

Fabrication, Physicochemical Characterization and Evaluation of In vitro Anticancer Efficacy of a Novel pH Sensitive Polymeric Nanoparticles for Efficient Delivery of Hydrophobic Drug against Colon Cancer

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ARTICLE INFO

Article history:

Received on: 15/09/2015

Revised on: 05/10/2015

Accepted on: 04/11/2015

Available online: 27/11/2015

Key words:

Camptothecin, nanoprecipitation, anticancer activity, stability studies.

ABSTRACT

Objective: The present investigation was aimed to overcome the limitations and to enhance the incorporation of the hydrophobic drug into polymeric nanoparticles and characterize the prepared nanoparticles and also to evaluate the *in vitro* anticancer efficacy of prepared nanoparticles.

Method: Nanoprecipitation method was used to prepare plain and hydrophobic drug (Camptothecin) loaded polymeric nanoparticles. Prepared nanoformulations were evaluated for average particle size, particle size uniformity, surface area, zeta potential, surface morphology, drug content, encapsulation efficiency, drug loading, *in vitro* release, anticancer activity and stability studies at long term and accelerated storage conditions.

Results: Plain and Camptothecin loaded polymeric nanoparticles were successfully prepared by nanoprecipitation method using stirring technique. Prepared Camptothecin encapsulated polymeric nanoparticles were (a) spherical in shape with size < 100 nm, displayed excellent uniformity with <0.3 and zeta potential > 20 mV; (b) showed > 95% release in colonic environment; (c) demonstrated enhanced anticancer activity than pure Camptothecin; and (d) extremely stable at both long term and accelerated storage conditions.

Conclusion: In summary, the investigation concluded that the prepared Camptothecin encapsulated polymeric nanoformulations may be considered as an attractive and promising formulation which significantly overcome the limitations of Camptothecin and synergistically enhance its anticancer activity.

INTRODUCTION

Colon cancer is a multifactorial disease and has emerged as a major public hazard (Giftson *et al.*, 2001). It is the third most common malignancy and second most common cause of cancer with more than 1.4 million new cases and over half a million death worldwide each year (Terzic *et al.*, 2010). Surgery, chemotherapy and radiotherapy are the currently available predominant therapeutic strategies for the treatment of colon cancer. Among the modalities, chemotherapy is the major remedial approach for the treatment of localized and metastasized cancer (Xiao *et al.*, 2015). Well-designed drug delivery vehicle

is need to be developed to enable cancer chemotherapy that fundamentally enhances the therapeutic efficacy by minimizing drug release in undesirable sites (Eun-Kyung *et al.*, 2013). Furthermore, oral colonic drug delivery is convenient to administers and painless to patients when compared with surgery and radiotherapy (Wang *et al.*, 2013). Investigations revealed that encapsulation of anticancer drug into colloidal drug delivery vehicle minimize the tissue distribution and improves therapeutic performance (Averineni *et al.*, 2012). Hydrophobic drug compounds account for 40% of the new drug candidates under FDA review (Kumar and Prud'homme, 2009). Many therapeutic anticancer drugs are limited in their clinical applications because of their toxicities and low solubility in aqueous media (Eun-Kyung Lim *et al.*, 2013).

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For instance, Camptothecin (CPT), is one of the distinguished antineoplastic agent reported to possess promising anticancer activity that targets the nuclear enzyme: DNA topoisomerase I and inhibits the relegation of the cleaved DNA stand, results in tumor cell death. However, the clinical application of Camptothecin is hindered by its poor aqueous solubility and severe systemic toxicities (Manikandan and Kannan, 2015). Solubility of the active ingredient is a major pharmaceutical concern in developing a novel drug delivery system and is also a key parameter when it comes to screening, drug designing, formulating and improving the modeling of oral bioavailability of the drug (Averineni *et al.*, 2012, Prakash *et al.*, 2008). Delivering the drug precisely and safely to its target site at the right period of time to have a controlled release and achieve the maximum therapeutic effect remains a yardstick in the design and development of novel drug delivery systems (Mishra *et al.*, 2010).

Nanotechnology is a rapidly expanding field and the novel properties of nanomaterial's offer great promise to provide new technological breakthroughs (Gregory Morose, 2010). Nanomaterial based drug delivery systems have received attention in overcoming the drawbacks. In particular, the use of nanomaterial's as anticancer drug nanovectors is expected to overcome some of the issues inherent to conventional chemotherapy, including the poor pharmacokinetic profiles of the anticancer drugs and their lack of tumor specificity (Emilie Secret *et al.*, 2013).

Nanoparticulate systems are new tools that promise a revolution in the field of drug delivery. According to the Ostward-Freundlich and Noyes-Whitney equation, the saturation solubility and dissolution rate of a drug can be increased by reducing the particle size to increase the interfacial surface area (Ji-Yao Zhang *et al.*, 2006). Nanocarriers, on account of their higher ratio of surface area to volume, show improved pharmacokinetics and biodistribution of therapeutic agents and thus minimize toxicity by their preferential accumulation at the target site. They improve the solubility of hydrophobic drug and render them suitable for oral administration (Alexis *et al.*, 2008). Conventional technique to increase the bioavailability of these hydrophobic drugs is by formulation nanoparticles with high surface-to-volume ratios (Kumar and Prud'homme, 2009).

Nanoparticles (NPs) are customized drug delivery vectors capable of preferentially targeting large doses of chemotherapeutic agents into malignant cells while sparing the healthy cells (Sinha *et al.*, 2006). Generally, nanoparticles that have a mean diameter of approximately 100 nm, bearing a neutral and hydrophilic surface, exhibit prolonged blood circulation and an increased level of tumor delivery (Catarina *et al.*, 2010). Nanoparticle system with maximal drug loading and a high entrapment efficiency will reduce the quantity of carrier required for the administration of sufficient amount of drug to the target site as well as drug wastage during formulation (Thirumala Govender *et al.*, 1999).

The delivery of an anticancer drug to the target tissue can be achieved by NPs primarily in two ways: passive and ligand-

based targeting. Passive targeting approach exploits the pathophysiological conditions, such as leaky vasculature, pH, temperature and surface charge surrounding the tumor for specific delivery of NPs (Prabhu *et al.*, 2015). While nanoparticles tend to passively accumulate in tumor tissue due to the enhanced permeation and retention (EPR) effect, which is ascribed to enhanced vascular permeability in tumors, active endocytosis of nanoparticles targeting cancer cells is equally desired in order to further increase the concentration of the anticancer drug intracellularly and to limit its toxic effects in normal tissue (Torchilin, 2011).

