. Chitosan was dissolved in 50ml aqueous solution of acetic acid (1% v/v) to prepare various concentrations (0.5mg/ml, 1mg/ml, 1.5mg/ml). Under magnetic stirring at room temperature, 0.5 mg/ml, 0.75 mg/ml, and 1.0 mg/ml concentration of 20 ml TPP aqueous solution was added drop wise using syringe needle into chitosan solution containing 25 mg of capecitabine. pH was adjusted to 6.0 by adding 0.1 N NaOH. The stirring was continued for about 30 minutes. The resultant nanoparticles suspensions were centrifuged at 12000 rpm for 30 minutes. Particles were settled down and separated from clear supernatant. The Particles obtained after centrifugation were finally freeze dried and stored in air tight close container. The formation of the nanoparticles

was because of the interaction be-tween the negative groups of the TPP and the positively charged amino groups of chitosan (ionic gelation) [10, 11] (Table 1).

Table 1: 3^2 Factorial Design of Chitosan Nanoparticles

Batch	Capecitabine (mg)	Chitosan in 1% v/v acetic acid (mg/50ml)	TPP in water (mg/20ml)
CS-NPs -1	25	25	10
CS-NPs -2	25	25	15
CS-NPs -3	25	25	20
CS-NPs -4	25	50	10
CS-NPs -5	25	50	15
CS-NPs -6	25	50	20
CS-NPs -7	25	75	10
CS-NPs -8	25	75	15
CS-NPs -9	25	75	20

Solubility Studies

Solubility of the drug was determined by saturation equilibrium method. Excess quantity of capecitabine was added in to the 10ml volumetric flask and then volume was made up to 10ml mark with water, and then mixture was place in incubator shaker overnight, to get saturated solution of drug. Next day, undissolved drug was separated from the solution by filtering the mixture from whatman filter paper. Supernatant was diluted appropriately with water and the absorbance was determined using UV-visible spectrophotometer. Concentration of the drug was calculated from the standard calibration curve of drug taken in water at 240 nm.

Characterization

Capecitabinecontent

Estimation of drug content was done as per the method reported earlier[12]. Nanoparticles of known weights were soaked in 50ml of water for 30 mints and sonicated using a probe sonicator for 15 mints to break the nanoparticles and facilitate extraction of the drug. The whole solution was centrifuged using a centrifuge to remove the polymeric debris and polymeric debris was washed twice with fresh solvent (water) to extract any adhered drug. The clear supernatant solution was analyzed for capecitabine content by UV spectrophotometer (Shimadzu 1800) at λmax value of 240 nm. The complete extraction of drug was confirmed by repeating the extraction process on the already extractedpolymeric debris.

Entrapment efficiency

At the end of the formation of nanoparticles, it was separatedfrom medium by centrifugation at 12000 RPM for 30 minutes. Then, the nanoparticles pellets and supernatant was separated. Then after, supernatant was appropriately diluted in water and absorbance was taken against water as a

reference on UV-Visible Spectrophotometer at 240nm. Percentage entrapment was calculatedusing following formula.

$$\%\ \text{DrugEntrapment} = \frac{\text{TotalDrugtaken} - \text{druginsupernatant}}{\text{Totaldrugtaken}} \times 100$$

***In-vitro* drug release**

In vitro release study of capecitabine from nanoparticles was carried out in PBS medium, according to a reported procedure[12]. A 4-5 cm long portion of the dialysis bag was made into a dialysis sac by folding and tying up one end of the bag with thread. It was then filled up with phosphate-buffer pH 7.4 and examined for the leaks. The sac was then emptied and NPs dispersion (equivalent to 10 mg drug) was accurately transferred into sac which served as the donor compartments. The sac was once again examined for leak and then suspended in the glass beakers containing 50 ml phosphate-buffer pH 7.4, which become the receptor compartment. Aliquots were taken at 1,2,3,4,5,6,7,8 12, 24, 48 and 72 hours and analyzed spectrophotometrically at 240 nm. Fresh buffer was used to replenish the receptor compartment at each time to maintain sink condition.

Particle size

The average particle size of nanoparticles was measured using a Malvern particle size analyser[8].

Zeta potential

Particle charge is a stability determining parameter in aqueous nanoparticles it is measured by electrophoresis and expressed as electrophoretic mobility (or) converted to the zeta potential (mV). Zeta potential was measured with a combination of laser Doppler velocimetry and phase analysis light scattering (Malvern instruments UK). A Smoluchowsky constant F (Ka) of 1.5 was used to calculate zeta potential values from the electrophoretic mobility[9].

