ORIGINAL ARTICLE

Formulation and Characterization of Noscapine-loaded Polycaprolactone Nanoparticles

G. Ramesh, S. Sathesh Kumar

Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai, Tamil Nadu, India

Abstract

Objective: In this study, noscapine (Nos)-loaded polycaprolactone (PCL) nanoparticles (NPs) have been prepared for the treatment of glioblastoma multiforme (GBM). **Materials and Methods:** Nos-loaded PCL-NPs were prepared by double emulsion solvent evaporation method and characterized for particle size and its size distribution, zeta potential, surface morphology, drug entrapment efficiency, and drug release profile. *In vitro* cytotoxicity study was performed on U87MG cell lines. **Results:** Nos-loaded PCL-NPs were prepared for optimization of drug-polymer ratio, surfactant concentration, stirring time, and stirring revolutions per minute (RPM) and characterized for mean particles size range from 150 nm to 818 nm; the zeta potential values were in the range of −8.8 to −16.6 mV; the encapsulation efficiencies were between 38% and 79%; and the drug release for 96 h showed 85%. Scanning electron microscopy showed the smooth, spherical in shape. *In vitro* cytotoxicity showed reduced IC_{50} of Nos-loaded PCL-NPs when compared with pure drug. **Conclusion:** The Nos-loaded PCL-NPs were prepared successfully with target particle size around 200 nm for targeting the GBM with 31% reduced IC_{50} values.

Key words: Blood tumor, glioblastoma, noscapine, polycaprolactone nanoparticles

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive form of malignant primary brain tumor, <5% of patients will survive for 5 years after the diagnosis.[1] With the current best available treatment strategy of surgery, radiation and chemotherapy will increase the median survival rate to 15 months.[2] Recurrent primary brain tumor is common phenomenon due to resistant to standard chemotherapy including standard care drug temozolomide (TMZ) for GBM.[3] The blood–brain barrier (BBB) is a dynamic barrier protecting the brain against invading organisms and unwanted substances. It is also the most important barrier impeding drug transport into the brain through the blood circulation.[4] An important consideration for GBM therapy is the BBB, which prevents the drug to enter into the target site (brain) to achieve the therapeutic activity.[5]

Resistance to TMZ becomes major obstacle to GBM therapy. There is much need of new treatment for targeting TMZ-resistant primary brain tumors.[6]

For the treatment of GBM, drugs must penetrate this BBB. Using an *in vitro* BBB model, Landen *et al*.[7] demonstrated that noscapine (Nos) transverses this barrier. Thus, Nos is a potentially powerful agent for the treatment of central nervous system tumors with minimum cytotoxicity to normal cells.

Nos is a non-narcotic alkaloid form of opium, primarily used as a cough suppressant. Noscapine has been proposed as an anticancer agent due to its microtubule-destabilizing effects with minimal cytotoxicity to normal cells.[8] However, with short plasma half-life (0.39 h) through oral administration requires high concentration of Nos to effect antitumor

Address for correspondence:
Dr. S. Sathesh Kumar, Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai–600 041, India.
Mobile: +91-95514 12737. Fax: +91-44-2431-5542.
E-mail: Sathesh2000@gmail.com

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activity (ED50 = 300–600 mg/kg) with considerable first-pass metabolism activity. In addition, Nos has rapid renal clearance.[9]

Polymeric nanoparticles (NPs), a biodegradable controlled system, which can prolong the circulation time and increase the concentration of the drug at the target site with high drug-loading capacity, improved biological stability and long *in vivo* circulation.[10] Polymeric NPs enhance the bioavailability of therapeutic agents in brain tumors.[11] To prevent rapid renal filtration or elimination through reticuloendothelial system, minimum size of the NPs is also restricted. Based on the literature, size ranging between 50 nm and 300 nm and surface characteristics can take the advantage of EPR effect and at the same time minimize excretion or elimination. Stabilizer concentration as well as amount of polymer and drug could also affect size, entrapment efficacy% (EE %) of NPs.[8]

In this study, Nos-loaded polycaprolactone (PCL) NPs have been optimized using double emulsion solvent evaporation method for NP size, size distribution, zeta potential, entrapment efficiency, *in vitro* release profile, morphology, and *in vitro* cytotoxicity for crossing BBB.

MATERIALS

Nos hydrochloride was received as a gift sample from Biological E limited, Hyderabad. PCL was kindly provided as a gift sample from Evonik India limited. Polyvinyl alcohol (PVA), dialysis bag, was purchased from HiMedia. (L110). U87MG cell line procured from NCCS, Pune.