The propensity of nanoscale materials (compared to single small molecules) to accumulate in a tumor through the EPR effect provides many possibilities to design effective drug delivery nanosystems that vectorize poorly water soluble toxic anticancer drug to tumor sites (Danhier *et al.*, 2010). Vectorization of the hydrophobic alkaloid Camptothecin using polymeric nanoparticle is a highly promising route to avoid the drawbacks of this drug and develop a novel delivery system (Emilie Secret *et al.*, 2013).

Polymeric nanoparticles (PNPs) are solid colloidal systems in which the therapeutic agent is dissolved, entrapped, encapsulated or adsorbed onto the constituent polymer matrix (Prabhu *et al.*, 2015). Polymeric nanoparticles are biodegradable and biocompatible and have been adopted as a preferred method for nanomaterial drug delivery. The polymer matrix prevents drug degradation and may also provide management of drug release from these nanoparticles. The surface properties of these polymeric nanoparticles are also a vital component of their targeting characteristics (Faraji and Wipf, 2009). Drug delivery systems can be designed to release drugs triggered by environmental parameters such as pH, enzymes, and temperature. pH dependent solubility would significantly assists in improving the modeling of oral bioavailability of the drug (Eun-Kyung Lim *et al.*, 2013).

Methods of preparation of PNPs fall into two major classes: one deals with the polymerization of monomers (e.g., emulsion and dispersion polymerization), whereas the other essentially involves dispersion of polymers (e.g., salting out, emulsification-diffusion and nanoprecipitation) (Galindo-Rodriguez *et al.*, 2004). In order to produce small and low polydisperse nanoparticle population, the nanoprecipitation method developed by Fessi *et al.*, in 1989 is one of the most easy and reproducible technique and has been widely used by several research group to prepare polymeric nanoparticles (Thirumala Govender *et al.*, 1999, Eliana Leo *et al.*, 2004). This method is basically applicable to hydrophobic drugs due to the miscibility of the solvent with the anti-solvent (Jose Mario Barichello *et al.*, 1999).

The present investigation was aimed to overcome the limitations and to enhance the incorporation of the hydrophobic drug into polymeric nanoparticles and characterize the prepared nanoparticles and also to evaluate the *in vitro* anticancer efficacy of prepared nanoparticles.

MATERIALS AND METHODS

Materials

Camptothecin was commercially purchased from S.M Herbals, India. β -cyclodextrin and poloxamer (Grade 188) were procured from Sigma Aldrich, India. Poly (methacrylic acid-co-methyl methacrylate) was obtained from Evonik Industries, India. Dimethyl sulphoxide and propanol were obtained from E-Merck specialities Pvt. Ltd., India. 3-(4,5-dimethyl thiazol-2-yl) -5-diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., India. All other chemicals and reagents used were of analytical grade and used without further purification.

Fabrication of plain and Camptothecin loaded polymeric nanoparticles

Processing of hydrophobic drug into polymeric nanoparticle requires a conventional technique. Nanoprecipitation is the process with an attractive processing scheme based on rapid micro-mixing of two streams: solvent and anti-solvent. The solvent stream is composed of hydrophobic drug and copolymer dissolved in water miscible organic solvent and water containing surfactant is generally used as an anti-solvent. This technique is a rapid and direct process, which can be performed in ease. The particle size of the drug decreases with the increase in the stirring rate (Kumar and Prud'homme, 2009, Ji-Yao Zhang *et al.*, 2006). Camptothecin loaded polymeric nanoparticles were prepared by a novel nanoprecipitation technique.

Briefly, about 100 mg of poly (Methacrylic acid-co-methyl-methacrylate) polymer with and without (plain nanoparticles) 10 mg of Camptothecin were dissolved in 10 ml of dimethyl sulphoxide. The prepared organic phase was transferred at once into 500 ml beaker containing 50 mg of β -cyclodextrin, 100 mg of poloxamer 188 and 20 ml of distilled water under mechanical stirring (Remi, India) at 500 rpm. Polymeric nanoparticles were formed spontaneously but the stirring process is continued for 50 mins to aid the size reduction and to evaporate the residual solvents (Table 1). The fabrication experiments were performed in triplicate. Prepared polymeric nanoformulations were stored at room temperature for one month to identify any aggregation and post-formulation degradation.

Physicochemical characterization of prepared polymeric nanoparticles

Average Particle size, Particle size uniformity, Surface area and Zeta potential

Prepared plain and Camptothecin loaded polymeric nanoparticles were characterized for average particle size, particle size uniformity, surface area, as these parameters decides the performance such as solubility, dissolution, stability, circulation half-life, drug release, cellular uptake and bio-distribution (Manikandan and Kannan, 2015, Xie and Smith, 2010).

The average particles size, particle size uniformity and surface area of prepared polymeric nanoparticles were measured based on quasi-elastic light scattering principle using mastersizer (Malvern Instrument, UK). Briefly, prepared Camptothecin loaded polymeric nanoparticle formulations was added drop wise into the water maintained in the sample dispersion unit of particle size analyzer, where the nanoparticles scattered using simple shaft pump an stirrer and re-circulated continuously around the measurement zone of particle size analyzer. The experiments were performed in triplicate (Mohanty and Sahoo, 2010).

Zeta potential of the prepared polymeric nanoparticles was measured using zetasizer (ZEN3600, Malvern). The zeta potential is commonly used to characterize and measure the surface charge as it plays a significant role in aggregation. Aggregation in nanoparticles in formulation reduces the physical stability of the nanosuspension and leads to decreased oral bioavailability. In a sense, zeta potential represents an index for particle stability. Higher number of either positive or negative charge increase the repulsive interaction leading to stable particles which in turn prevents aggregation. A nanosuspension stabilized by electrostatic repulsion must have a minimum zeta potential of ± 30 mV (Catarina *et al.*, 2010, Muller *et al.*, 2001). Briefly, about 1 ml of polymeric nanoparticles was diluted approximately using ultra-pure water (Milli Q Academic Milli Pore). Diluted samples were loaded separately in a disposable zeta cell and measured for zeta potential.