Polydispersity index

The polydispersity index was determined using non-invasive back scatter technology which allows samples measurement in the range of 0.6 nm - 6 µm, freshly prepared capecitabine nanoparticles (800 µl) was placed in a folded capillary cell without dilution. The measurement was carried out using 4MW He-Ne laser as light source at a fixed angle of 173^{o}C the parameters were used for the experiments like medium temperature 25^{o}C.

Fourier transformer infrared spectroscopy

FT-IR spectra was taken to identify changes in chemical structure of the capecitabine nanoparticle. The samples were first lyophilized in freeze drier, and then grounded into homogeneous powders.

The spectra were acquired at 400–4000cm^{-1} wave numbers with a 4cm−1 resolution utilizing a Shimadzu FT-IR spectrophotometer.

Differential scanning calorimetry

Differential scanning calorimetry (Shimadzu DSC-60) analysis was performed using an automatic thermal analyzer. Aluminum pans were employed in the experiments for all samples and an empty pan, prepared in the same manner, was used as a reference. Samples of 3 mg were weighted directly into the aluminum pan which was firmly crimped around the lid to provide an adequate seal. The thermal analyses were conducted from ambient temperature to 300^{o}C at a pre-programmed heating rate of 10^{o}C per min.

Scanning electron microscopy

The morphology of optimizedCS-NPs-8 nanoparticles was examined by scanning electron microscopy (SEM) operated at an acceleration voltage of 10 kV. The samples were attached to aluminum stubs with double side adhesive carbon tape then gold coated and examined using a scanning electron microscope (Leo 1430 VPGermany).

MTT assay

Capecitabine, blank CS-NPs and capecitabine loaded CS-NPs was evaluated for its anti-colon cancer activity by using three different cell lines; HT-29, MCF-7 and Vero.Cells were preincubated at a concentration of 1×106 cells/ml in culture medium, taken in T flask, for 3 h at 37°C and 6.5% CO2. Then after cells were seeded at a concentration of 5×104 cells/well in 100µl culture medium and various amounts of compound (final concentration e.g. 100µM - 0.005µM) were added into microplates (tissue culture grade, 96 wells, flat bottom). Then cell cultures were incubated for 24 h at 37°C and 6.5% CO2 after which 10 µl MTT labeling mixture was added and incubatefurther for 4 h at 37°C and 6.5% CO2. Then in the last the formazan crystals that formed were solubilized by adding 150 µl solubilization solution (isopropanol containing 0.01N HCl)in each well and incubated for overnight and number of viable cells in each well was determined from the absorbance at 570 nm in a plate reader.

Statistical analysis

Data were expressed as means of three separateexperiments, and were compared by analysis of variance(ANOVA). A p-value <0.05 was considered statisticallysignificant in all cases.

Stability study

The stability study was carried out for Capecitabine loaded CS NPs and FA conjugated CS NPs as per ICH guidelines. Nanoparticles of the optimized formulation were placed in screw capped glass container and stored at various ICH storage condition which are 250 C ± 20C (60%± 5%RH) and

400 C ± 20C (75%± 5%RH) for a period of 90 days. The samples were analyzed for physical appearance and for the entrapement efficiency at regular interval of 15 days. Drug release was performed after 90 days.

RESULTS AND DISCUSSIONS

CS (Chitosan) nanoparticles can easily be prepared upon the incorporation of TPP solution to the CS solution under magnetic stirring, since the creation of nanoparticles depends mainly on the evolved ionic interaction of CS with TPP that eventually leads to the reduction of aqueous solubility of CS. The ratio between CS and TPP is critical and controls the size and the size distribution of the nanoparticles. The size characteristics have been found to affect the biological performance of CS nanoparticles. For this reason before the drug encapsulation into CS nanoparticles, the effect of CS/TPP ratio on the size characteristics of the nanoparticles was studied in order to find the optimum ratio that result to nanoparticles of low size and narrow size distribution.

Solubility

The solubility of capecitabine in water was found to be 25.32±0.12mg/ml which is very nearer to reported solubility of capecitabine. The standard calibration curve of the capecitabine was obtained by plotting the concentration from 5, 10, 20, 30 and 40µg/ml against its respective absorbance at 240nm (Figure 2).

