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FORMULATION AND CHARACTERIZATION OF TEMOZOLOMIDE LOADED CHITOSAN NANOPARTICLES

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ABSTRACT

The aim of current study was to encapsulate Temozolomide into chitosan-based nanoparticles using a modified ionic gelation method. Different ratios of Temozolomide to chitosan were prepared. The highest encapsulation efficiency of Temozolomide into chitosan nanoparticles was found to be about 46-53 %. The prepared nanoparticles were characterised for particle size analysis and zeta potential. The values of zeta potential of TMZ loaded nanoparticles ranged from -21.7 to 30.1 were sufficient to keep the particles stable. polydispersity indices PDI of all the nanoparticles was in the range of 0.187 to 0.229 indicating all the formulation were homogenous in distribution. In vitro drug release studies showed initially rapid drug release was observed due to surface attached drug that varies from 28 to 37 % and later on the drug release was consistent in all the formulations, The drug release was studies for 48 hours and the release was found to be around 90% from all four formulations. The in vitro release study revealed that the release of Temozolomide could be better sustained from chitosan nanoparticles. Drug and excipient compatibility was established by DSC nd FTIR study and showed no drug polymer interaction. Surface morphology of nanoparticles were characterized by SEM study the prepared nanoparticles are separate and free flowing and spherical in nature without any aggregates.

KEY WORDS

Chitosan, ionic gelation method, nanoparticles, Temozolomide, DSC, FTIR, SEM

INTRODUCTION

Today, nanotechnology is applied in a wide range of applications in the pharmaceutical industry [1]. Tremendous advances in nanotechnology, made it possible to produce drug nanoparticles that can be utilized in a variety of innovative ways [2]. Polymeric nanoparticles have been widely investigated recently, as a carrier for drug delivery system [3]. Nanoparticles do have a special role in targeted drug delivery system. Nanoparticles have a long shelf life and have the ability to entrap more drugs. Some investigators have observed that the number of nanoparticles can cross the intestinal epithelium greater than that of the microspheres [4].

Polymeric nanoparticles formulated with biodegradable and biocompatible polymers are found to be good candidates for drug carrier to deliver drugs. As they are expected to be adsorbed in intact form in the gastrointestinal tract after oral administration [5]. They offer several advantages like sustained release of drug over longer period of time, retention of dosage form in entire length of gastrointestinal tract, increased bioavailability, site

specific drug delivery and better patient compliance due to reduction in dosing frequency [6]. The hydrophilic nanoparticles have gained considerable recognition to deliver therapeutic peptide, protein, antigen, oligonucleotide, and genes by oral, intravenous, and mucosal route[7]. This information emphasized the importance of size and revealed the superior properties of nanoparticles over the microspheres[8]. It has also been reported that the number of nanoparticles can cross the epithelium at a greater rate than the number of microspheres.

Chitosan is a naturally occurring biopolymer and consists of 1, 4 -linked glucosamine units. It is a biodegradable, biocompatible and bio adhesive polysaccharide. Chitosan is found to be non-toxic and soft tissue compatible in a range of toxicity tests [9]. It is produced by deacetylation of chitin which is extracted from shells of crabs, krills and shrimps [10, 11]. Chitosan has been widely used in the development of controlled drug release systems including chitosan film, chitosan sponge [12-15], chitosan nanoparticle, etc. [16]. Chitosan microbead (microsphere) [17], chitosan bead [18]. Nanoparticles using a tripolyphosphate (TPP) cross-linking method, as protein carrier were first developed by Calvo et al. [19]. This formulation was evaluated as a carrier for therapeutic peptides such as insulin, proteins and vaccine [20-22]. It has wide applications in pharmaceutical research as a carrier for drug delivery and as biomedical material [23].

Chitosan nanoparticles were developed due its mucoadhesivity and ability to enhance the penetration of larger molecules across mucosal surface [24]. Chitosan nanoparticles can be formulated by the Ionotropic gelation method based on the interaction between the negative groups of sodium tripolyphosphate (TPP) and the positively charged amino groups of chitosan. This process has also been reported to prepare Chitosan nanoparticles for the delivery of proteins and peptides including cyclosporine[25] and insulin [26].