Particle surface morphology analysis

Prepared plain and Camptothecin loaded polymeric nanoparticles were characterized for surface morphology, as it decides the basic function of particles, degradation, release of drug from polymer, transport of particles in the body and internalization of drug (Xie and Smith, 2010, Mohanty and Sahoo, 2010).

Table 1: Fabrication of Camptothecin loaded polymeric nanoparticles .

Trials	A (mg)	B (mg)	C (mg)	D (mg)	E (ml)	F (ml)	G (Min)	H (rpm)	J	K	L
Plain	-	100	50	100	10	20	50	500	At once	Or. to Aq.	Blade
CPT	10	100	50	100	10	20	50	500	At once	Or. to Aq.	Blade

A: Concentration of Drug; B: Concentration of Poly (Methacrylic acid-co-methyl-methacrylate); C: Concentration of β -Cyclodextrin; D: Concentration of Poloxamer 188; E: Volume of Organic Phase; F: Volume of Aqueous Phase; G: Stirring Time; H: Stirring Speed; J: Mode of Addition; K: Process; L: Stirring Mode; Or.: Organic Phase; Aq.: Aqueous Phase.

The morphological examination of the prepared polymeric nanoparticles was performed by Field Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM). FESEM (Hitachi SU 660) was used to analyse the morphology by accelerating voltage of 15 kv. The spot size in FESEM is smaller than conventional SEM and it can produce very high resolution image. The TEM is also used to analyse the shape of the prepared nanoparticles.

Briefly, the prepared Camptothecin loaded polymeric nanoparticles were dropped onto Formvar-coated copper grids and air dried. The samples were then negatively stained with 1% uranyl acetate for 10 minutes and air dried again. The samples were then imaged using transmission electron microscope (Hitachi H7500, India) at 20,000 magnifications (Champion *et al.*, 2007, Jung *et al.*, 2002).

Encapsulation efficiency and drug loading estimation

Camptothecin loaded polymeric nanoformulations were centrifuged using a cooling centrifuge (C-24, Remi) for 45 min at 19 000 rpm at -20 °C and supernatant was separated. To 1 ml of supernatant, an equal volume of methanol was added (Verma and Garg, 2005) and sonicated (Ultrasonic cleaner, Lark) for 5 min followed by filtration through 0.45 µm membrane. Samples were analyzed using the developed HPLC methods as mentioned below. Estimated amount of free drugs was expressed as W_{free} . The experiments were performed in triplicate.

For the analysis, Shimadzu HPLC system was used with the best chromatographic conditions equipped with C18 column (ODS 250 mm X 4.6 mm with 5 micron pore size, Phenomenax) using a mobile phase combination of 0.5 % W/V of ammonium acetate aqueous solution and acetonitrile (85:15, v/v) in an isocratic mode elution with a flow rate of 1 ml min⁻¹ at the column oven temperature of 35 °C and the samples were analyzed by PDA detector at a wavelength of 368 nm (Karin Liltorp *et al.*, 2011, Stecanella *et al.*, 2013).

Encapsulation efficiency (EE) and drug loading (DL) were estimated as follows

$$EE (\%) = \frac{[\text{Drug Content } (W_{total})] - [\text{Drug in the supernatant } (W_{free})]}{[\text{Drug Content } (W_{total})]} \times 100$$

$$DL (\%) = \frac{[\text{Drug Content } (W_{total})] - [\text{Drug in the supernatant } (W_{free})]}{[\text{Weight of the polymer used in the formulation } (W_{polymer})]} \times 100$$

In vitro drug release study

In vitro drug release of Camptothecin from polymeric nanoparticles was evaluated by dialysis bag diffusion technique. The prepared Camptothecin loaded polymeric nanosuspension (weight equivalent to 10 mg of drug) was placed in cellulose dialysis bag (cutoff 12 000; HIMEDIA, Mumbai, India) and sealed at both ends. The dialysis bag was immersed in the receptor compartment containing dissolution medium maintained at 37 °C ± 0.5 °C with a rotating speed of 100 rpm (Reddy and Murthy, 2005). The release characteristic of Camptothecin from the

prepared nanoparticle formulation was investigated using USP dissolution apparatus 2 (Electrolab, Mumbai, India). To achieve simulated gastrointestinal transit condition the release profile of nanoformulation was studied with the dissolution medium of changing pH at various time intervals. Initially, the dissolution medium was maintained at pH 1.2 with 350 ml of 0.1N HCl for 0-2 h. At the end of second hour, the pH of the dissolution medium was raised to 4.5 by the addition of 250 ml of solution composed of 3.75 g of KH₂PO₄ and 1.2 g of NaOH and the total volume of dissolution medium was 600 ml. At the end of fourth hour, pH of medium was raised to 7.4 by addition of 300 ml of phosphate buffer concentrate (2.18 g of KH₂PO₄ and 1.46 g of NaOH in distilled water) (Seema Badhana *et al.*, 2013, Ahmed Abd El-Bary *et al.*, 2012). At predetermined time intervals 5 ml of sample was withdrawn and replaced with fresh dissolution media. The collected samples were filtered through 0.45 µm membrane filter (Millipore). After appropriate dilution, the concentration of drug in the sample was analyzed using HPLC.

Kinetic Analysis of Dissolution data

The data obtained from *in vitro* release studies were kinetically analysed to find out the mechanism of drug release rate kinetics of dosage form. The obtained data was fitted with zero order, first order, Higuchi, Hixson-Crowell erosion equation, Korsmeyer-Peppas equation (Kannan *et al.*, 2012, Pandian *et al.*, 2012, Harris Shoab *et al.*, 2006).

Zero Order Kinetics

The graph was plotted as cumulative % drug release Vs time where the drug release rate is independent of its concentration (Hadjioannou *et al.*, 1993).

$$C = K_0t$$

Where,

K_0 = Zero order rate constant expressed in units of concentration/time,
t = Time in hours.

First order Kinetic model

The graph was plotted as log cumulative % of drug remaining Vs time, where release rate is concentration dependent (Uday S Rangole *et al.*, 2008).

$$\log C = \log C_0 - Kt / 2.3030$$

Where,

C_0 = Initial concentration of drug,

K = First order constant,

t = Time in hours.