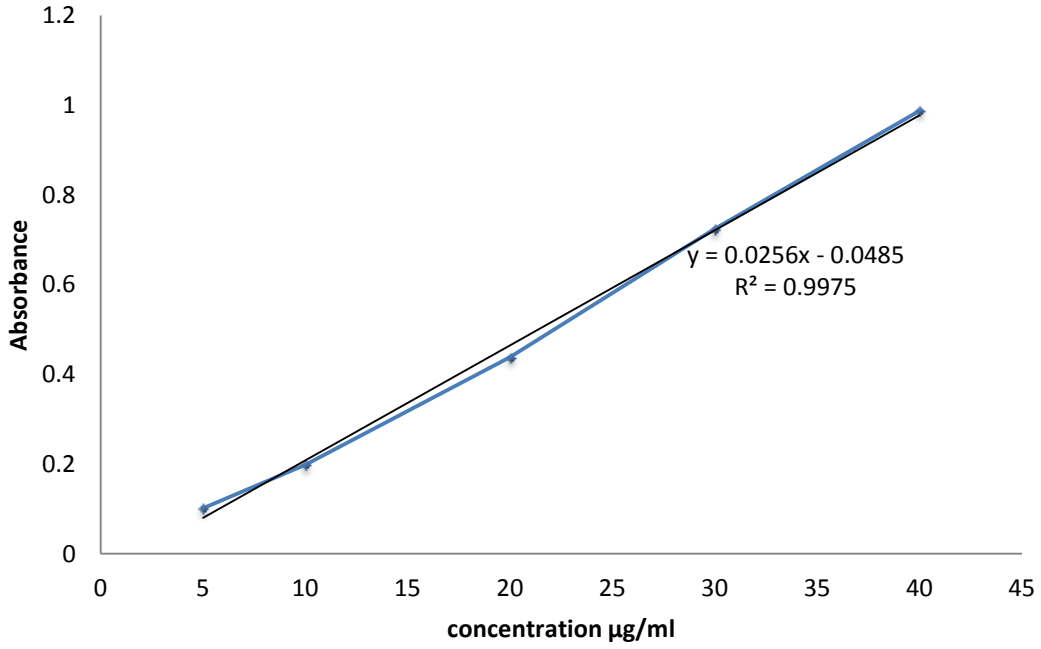

Figure 2: Calibration Curve of Capecitabine in Phosphate Buffer Solution pH 7.4

Table 2: Calibration Curve of Capecitabine in PBS pH 7.4 at λmax 240 nm

Sr. No.	Conc. (µg/ml)	Absorbance			Mean ± SD
		I	II	III	
1	5	0.105	0.099	0.101	0.101±0.003
2	10	0.199	0.201	0.196	0.198±0.002
3	20	0.438	0.441	0.435	0.438±0.003
4	30	0.724	0.72	0.728	0.724±0.004
5	40	0.987	0.99	0.985	0.987±0.002

n=3

Encapsulation efficiency and drug loading

The encapsulation efficiency and drug loading of capecitabine nanoparticles was measured using U.V spectroscopy. Entrapment efficiency was determined for all 09 batches and it was obtained in the range of 45 to 85%. The maximum entrapmentwas in batch CS-NPs-8, 85.74±2.36.This may be because of optimum concentration of chitosan as well as of TPP. Initially as concentration of TPP increases, the entrapment was also found to be increasing up to certain limit,but as concentration further increases, the entrapment was decreasing. This suggest that at lower level TPP is not sufficient enough to crosslink all chitosan used, and at higher concentration TPP might be causing the over cross linking which is reducing the entrapment. For chitosan, higher level was observed to cause maximum entrapment, which suggests that the 1:3 capecitabine chitosan ratio is optimum(Table 3 and figure 3).