Temozolomide is an oral alkylating agent used to treat refractory anaplastic astrocytoma in adult patients whose disease has progressed after therapy with nitrosourea and procarbazine, as well as concomitantly with radiation therapy for treatment of newly diagnosed glioblastoma multiforme. Temozolomide is not active until it is converted to the active form, 5(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) at physiologic pH. It is a prodrug that has little to no pharmacological activity. Absorption is rapid in the gastrointestinal tract. It is suggested that MTIC alkylates DNA at the N7 position of guanine (most common site), O3 position of adenosine, and O6 position of guanosine. methylation of guanine residues breaks single and double-strand DNA and leads subsequent apoptotic cell death. The N7-methylguanine plays a critical role in the antitumor activity of the drug. There is a correlation between the temozolomide to sensitivity of tumor cell lines and the activity of O6-alkylguanine alkyltransferase (DNA repair protein) that specifically removes alkyl groups at the O6 position of guanine [27-31].

MATERIALS AND METHOD

Materials

Temozolamide was obtained as a gift sample from Dr Reddys lab, hyd. Sodium TPP was obtained from Himedia Laboratories Pvt Ltd. Chitosan was obtained from Sd Fine Chem. Limited, Mumbai. All other chemicals and reagents used in the study were of analytical grade.

Methods

Preparation of Temozolomide drug Loaded Chitosan Nanoparticles

Preparation of blank Chitosan Nanoparticles

Blank chitosan nanoparticles were prepared by ionic gelation method. Different concentrations of

polymer, ranging from 0.10 to 0.75 %w/v, were dissolved in 1.5 % V/V acetic acid solution.

Sodium tripolyphosphate solution was also prepared in distilled water in concentrations ranging from 0.10 to 0.75% w/v. Sodium tripolyphosphate solution was added dropwise with a syringe to chitosan solution while stirring, followed by sonication for 10 min. The resulting suspension was subsequently centrifuged at 15000 rpm for 10 min. The pellets obtained were re-suspended in deionized water by sonication, centrifuged and dried at room temperature (about 25 ^{0}C).

Optimized blank chitosan nanoparticles were prepared by ionotropic gelation method. 20 ml of 0.35 % w/v of chitosan solution is taken into the beaker under the magnetic stirring and add 12 ml of 0.4 % w/v of sodium TPP drop wise to the chitosan solution under the magnetic stirring until uniform suspension is formed. After completion of magnetic stirring the resultant suspension is sonicated for 10 min by using probe sonicator (Frontaline).

The resultant suspension is centrifuged at15000 rpm for 15 min and the segregated nanoparticles are separated from the supernatant liquid by decanting. The resultant nanoparticles are collected in a petri dish and dried at room temperature.

Preparation of Chitosan Temozolomide drug solutions.

Known amount ofTemozolomide drug is dissolved in 20 ml 0.35 % w/v of chitosan solution.Temozolomide drug was weighed in the concentrations ranging from 0.2 to 0.5 % w/v were prepared using above chitosan solution. The solutions were kept under constant stirring for the complete solubility of drug in the chitosan solution. The solutions were kept a side until further use.

Chitosan Timozolamide nanoparticles are prepared by Ionotropic gelation method. TMZ nanoparticles are prepared with different concentration of Drug i.e TMZ is dissolved in 0.35% w/v of chitosan solution. Timozolamide drug loaded chitosan nanoparticles were formed by spontaneously upon drop wise addition of 12 ml of 0.4% w/v aqueous sodium TPP solution to 20 ml of 0.35% w/v chitosan solution containing TMZ drug under magnetic stirring until the uniform suspension is formed. After completion of magnetic stirring the resultant suspension is sonicated for 10 min using probe sonicator (Frontline).

The resulting nanoparticle suspension is centrifuged at 15000 rpm for 15min, and the segregated nanoparticles are separated from the supernatant liquid by decanting. The resultant nanoparticles are collected in a Petri dish and dried at room temperature. The prepared nanoparticles were analysed for various physicochemical properties, such as Entrapment efficiency, Particle size distribution, DSC, FTIR and SEM studies.

Table 1: Formulation of TMZ- chitosan nanoparticles by Ion tropic gelation method.

Formulation	TMZ (mg)	TPP (0.4%) (ml)	Chitosan (0.35%) (ml)
TMZCN1	20	12	20
TMZCN2	30	12	20
TMZCN3	40	12	20
TMZCN4	50	12	20

Characterization of nanoparticles.