Higuchi kinetics

Higuchi describes the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion. The graph was plotted as cumulative % drug released Vs square root of time (Higuchi, 1963).

$$Q = Kt^{1/2}$$

Where,

K = Constant reflection design variable system,

$t^{1/2}$ = Time in hours.

Hence, drug release rate is proportional to the reciprocal of square root of time. If the plot yields a straight line, and the slope is one then the particular dosage form is considered to follow Higuchi kinetics of drug release.

Hixson-crowell erosion equation

It describes the drug release with changes in the surface area and the diameter of particles the data were plotted using the Hixson crowell rate equation. The graph was plotted by cube root of % drug remaining in matrix Vs time (Hixson and Crowell, 1931).

$$Q_0^{1/3} - Q_t^{1/3} = KHC t.$$

Where,

Qt = Amount of drug released in time t,

Q0 = Initial amount of drug in tablet,

KHC = Rate constant for Hixson crowell rate equation.

Korsmeyer-Peppas equation

To find out the mechanism of drug release, it was further plotted in peppas equation as log cumulative % of drug released Vs log time (Korsmeyer *et al.*, 1983, Korsmeyer *et al.*, 1986a).

$$Mt / M_\infty = Kt^n,$$

$$\text{Log } Mt / M_\infty = \text{log } K + n \text{ log } t$$

Where,

Mt / M_∞ = Fraction of drug released at time t,

K = Kinetic rate constant,

t = Release time,

n = Diffusion exponent indicative of the mechanism drug release.

This model is used to analyze the release of pharmaceutical polymeric dosage forms when the release mechanism is not known or more than one type of release phenomenon was involved. The n value could be obtained from slope of the plot of log cumulative % of drug released Vs log Time (Table 2).

Table 2: Diffusion exponent and solute release mechanism for cylindrical shape.

Diffusion exponent (n)	Overall solute diffusion mechanism
0.45	Fickian diffusion
0.45 < n < 0.89	Anomalous (non-fickian) diffusion
0.89	Case-II transport
n > 0.89	Super case-II transport

In vitro anticancer activity

Prepared polymeric nanoformulation was evaluated for anticancer activity using (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (MTT) assay on HT-29 (Human colon carcinoma) (Francis and Rita, 1986). Briefly, prepared polymeric nanoformulation was diluted with DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS) to obtain a stock solution of 5 mg/ml concentration, which was sterilized by filtration and finally centrifuged. Serial dilutions

(1000, 500, 250, 125 and 62.5 µg/ml) were made from the stock solution. About, 0.1 ml of the diluted HT-29 cell suspension (approximately 10 000 cells) was added each well of the 96 well microtitre plate. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added. The plates were then incubated at 37°C for 3 days in 5 % CO₂ atmosphere and microscopic examination was carried out and observations were noted every 24 h intervals. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5 % CO₂ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm (Pavan Kumar Bellamakondi *et al.*, 2014). The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line. The experiments were performed in triplicate.

$$\text{Growth Inhibition (\%)} = 100 - \left\{ \frac{\text{Mean optical density of test}}{\text{Mean optical density of control}} \right\} \times 100$$

Stability Studies

Stability study was performed as per International Conference on Harmonisation guidelines Q1A(R2) to assess the prepared hydrophobic drug loaded polymeric nanoformulation for its stability and potential to withstand atmospheric/environmental changes. Prepared plain and Camptothecin loaded polymeric nanoformulation were packed in a clear glass vial and stored at long term condition (25°C ± 2°C/60% RH ± 5% RH) for 12 months and accelerated stability condition (40°C ± 2°C/75% RH ± 5% RH) for 6 months in a stability chamber (SS134, Shivani Scientific). The samples were evaluated at time intervals of 3 months. The stored samples were analysed for average particle size, particle size uniformity, surface area, zeta potential, drug content and drug release (Ahmed Abd El-Bary *et al.*, 2012). The experiments were performed in triplicate.

RESULTS AND DISCUSSION

Fabrication of plain and Camptothecin loaded polymeric nanoparticles

Plain and Camptothecin loaded Poly (Methacrylic acid-co-methyl-methacrylate) nanoparticles were prepared based on the principle of nanoprecipitation under the influence of stirring. In nanoprecipitation method, the solvent stream contains Camptothecin and Poly (Methacrylic acid-co-methyl-methacrylate) in water miscible organic solvent dimethyl sulphoxide and anti-solvent stream contains poloxamer 188 as a surfactant and β cyclodextrin as a stabilizer in water. Addition of solvent stream into the anti-solvent stream results in the miscibility of dimethyl sulphoxide with water, which leads to the increase in

the polarity of dimethyl sulphoxide, which in turn decreases the solubility of polymer. However, nucleation of polymer gets initiated when the equilibrium concentration surpasses the solubility threshold of polymer. Stirring process aid the size reduction of polymer at the initial stage but in the later stages, anionic nature of polymer provided anionic charge to the nanoparticle surface and higher number of likely charged nanoparticles repels each other and creates an electrostatic repulsive force and maintains the nanoparticles in Brownian motion, which is expected to overcome the van der Waals attractive force arising from induced dipole-dipole interaction between nanoparticles and gravitational force, thereby stabilize the nanoformulation by preventing the aggregation.

Average particle size, Particle size uniformity, Surface area and Zeta potential analysis

After fabrication, prepared plain and Camptothecin loaded polymeric nanoparticles were stored at room temperature for one month to identify any aggregation and post-formulation degradation. Prepared plain and Camptothecin loaded polymeric nanoparticles were characterized for average particles size, particle size uniformity, surface area and zeta potential as per the procedure mention above. The results were summarized in table 3 and characterization spectrums were displayed in figure 1 and figure 2.