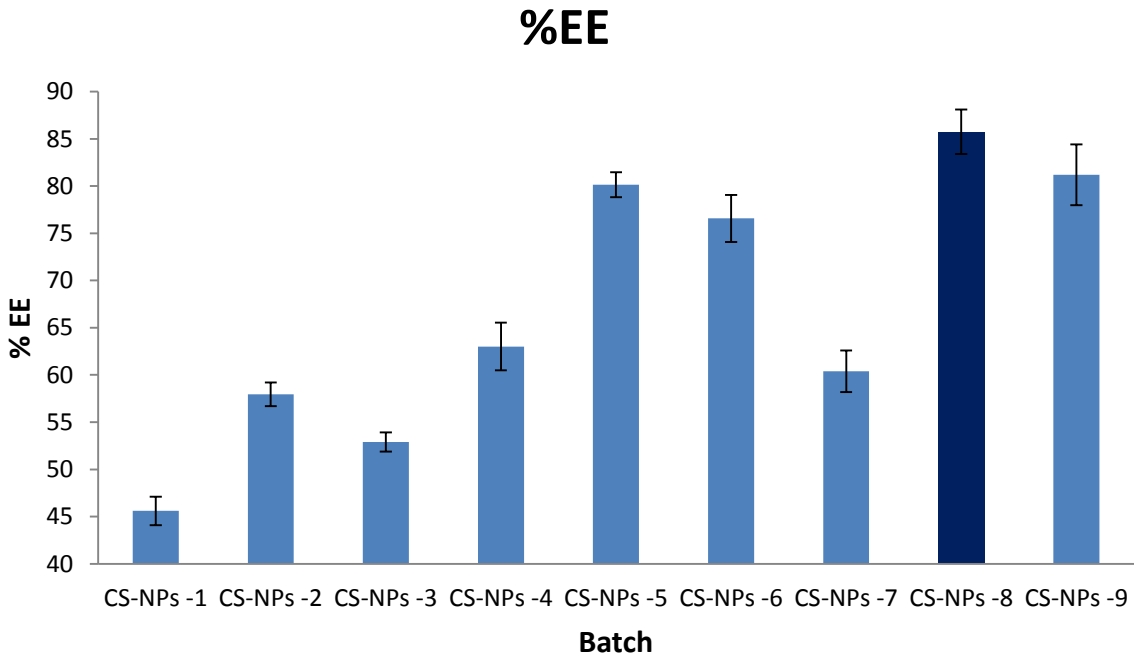

Figure 3: % Entrapment efficiency of capecitabine CS-NPs

Table 3. Data of % yield, % entrapment efficiency (EE) and % drug loading of capecitabine CS-NPs

Batch No.	%Yield	%EE	%Drug Loading
CS-NPs -1	69.6±1.4	45.6±1.5	25.18±0.62
CS-NPs -2	66.38±1.21	57.94±1.25	27.2±0.76
CS-NPs -3	62.07±0.95	52.9±1.023	24.85±.54
CS-NPs -4	65.2±2.06	63.01±2.53	27.56±0.91
CS-NPs -5	70.1±1.3	80.15±1.32	28.67±1.04
CS-NPs -6	73.9±0.96	76.57±2.5	25.87±0.82
CS-NPs -7	54.17±2.5	60.39±2.21	24.66±0.85
CS-NPs -8	69.64±1.11	85.74±2.36	29.9±0.91
CS-NPs -9	70.06±0.93	81.19±3.21	23.03±0.43

n=3

Optimization of formulation

Prepared chitosan nanoparticles were optimized by plotting the contour plots by considering the %EE as a response. Contour plot was drawn by Sigmaplot version 11.0. From the plot it was observed that the maximum entrapment ~85% was attributed to the +1 level of chitosan and 0 level of TPP variables, which represents the batch CS-NPs-8 (Figure 4).