Determination of encapsulation efficiency

The encapsulation efficiency of nanoparticles was determined by first separating the nanoparticles formed from the aqueous medium by ultracentrifugation at 15000 rpm for 30 min. The amount of free TMZ in the supernatant was measured by UV spectrophotometery at 329 nm using UV Spectrophotometer.

***In vitro* Drug release Studies**

The in vitro diffusion studies were performed for the prepared Nanoparticles. Twenty-five milligrams of freeze-dried nanoparticle powder were dispersed in 45mL of 0.1N HCl solution. This suspension was incubated at 37°C. Two millilitres of dispersion was withdrawn from the system at appropriate time interval and centrifuged (14,600 g for 5 min). The drug concentration in supernatant was determined by using UV spectrophotometer at 329 nm.

Determination of zeta potential

The zeta potential of the drug-loaded chitosan nanoparticles was measured on a zeta sizer (Malvern Instruments) by determining the electrophoretic mobility in a microelectrophoresis flow cell. All the samples were measured in water at 25 °C in triplicate.

Measurement of mean particle size

The mean size of the nanoparticles was determined by photo correlation spectroscopy (PCS) on a submicron particle size analyser (Malvern Instruments) at a scattering angle of 90°. A sample (0.5mg) of the nanoparticles suspended in 5 ml of distilled water was used for the measurement.

Fourier transforms infrared spectroscopy:

The FT-IR spectrum of pure drug and formulation were determined A FTIR (Thermo nicolet 670 spectrometer) was used for the analysis in the frequency range between 4000 and 400cm^{-1}, and 4cm-1 resolution. The reagents were the means of 6 determinations. A quality equivalent to 2mg of pure drug was used for the study.

Differential scanning calorimetry:

Thermal properties of pure drug and the formulation were evaluated by Differential scanning calorimetry (DSC) using a diamond (DSC) (Mettler star sw8.10). The analysis was performed at a rate 50 C min^{-1} to 200^{0}C temperature range under nitrogen flow of 25ml min^{-1}.

Results and discussion

Timozolamide nano particles were prepared by chitosan alginate. Prepared nanoparticles were evaluated for invitro characteristics. Results regarding particle size, zeta-potential, polydispersity index and percent entrapment of TMZ in nanoparticles are presented in Table1. The mean particle size of prepared nanoparticles ranges from 163-317 nm.

It has been found that particle size and polydispersity indices (PDI) increased with raise in polymer concentration, PDI of all the nanoparticles was in the range of 0.187 to 0.229 indicating all the formulation were homogenous in distribution. Zeta potential is a main factor for evaluation of the stability of nano particles. It was currently stated that zeta potentials over 30 mV were being necessary for full electrostatic stabilization.Temozolomide nanoparticles, which are slightly negatively charged. The values of zeta potential of TMZ loaded nanoparticles ranged from -21.7 to 30.1 were sufficient to keep the particles stable.

The encapsulation efficiency of nanoparticles was determined by first separating the nanoparticles formed from the aqueous medium by ultracentrifugation at 15000 rpm for 30 min. The amount of free TMZ in the supernatant was measured by UV spectrophotometry at 329 nm using UV Spectrophotometer.

The entrapment efficiency of prepared nanoparticles was found in the range of 46 to 53 %. The entrapment efficiency was increased with increased drug content in the nanoparticles.

Table 2: Particle size, Zetapotential, Polydispersity index and % drug entrapment efficiency ofTemozolomide nanoparticles.

Formulation code	Particle size (nm)	Polydispersity index	Zeta potential	% Entrapment Efficiency
TMZCN1	163 ±1.17	0.187 ±0.02	-21.7 ±0.4	46.15 ± 2.15
TMZCN2	217 ±1.12	0.192 ±0.03	-25.4 ±0.6	49.55 ± 2.69
TMZCN3	246 ±1.28	0.207 ±0.04	-27.4 ±0.7	52.15 ± 1.25
TMZCN3	317 ±1.25	0.219 ±0.02	-30.1 ±0.6	53.13 ± 0.75

The *in vitro* diffusion studies were performed for the prepared Nanoparticles. Twenty-five milligrams of freeze-dried nanoparticle powder were dispersed in 45mL of 0.1N HCl solution. This suspension was incubated at 37°C. Two millilitres of dispersion was withdrawn from the system at appropriate time interval and centrifuged (14,600 g for 5 min). The drug concentration in supernatant was determined by using UV spectrophotometer at 329 nm.