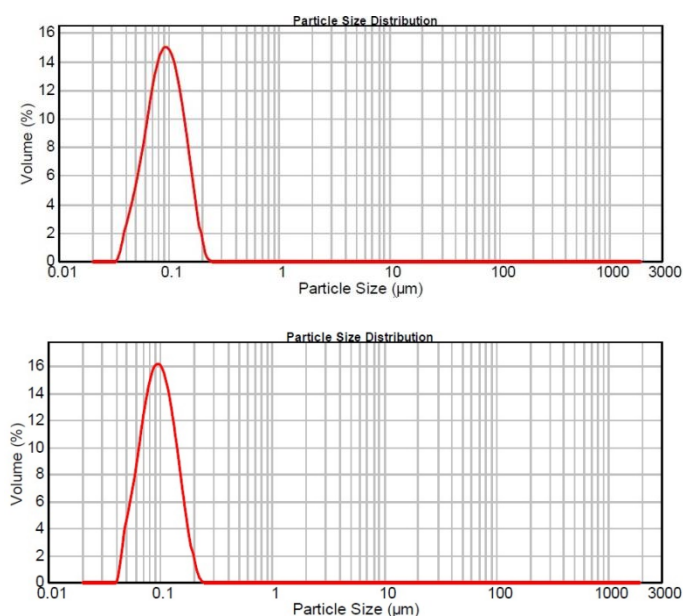


Fig. 1: Particle size spectrum of Plain (a) and Camptothecin (b) loaded polymeric nanoparticles.

After fabrication, prepared plain and Camptothecin loaded polymeric nanoparticles were stored at room temperature for one month. However, there was no visual aggregation and post formulation degradation. In stirring technique, plain polymeric nanoparticles has shown an average particle size of 97 nm, particle size uniformity of 0.268, surface area of $74.2 \text{ m}^2\text{g}^{-1}$ and zeta potential of -28.5 mV . Nevertheless, encapsulation of

Camptothecin has increased the average particle size to 100 nm, decreased particle size uniformity to 0.242, decreased the surface area to $65.2 \text{ m}^2\text{g}^{-1}$ and decreased the zeta potential to -22.41 mV respectively. After encapsulation of Camptothecin, average particle size increased due to the accommodation of drug in polymeric nanoparticles. Average particle size $\leq 100 \text{ nm}$, particle size uniformity < 0.3 , surface area $> 50 \text{ m}^2\text{g}^{-1}$ and zeta potential $> -20 \text{ mV}$ were set as acceptance criteria (Feng-Lin Yen *et al.*, 2010). However, Camptothecin loaded nanoparticles prepared by stirring method satisfied all acceptance criteria.

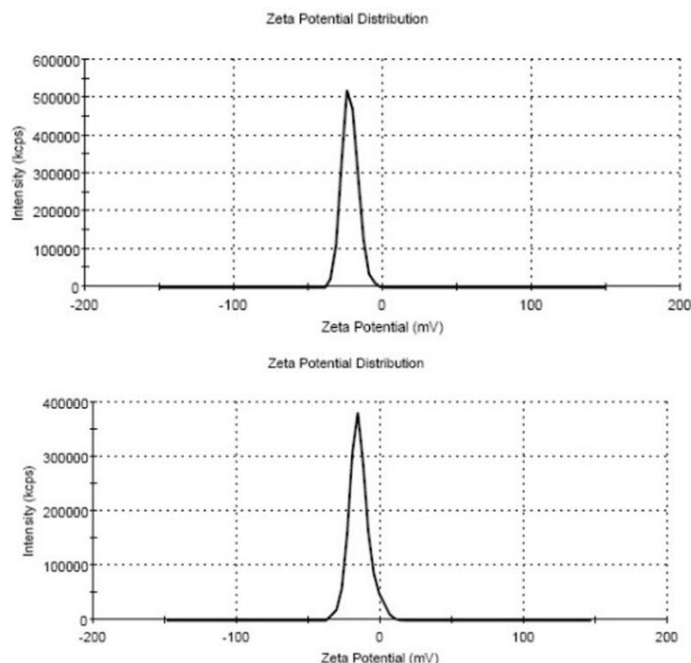


Fig. 2: Zeta potential spectrum of plain (a) and Camptothecin (b) loaded polymeric nanoparticles.

Table 3: Average particle size, particle size uniformity, surface area and zeta potential analysis of plain and Camptothecin loaded polymeric nanoparticles.

Trails	Average particle size (nm)	Particle Size Uniformity	Surface Area ($\text{m}^2\text{g}^{-1} \pm \text{SD}$)	Zeta Potential (mV)
Plain	97 ± 0.18	0.268 ± 0.11	74.2 ± 0.19	-28.5 ± 1.25
CPT	100 ± 0.25	0.242 ± 0.04	65.2 ± 0.39	-22.4 ± 1.36

The values are expressed as Mean \pm SD; n=3;

Particle surface morphology analysis

Particle surface morphology decides the basic function of particle degradation, release of drug from polymer matrix, transport of particles in the body, internalization of drug. The surface morphology analysis for the prepared plain and Camptothecin loaded polymeric nanoparticles were performed by field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) as per the procedure mentioned above and the TEM images were displayed in figure 3 & 4 as well as the FESEM images were displayed in figure 5 & 6. Prepared Camptothecin loaded polymeric nanoparticles were spherical in shape. Hence, Camptothecin encapsulated in the polymer matrix will also be in spherical shape and expected to

enhance the basic function, release of Camptothecin from the polymer matrix, transport of Camptothecin in the body and internalization of Camptothecin by many folds than the free Camptothecin.

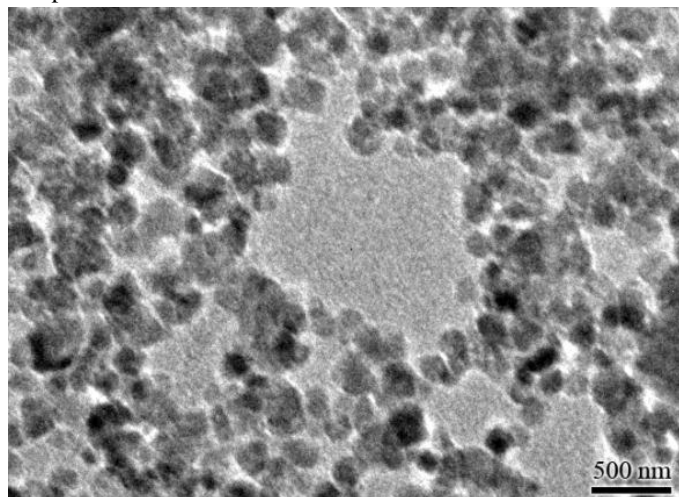


Fig. 3: TEM image of plain polymeric nanoparticles.