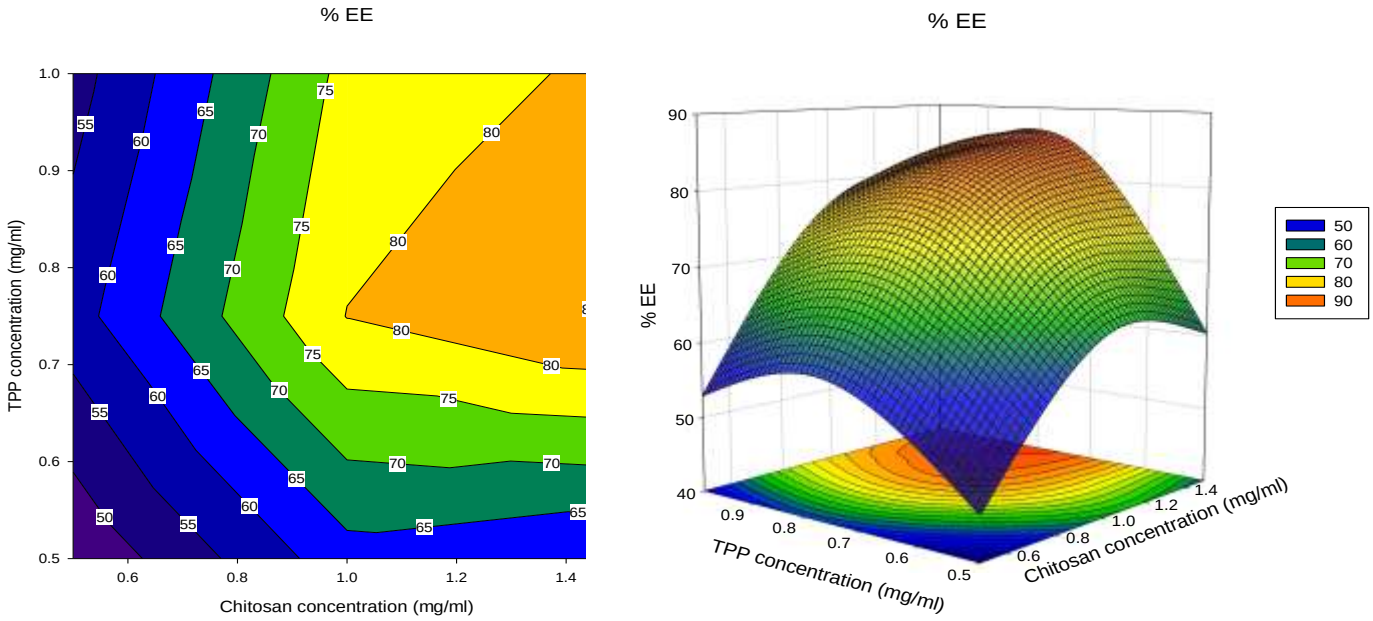

Figure 4: 2D and 3D Contour plots of variables on %EE

Particle size and PDI

Particle size is one of the most important parameters determining biocompatibilities and bioactivities of nanoparticles. Small nanoparticles have a higher intracellular uptake than large ones[14]. Reported that the gastrointestinal uptake of particles of 100nm was 15–250 folds greater than larger size microparticles. Since particle size plays a vital role in mucosal and epithelial tissue

uptake and intracellular trafficking of nanoparticles[13], it is possible to enhance the mucoadhesive properties of CS nanoparticles by decreasing its particle size, and thus to improve mucosal uptake of capecitabine-loaded nanoparticles. Average particle size distribution of CS-NPs-8 was found to be 87.23nm which quite good as per the nano perspective of the formulation and PDI was found to be 0.113 which shows that the optimized nanoparticles were exhibiting narrow size distribution which drastically reduce the possibility of the aggregation of the nanoparticles (Figure 5).

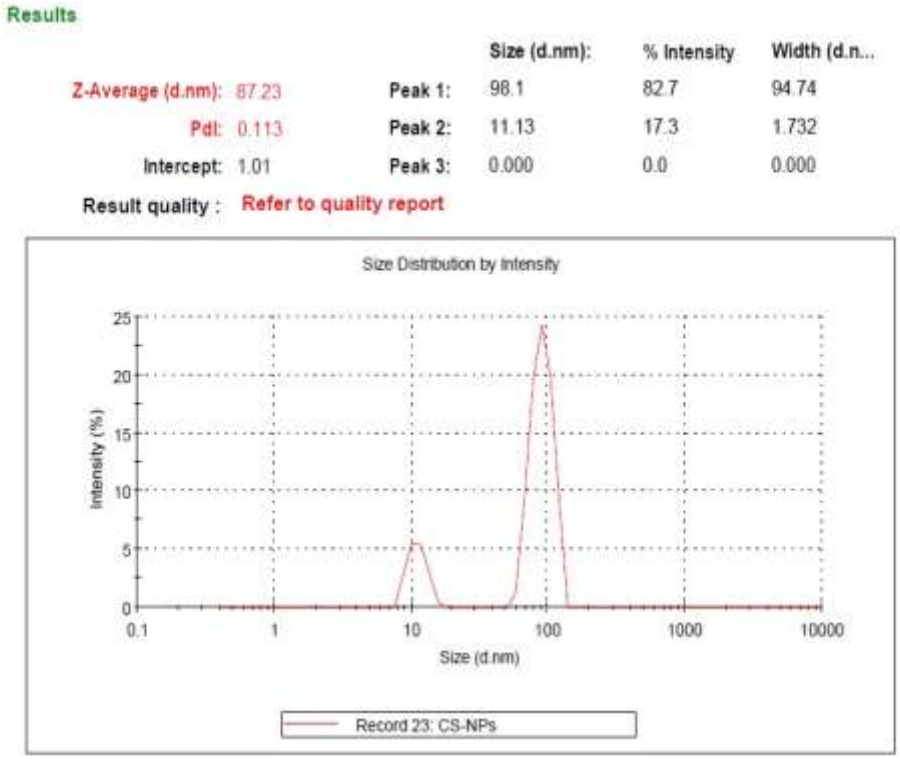

Figure 5: Particle size distribution of optimized formulation CS-NPs-8

Zeta potential

Sufficient zeta potential must exist at the surface of the nanoparticles in order to prevent the aggregation during the storage period. Zeta potential of the optimized bath CS-NPs-8 was analysed and was found to be -35.9mV which is sufficient enough to work as repulsion force between particles so that the stability of the nanoparticles will not be affected and particles will not undergoe the aggregation (Figure 6). Zeta potential is another key parameters contributing to various nutritional properties of CS nanoparticles. It has been well documented that CS possesses mucoadhesive properties, due to molecular attractive forces formed by an electrostatic interaction between positively charged CS and negatively charged mucosal surfaces. Since most tumor cell membranes are negatively charged, CS nanoparticles have recently been studied to develop tumor-specific delivery of anticancer drugs.