The cumulative percent drug release vs time was calculated, and a graph was plotted against cumulative percent drug diffused vs time. Initially rapid drug release was observed due to surface attached drug that varies from 28 to 37 % and later on the drug release was consistent in all the formulations, The drug release was studies for 48 hours and the release was found to be around 90% from all four formulations. There is no much variation in drug release at later stage because of similar polymer content present in the formulations.

To determine the process of drug, release the experimental data had been fitted into various kinetic equations. The R2 values range between 0.9189–0.9476 (Zero order), 0.9901–0.9944 (First order), 0.9294–0.9625(Higuchi), 0.9155–0.9681 (Peppas). Drug release from all these formulations could be best stated by first order release. From Higuchi model, it is evident that the drug is released by diffusion process. For confirmation the data was fitted into Korsmeyer–Peppas formula. The slope (n) values for the peppas model ranged from 0.3921 to 0.5034 for formulation followed fickian diffusion. This indicates the release may be due to diffusion mechanism.

Table 3: Release kinetic models ofTemozolomide nano particles

Formulation code	Zero order	First order	Higuchi model	Korsemeyer Peppas model	
	R^2	R^2	R^2	R^2	n
TMZNP1	0.9189	0.9924	0.9625	0.9681	0.5034
TMZNP2	0.9241	0.9236	0.9539	0.9486	0.4561
TMZNP3	0.9408	0.9944	0.9395	0.9235	0.4164
TMZNP4	0.9476	0.9901	0.9294	0.9155	0.3920

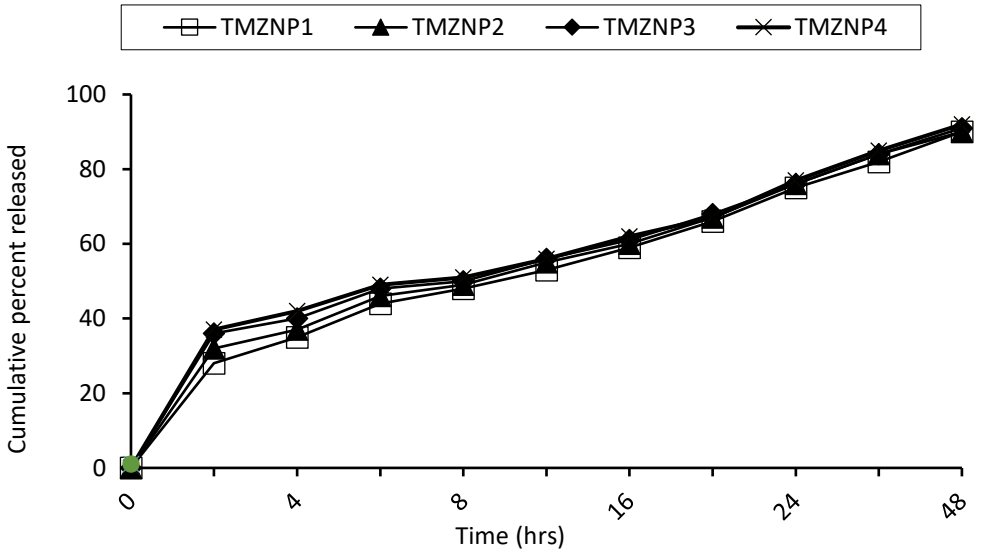

Figure 1: Cumulative % drug release of the Timozolamide nanoparticles

The DSC study of the pure drug, excipients and the physical mixture of drug and excipients did not reveal any incompatibility between the drug and the excipients as shown in Figure. The exothermic peak of TMZ was well observed in pure drug at 215 ^{0}C. The peak shape and enthalpy changes in the nanoparticles was due to the presence of impurities in the sample and it was observed at 205 ^{0}C. Hence, these minor changes in the drug exotherm might be attributed to the blend of drug and excipients which lowers the purity of each component in the mixture and doesn't necessarily indicate incompatibility.