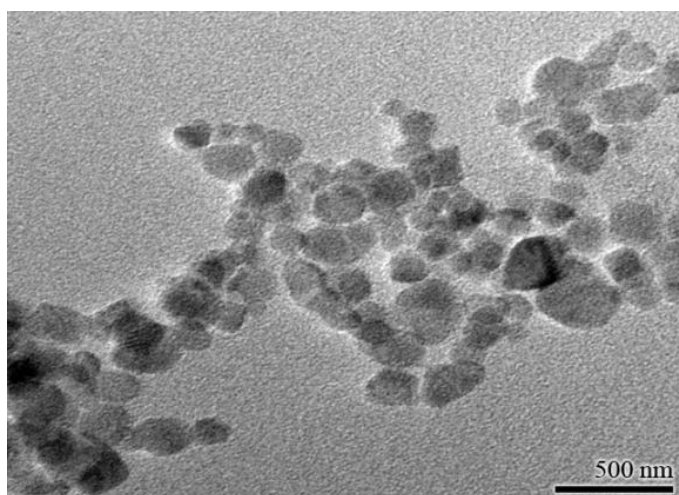


Fig. 4: TEM image of Camptothecin loaded polymeric nanoparticles

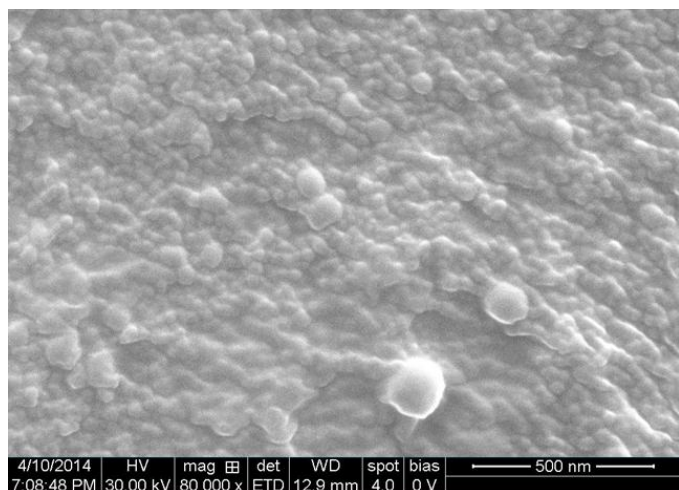


Fig. 5: FESEM image of plain polymeric nanoparticles.

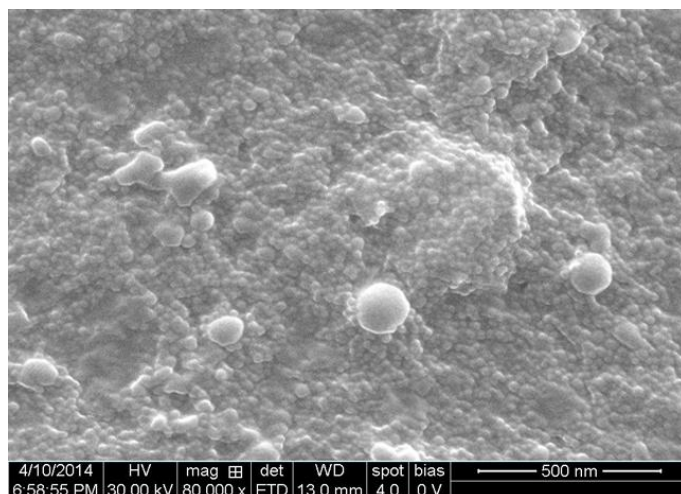


Fig. 6: FESEM image of Camptothecin loaded polymeric nanoparticles.

Drug content, encapsulation efficiency & drug loading estimation

The amount of Camptothecin encapsulated in polymeric nanoparticles determines the effectiveness of prepared nanoformulations. Hence, drug content, encapsulation efficiency and drug loading estimation were performed as per procedure mentioned above. Drug content was estimated by performing an assay whereas encapsulation efficiency and drug loading were calculated by measuring the free Camptothecin in the nanoformulation. The results were summarized in table 4.

Table. 4: Drug Content, Encapsulation efficiency and Drug loading of the prepared Camptothecin loaded polymeric nanoparticles.

Trial	Drug content (%)	Encapsulation efficiency (%)	Drug loading (%)
CPT	97.96 ± 0.59	93.17 ± 0.72	9.13 ± 0.38

The values are expressed as Mean ± SD; n=3;

Drug content in the formulations was in the range of 96 to 100, which shows that there was no post-formulation degradation or drug loss. The drug content, encapsulation efficiency and drug loading of the prepared Camptothecin loaded polymeric nanoparticles were found to be 97.96%, 93.17% and 9.13% respectively. The stirring approach displayed excellent encapsulation efficiency and drug loading only insignificant amount of Camptothecin were seen as free drug. Hence, prepared Camptothecin loaded polymeric nanoparticles is expected to display superior pharmacological activities.

In vitro drug release study

In vitro drug release from the Camptothecin loaded polymeric nanoparticles prepared by nanoprecipitation method using stirring approach was assessed in simulated gastrointestinal conditions. The pH condition used was pH 1.2 for a period of 2 hrs (stomach), pH 4.5 for 2 hrs (duodenum) followed by pH 7.4 (distal ileum and colon) for the remaining period of the study using a USP dissolution test apparatus (Apparatus type 2) (Chandran *et al.*, 2009) and *in vitro* drug release were shown in figure 7. The

drug release was found to be less than 5% upto 4 hrs and the drug release increased when the pH of the medium was adjusted to 7.4. Poly (Methacrylic acid-co-methyl-methacrylate) is an anionic polymer, the ratio of free carbonyl groups to the ester groups is approximately 1:2. It exhibits a dissolution threshold pH slightly above 7.2 (Sinha and Kumari, 2003). Due to the pH sensitive property, it was selected to avoid the rapid dissolution of Camptothecin during the initial transit of the nanoparticles through gastric cavity and the upper small intestine. It was observed that polymer coated Camptothecin nanoparticles gave no release in the simulated gastric fluid, negligible release in the simulated intestinal fluid and maximum release in the colonic environment. Hence, released nanosized Camptothecin are expected to display enhanced aqueous solubility and permeability in the colon.