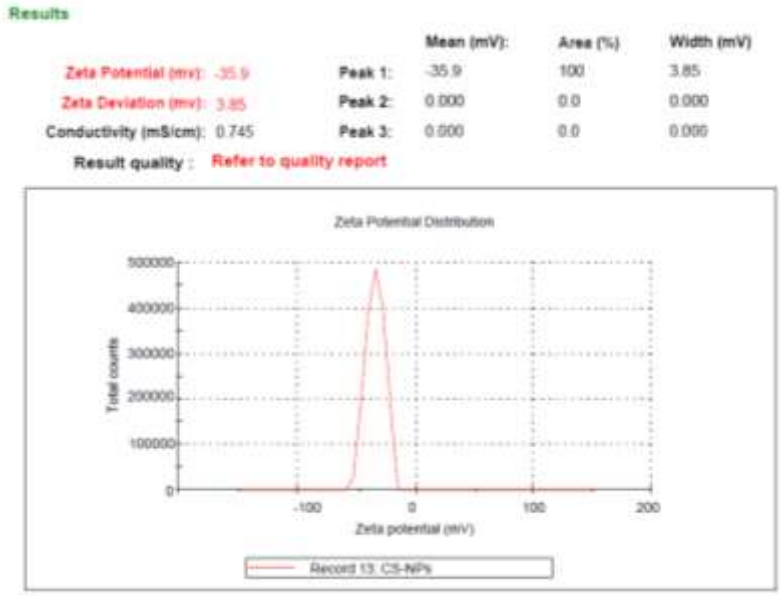

Figure 6: Zeta potential distribution of optimized formulation CS-NPs-8

Fourier transformer infrared spectroscopy

The intermolecular interaction between drug and excipients in nanoparticles was characterized by FT-IR.. first the FT-IR spectra of the capecitabine alone was taken and then the FT-IR spectra of the optimized formulation was taken and then both spectra was compared for the presence of the all the characteristic peaks of the capecitabine. From the comparison it was revealed that the capecitabine is not interacting with the chitosan or TPP as all the characteristic peaks of the drug was preserved in the formulation CS-NPs-8 (Figure 7).