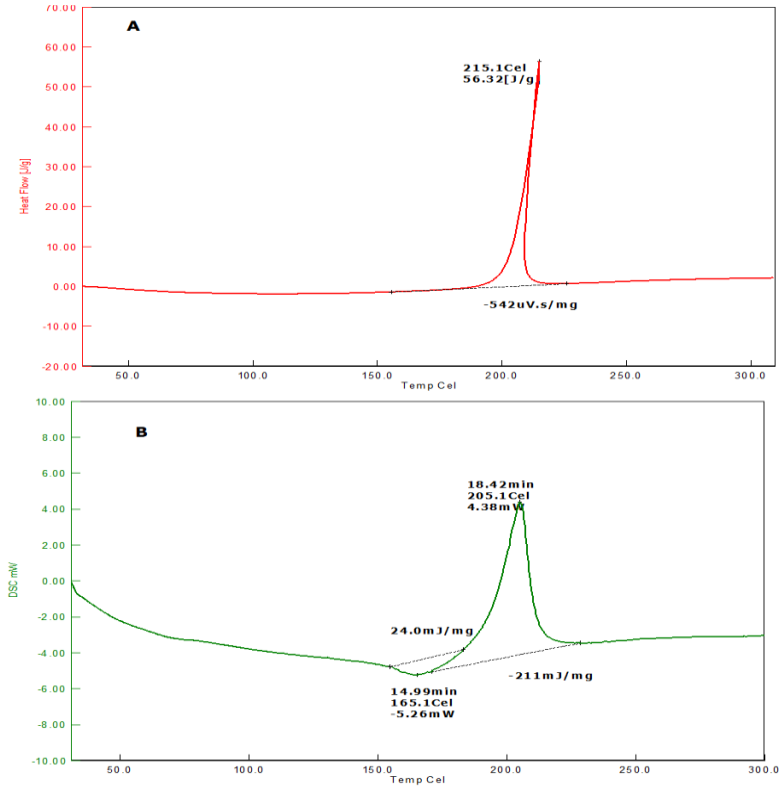

Figure 2: DSC thermograms of (A) pure timozolamide (B) timozolamide nanoparticles.

FTIR study was conducted on pure TMZ and Timozolamide- chitosan nanoparticles. The spectrum shows the characteristic vibrational modes of the drug molecule. The bands at 1760, 1735 and 1680 cm^{-1} are attributed to the carbonyl groups (C=O) stretching. The band at 1600 cm^{-1} is attributed to ν(N-H) deformation. The bands at 1450 cm^{-1} are attributed to C–N stretching and the C–C stretching vibration band is observed at 1357 cm^{-1}. The bands at 3387, 3287 and 3228 cm^{-1} are attributed to (N-H) stretching. Similar bands were observed in the prepared nanoparticles showed no drug polymer interaction.