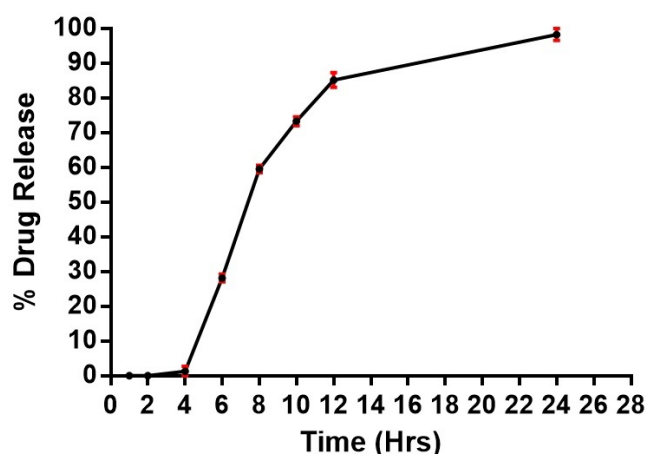


Fig. 7: *In vitro* release profile of Camptothecin from prepared polymeric nanoparticles.

Kinetics Analysis

The results of *in vitro* release profile obtained from the formulations were plotted to know the mechanism of drug release. The data were treated according to zero order release, first order release, Higuchi model, Korsmeyer Peppas model and Hixson Crowell cube root law. The release rate kinetics data of the formulation is shown in table 5.

Table 5: Determination coefficients (r^2) and release exponent (n) of kinetic data analysis of Camptothecin release from polymeric nanoparticles.

Trial	Zero order	First Order	Higuchi model	Korsmeyer-peppas model		Hixson-crowell cube root law
	r^2	r^2	r^2	r^2	n	r^2
CPT	0.7211	0.7157	0.6255	0.546	1.5289	0.9452

It is concluded that the Camptothecin loaded polymeric nanoparticles prepared by nanoprecipitation method using stirring approach gave a good fit to the Hixson Crowell cube root law. The diffusion exponent (n) value were greater than 0.89, this result

indicated that the release of drug from the polymer matrix formulations was found to be super case-II transport, i. e., drug release by both diffusion and relaxation of polymer chain.

In vitro anticancer activity

Prepared Camptothecin loaded polymeric nanoformulation was studied for its *in vitro* anticancer efficacy against human colon cancer HT-29 cell line and the results were summarised in table 6 and figure 8.

Pure Camptothecin displayed very poor anticancer activity on HT-29 cell at 1000 $\mu\text{g/ml}$ (CTC_{50} : >1000 $\mu\text{g/ml}$). Camptothecin loaded polymeric nanoformulation displayed good anticancer activity on HT-29 cells at 1000 $\mu\text{g/ml}$ (CTC_{50} : 170.00 $\mu\text{g/ml}$). However, prepared Camptothecin loaded polymeric nanoformulations displayed enhanced anticancer activity against HT-29 cells in comparison with pure Camptothecin.

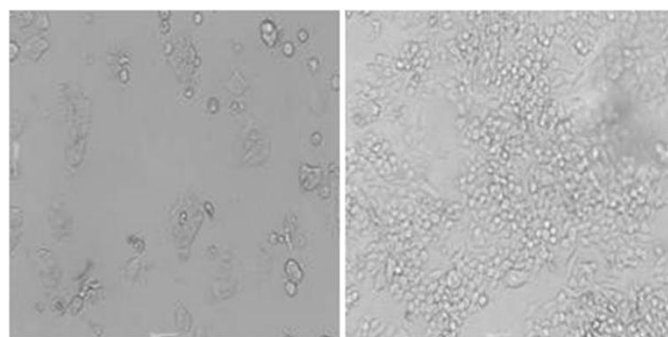


Fig. 8: *In vitro* anti cancer activity of pure Camptothecin (a) and Camptothecin loaded polymeric nanoparticles (b) on HT-29 cells.

Table 6: *In vitro* anticancer activity of prepared Camptothecin loaded polymeric Nanoparticles on HT-29 cells.

Samples	Percentage control growth					
	1000 ($\mu\text{g/ml}$)	500 ($\mu\text{g/ml}$)	250 ($\mu\text{g/ml}$)	125 ($\mu\text{g/ml}$)	62.5 ($\mu\text{g/ml}$)	CTC_{50} ($\mu\text{g/ml}$)
Pure Camptothecin	20.18 ± 0.24	16.21 ± 0.29	14.12 ± 0.25	12.24 ± 0.19	9.57 ± 0.15	>1000
Camptothecin loaded polymeric nano-formulation	95.62 ± 1.7	85.66 ± 0.7	57.77 ± 0.7	45.00 ± 0.68	44.9 5 ± 1.3	170.00 ± 0.7

The values are expressed as Mean \pm SD; n=3.

Stability studies

Prepared plain and Camptothecin loaded polymeric nanoformulation were subjected to stability studies as per the mentioned above. At the regular intervals the stored samples were evaluated for average particle size, particle size uniformity, surface area, zeta potential, drug content and drug release. The results were summarized in table 7 - 10.

Prepared plain and Camptothecin loaded polymeric nanoparticles showed insignificant change in average particle size, particle size uniformity, surface area, zeta potential, drug content and drug release after stability storage at both long term and accelerated conditions.

Table 7: Average particle size, particle size uniformity, surface area, zeta potential and drug content estimation of prepared polymeric nanoparticles subjected to long term stability study as per ICH guidelines (25°C ± 2°C/60% RH ± 5% RH)

Trial	Period (Months)	Average particle size (nm)	Particle Size Uniformity	Surface Area (m ² g ⁻¹ ± SD)	Zeta Potential (mV)	Drug Content (%)
Plain	0	97 ± 0.18	0.268 ± 0.11	74.20 ± 0.19	- 28.5 ± 1.25	-
	3 rd	98 ± 0.61	0.270 ± 0.71	74.80 ± 0.63	- 28.1 ± 0.78	-
	6 th	99 ± 0.93	0.279 ± 0.58	75.06 ± 0.93	- 27.8 ± 0.43	-
CPT	0	100 ± 0.25	0.242 ± 0.04	65.2 ± 0.39	- 22.4 ± 1.36	97.86 ± 0.98
	3 rd	101 ± 0.59	0.245 ± 0.55	64.9 ± 0.55	- 21.9 ± 1.23	97.15 ± 1.24
	6 th	101 ± 0.61	0.249 ± 0.73	64.1 ± 0.43	- 21.4 ± 1.24	96.89 ± 1.19

The values are expressed as Mean ± SD; n=3;

Table 8: Average particle size, particle size uniformity, surface area, zeta potential and drug content estimation of prepared polymeric nanoparticles subjected to accelerated stability study as per ICH guidelines(40°C ± 2°C/75% RH ± 5% RH).