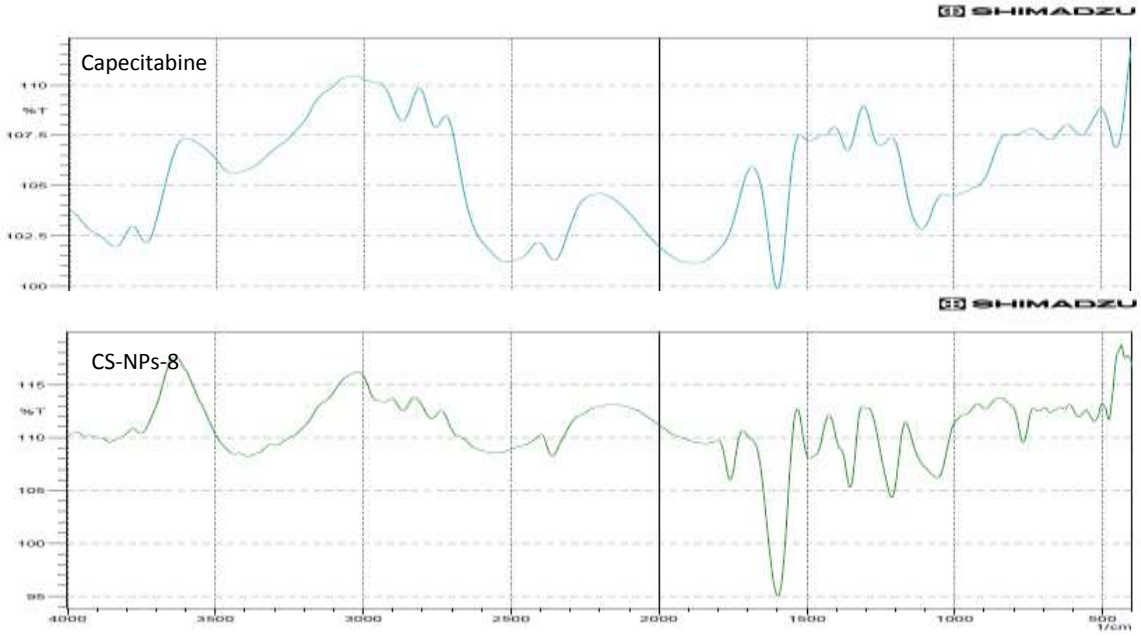

Figure 7: FTIR spectra of Capecitabine and CS-NPs-8

Differential scanning calorimetry

DSC thermogram of capecitabine exhibited a single endothermic peak at 123^{o}C, which corresponded to its intrinsic melting points. similarly, melting peak of capecitabine was identified in the DSC curves obtained from CS-NPs-8. The presence of phase transitions owing to capecitabine in the DSC analysis is evidence that capecitabine is physically compatible within the nanoparticles and exhibited thecrystalline state(Figure 8).

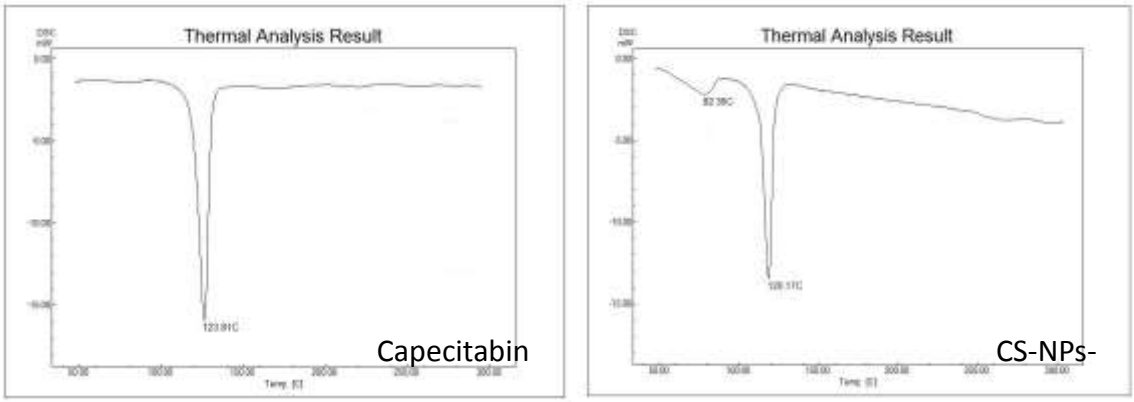

Figure 8: DSC thermogram of Capecitabine and CS-NPs-8

Scanning electron microscopy

Figure 9 represents the morphologies of the nanoparticles observed on SEM. It could be seen that all nanoparticles were regular spheres with smooth surface (diameters <100 nm).

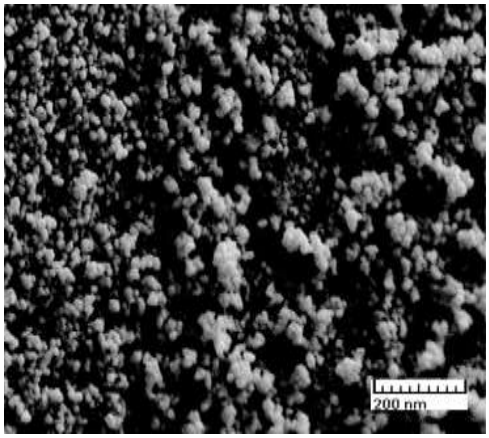

Figure 9: SEM of CS-NPs-8

***In-vitro* drug release of Capecitabine chitosan coated nanoparticles**

The cumulative percentage release of capecitabine from the prepared nanoparticles was obtained from 73.5±2.15 to 94.4±2.15 up to the 48 hours of duration.Highest drug release found in CS-NPs-8 might be because of the better through entrapment of the drug in chitosan network within nanoparticle. From the result of the drug release kinetic model drug release was found to be following first order and diffusion controlled. Initially the burst release was observed up to the

period of 8h and then after slow and gradual release was observed and after 48h the release was observed becoming linear(Figure 10).