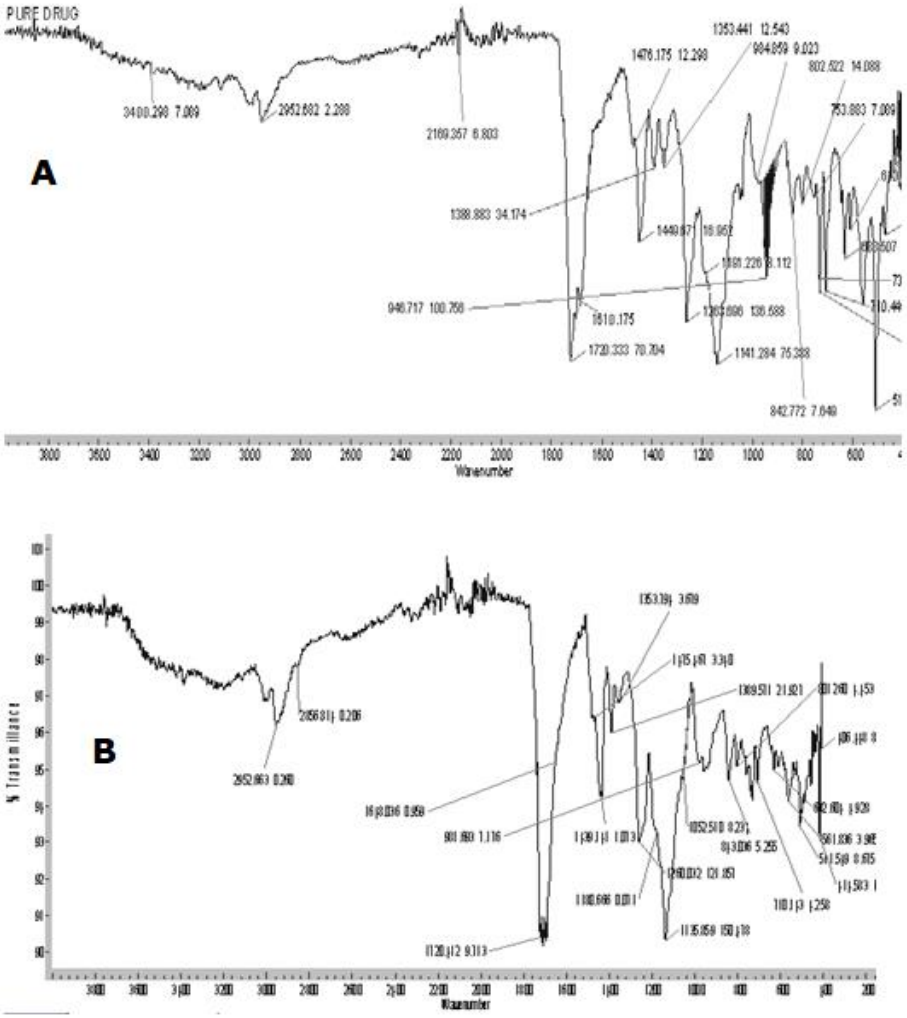

Figure 3: FTIR spectra of (A) pure timozolamide (B) timozolamide nanoparticles.

Scanning electron microscopic study of the prepared nanoparticles was performed. The prepared nanoparticles are separate and free flowing and spherical in nature without any aggregates. The following fig showed the sem images of the prepared TMZ nanoparticles with Chitosan.

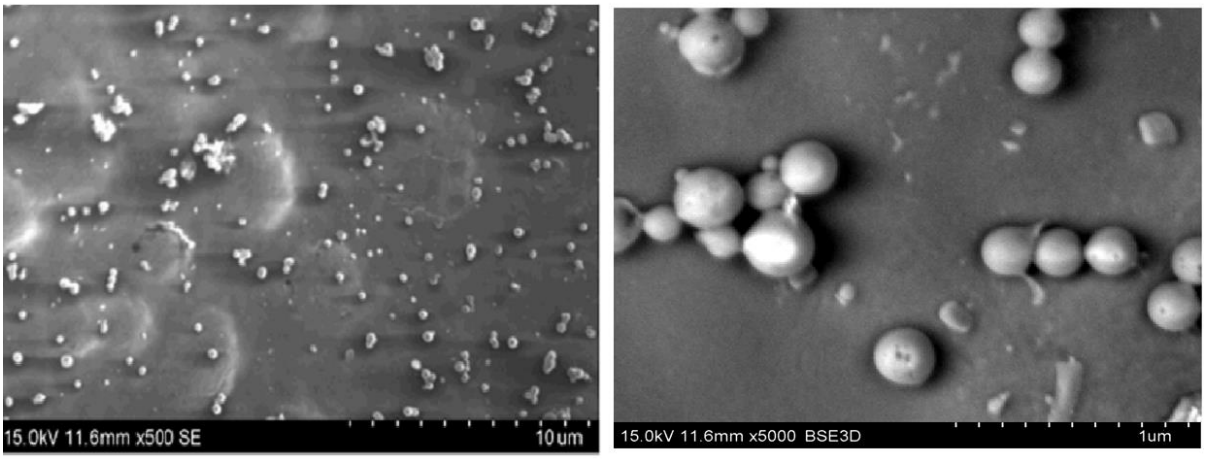

Figure 4: SEM image of timozolamide nanoparticles.

CONCLUSION

In this present study, chitosan nanoparticles loaded withTemozolomide were prepared based on ionotropic gelation method employing TPP as the crosslinker to investigate the physicochemical properties of nanoparticles. The prepared formulations showed a negative zeta potential value. The average particle size was found to be in nano range. This clearly further confirms the lower particle size of the nanoparticles. The in vitro release study revealed that the release ofTemozolomide could be better sustained from chitosan nanoparticles. The FTIR and DSC study confirmed that there was no interaction between the drug and polymer.

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