Trial	Period (Months)	Average particle size (nm)	Particle Size Uniformity	Surface Area (m ² g ⁻¹ ± SD)	Zeta Potential (mV)	Drug Content (%)
Plain	0	97 ± 0.18	0.268 ± 0.11	74.2 ± 0.19	- 28.5 ± 1.25	-
	3 rd	97 ± 0.25	0.271 ± 0.15	75.12 ± 0.26	- 27.1 ± 0.84	-
	6 th	99 ± 0.61	0.284 ± 0.19	75.02 ± 0.35	- 27.2 ± 0.57	-
CPT	0	100 ± 0.25	0.242 ± 0.04	65.2 ± 0.39	- 22.4 ± 1.36	97.86 ± 1.67
	3 rd	100 ± 0.35	0.246 ± 0.41	64.1 ± 0.26	- 22.1 ± 1.20	97.35 ± 1.42
	6 th	101 ± 0.64	0.251 ± 0.24	63.9 ± 0.12	- 21.2 ± 0.98	96.80 ± 1.26

The values are expressed as Mean ± SD; n=3;

Table 9: *In vitro* release profile of Camptothecin from the prepared polymeric nanoparticles subjected to long term stability study as per ICH guidelines (25°C ± 2°C/60% RH ± 5% RH).

Periods	0 Months	3 Months	6 Months	9 Months	12 Months
0 hour	0	0	0	0	0
1 hour	0	0	0	0	0
2 hour	0	0	0	0	0
4 hour	1.28 ± 1.40	1.25 ± 0.34	1.22 ± 0.49	1.19 ± 0.67	1.24 ± 0.37
6 hour	28.14 ± 1.12	27.89 ± 1.31	28.01 ± 0.78	27.69 ± 1.26	27.51 ± 1.37
8 hour	59.55 ± 1.09	59.10 ± 1.86	58.94 ± 1.67	58.54 ± 1.43	57.25 ± 1.29
10 hour	73.25 ± 1.26	72.84 ± 1.67	72.12 ± 1.59	71.96 ± 1.46	71.32 ± 1.47
12 hour	85.14 ± 2.14	85.01 ± 1.83	84.78 ± 1.37	84.23 ± 1.51	83.87 ± 1.79
24 hour	98.22 ± 1.71	98.01 ± 2.45	97.78 ± 2.19	97.20 ± 2.37	97.06 ± 2.57

The values are expressed as Mean ± SD; n=3;

Table 10: *In vitro* release profile of Camptothecin from the prepared polymeric nanoparticles subjected to accelerated stability study as per ICH guidelines(40°C ± 2°C/75% RH ± 5% RH).

Periods	0 Months	3 Months	6 Months
0 hour	0	0	0
1 hour	0	0	0
2 hour	0	0	0
4 hour	1.28 ± 1.40	1.23 ± 0.54	1.29 ± 0.65
6 hour	28.14 ± 1.12	27.74 ± 1.12	28.12 ± 0.64
8 hour	59.55 ± 1.09	59.54 ± 1.72	58.82 ± 1.42
10 hour	73.25 ± 1.26	72.72 ± 1.94	72.02 ± 1.26
12 hour	85.14 ± 2.14	85.06 ± 1.74	84.81 ± 1.23
24 hour	98.22 ± 1.71	98.21 ± 2.62	97.54 ± 2.45

The values are expressed as Mean ± SD; n=3;

CONCLUSION

A novel pH sensitive polymeric nanoparticle of a hydrophobic drug Camptothecin was prepared by nanoprecipitation method with stirring technique. Prepared plain and Camptothecin loaded polymeric nanoparticles were characterized for average particle size, particle size uniformity, surface area and zeta potential. Nanoparticles prepared using stirring method was with average particles of < 100 nm, uniformity of < 0.3 and zeta potential of > 20 mV. Prepared Camptothecin loaded polymeric nanoparticles were spherical in shape. Hence, Camptothecin encapsulated in the polymer matrix will also be in spherical shape and expected to enhance the basic function, release of Camptothecin from the polymer matrix,

transport of Camptothecin in the body and internalization of Camptothecin by many folds than the free Camptothecin. Stirring approach displayed excellent encapsulation efficiency and drug loading and only an insignificant amount of Camptothecin were seen as a free drug. Hence, prepared Camptothecin loaded polymeric nanoparticles is expected to display superior pharmacological activities. It was observed that Poly (Methacrylic acid-co-methyl-methacrylate) coated Camptothecin nanoparticles gave no release in the simulated gastric fluid, negligible release in the simulated intestinal fluid and maximum release in the colonic environment. It is concluded that the formulation gave a good fit to the Hixson Crowell cube root law. Prepared Camptothecin loaded polymeric nanoparticles were studied for its *in vitro* anticancer efficacy against human colon cancer cells using MTT assay.

Prepared Camptothecin loaded polymeric nanoformulations displayed enhanced anti cancer activity against HT-29 cells in comparison with pure Camptothecin. Thus, the results indicate the potential for *in vivo* studies of the developed pH sensitive polymeric nanoparticles of a hydrophobic drug Camptothecin to establish their clinical application. The prepared plain and dual drug loaded nanoformulation were subjected to stability studies at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$ for 112 months and $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \text{ RH} \pm 5\% \text{ RH}$ for 6 months. At the end of the storage period, it showed insignificant changes in average particle size, particle size uniformity, surface area, zeta potential, drug content and drug release. In summary, the investigation concluded that the prepared Camptothecin encapsulated polymeric nanoformulations may be considered as an attractive and promising formulation which significantly overcome the limitations of Camptothecin in the treatment of colon cancer and synergistically enhance its anticancer activity.

ACKNOWLEDGEMENT

The authors are thankful to UGC, Government of India, for providing UGC-BSR Fellowship.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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How to cite this article:

Manikandan Mahalingam, Kannan Krishnamoorthy. Fabrication, Physicochemical Characterization and Evaluation of *In vitro* Anticancer Efficacy of a Novel pH Sensitive Polymeric Nanoparticles for Efficient Delivery of Hydrophobic Drug against Colon Cancer. *J App Pharm Sci*, 2015; 5 (11): 135-145.