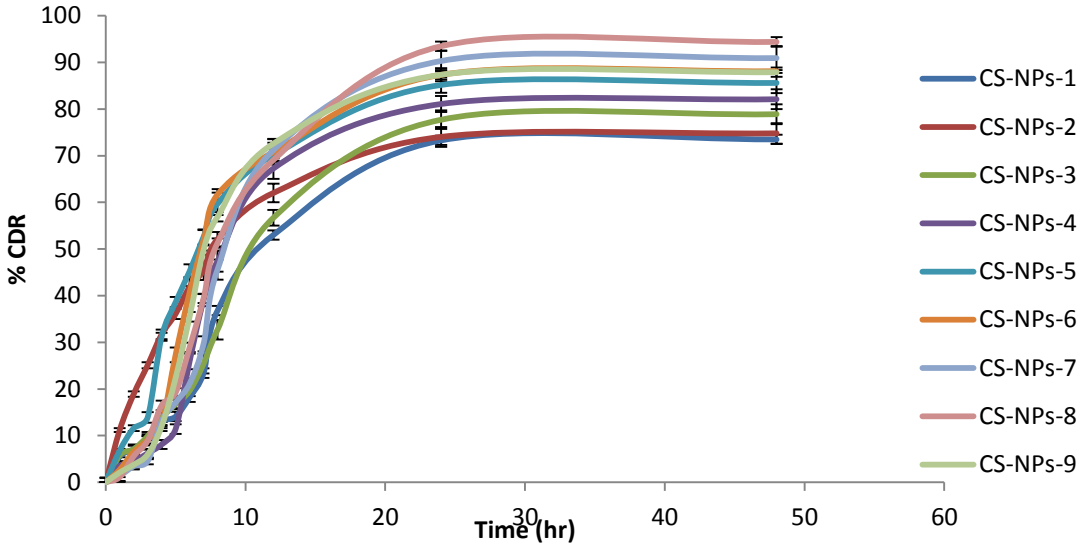

Figure 10: % cumulative drug release of CS-NPs

MTT assay

From the result of the MTT assay it was observed that the capecitabine loaded CS-NPs-8 was showing almost 50% more inhibition than the capecitabine alone in both the cancer cell line HT-29 and MCF-7, which is because of the nano size of the particles enabling the more ingestion the drug within the cells. However, growth inhibition was less in MCF-7 as compared to the HT-29 cell line which shows the better effect of drug in colon cancer than breast cancer. None of the samples have shown any cytotoxicity to the normal cell line Vero. This outcome suggest that the chitosan nanoparticles can be an important way to potentiate the anticancer effect in colon cancer (Figure 11).

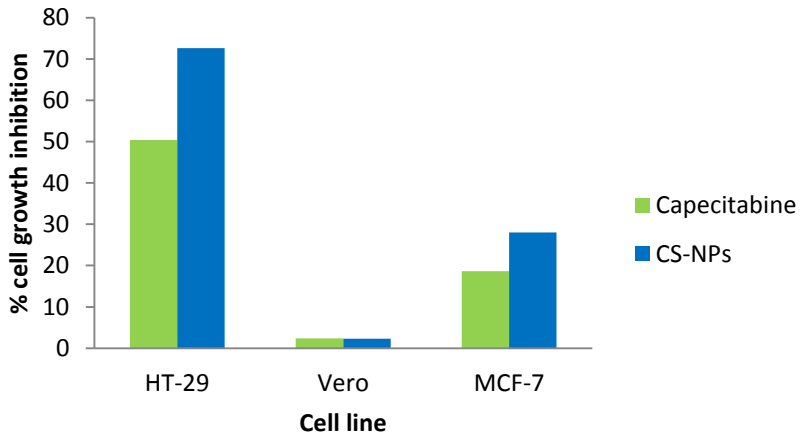

Figure 11: % inhibition of the cell growth by capecitabine and CS-NPs

CONCLUSION

Capecitabine loaded CS nanoparticles were successfully prepared under mild conditions by cross-linked with tripolyphosphate (TPP). The nanoparticles formulation of capecitabine was successfully optimized based on entrapment efficiency(85%) and sustain drug release up to 48h (92%), and optimized batch CS-NPs-8 was found to be having 87nm particle size, minimum Polydispersity (0.113), sufficient zeta potential (-35.9mV), spherical shape, and smooth surface and physical compatibility of drug in nanoparticles. Results from the stability studies at 25°C/60 ± 5% RH and 40°C/70 ± 5% RH indicated good stability of the optimized formulation as there was no significant change in the physical appearance, drug content and drug release. MTT assay shows the 50% rise in the anticancer activity of capecitabine as in chitosan nanoparticle form. So it can be concluded that the chitosan nanoparticles can be effectively used to drastically increase the anti cancer potency of the capecitabine in colon cancer in order to reduce its dose and hence its side effects.

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