METHODS

Ultra Violet (UV)–Visible Spectroscopy

UV–visible spectroscopy is to determine the wavelength of maximum absorption of Nos hydrochloride by dissolving in water at a concentration of 20 µg/mL and scanned in UV–visible region from 200 to 800 nm. Calibration curves were generated using five concentrations (in triplicates) in the range of 0–100 µg/mL with 20 µg/mL increment in each concentration. The linearity was calculated using coefficient of determination (r^2) of the calibration curves.[12]

Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. Qualitative analysis of Nos hydrochloride was done using FTIR. The raw Nos powder, PCL polymer, and Nos with PCL polymers (mix) were mixed with KBr and compressed to form a pellet which was analyzed by FT-IR spectroscopy.[13]

Differential Scanning Calorimetry (DSC)

DSC measures the amount of energy absorbed or released by a sample when it is heated or cooled, providing quantitative and qualitative data on endothermic (heat absorption) and exothermic (heat evolution) processes. The powder of Nos hydrochloride with PCL polymer was subject to DSC with heating rate of 10°C/min from 25°C to 250°C; the heat absorbed or evolved was recorded as exotherm or endotherm.[14]

PREPARATION OF Nos HYDROCHLORIDE-LOADED PCL NPs

Method of Preparation: Double Emulsion Solvent Evaporation Method

Nos hydrochloride dissolved in water (2 mL) was homogenized with different concentrations of PCL polymer dissolved in dichloromethane (DCM) (10 mL) at 14,000 RPM using high-speed homogenizer (POLYTRON, Kinematica, Switzerland) for 15 min in an ice-water bath. The resulting primary emulsion was further size reduced to form NPs by adding it dropwise to the 50 mL of phosphate buffer (pH 7.4) containing different concentrations of surfactant PVA, using high-speed homogenizer at 14,000 RPM followed by probe sonication at 80% amplitude for 8 min. During this procedure, the sample is placed in an ice bath. Complete evaporation of DCM from the emulsion (w/o/w) was affected by stirring up to 4 h.

NPs separated by subjecting the suspension for centrifugation at 20,000 RPM (10,000 RCF) for 30 min.[15] After centrifugation, incubate the NPs into 1% Pluronic F68 for 30 min at room temperature for surface coating of NPs.[16]

The effect of various experimental parameters such as drug-to-polymer ratio (1:1, 1:3, 1:5), percentage of surfactant concentration (1%, 2%, 3% w/v), stirring speed (6,000, 10,000, 14,000, and 16,000 RPM), stirring time (60 min, 120 min, 180 min, and 240 min) on mean particle size, polydispersity index (PDI), and entrapment efficiency of Nos hydrochloride NPs were also studied as per the Tables 1 and 2.

Optimization of product parameters (drug-to-polymer ratio, surfactant concentration (%)).

Optimization of process parameters (stirring speed, stirring time).

Characterization of Nos-Loaded NPs

Particle size, PDI, and zeta potential

The Nos-loaded PCL-NPs were analyzed for its particle size, PDI, and zeta potential using Zetasizer (Nano ZS, Malvern

Table 1: Study of effect of various product and process parameters on the size of Nos loaded nanoparticles

Formulation code	Drug: Polymer ratio	Surfactant concentration (%)	Stirring speed (RPM)	Stirring time (Min)
CAX3	A (1:1)	X (1)	14000 (3)	120 (H2)
CAY3		Y (2)		
CAZ3		Z (3)		
CBX3	B (1:3)	X (1)		
CBY3		Y (2)		
CBZ3		Z (3)		
CcX3	c (1:5)	X (1)		
CcY3		Y (2)		
CcZ3		Z (3)		

C- PCL polymer, A- 1:1 Drug-polymer ratio, B- 1:3 Drug-polymer ratio, c- 1:5 Drug-polymer ratio, X – 1% w/v PVA, Y- 2% w/v PVA, Z – 3% PVA, 3 – denotes stirring speed 14000 RPM. H2-120 min. Nos: Noscapine, PCL: Polycaprolactone, NPs: Nanoparticle, RPM: Revolutions per minute

Table 2: Nos-loaded PCL NPs

Formulation code	Drug: Polymer Ratio	Surfactant concentration (%)	Stirring speed (RPM)	Stirring time (min)
CBY1	B (1:3)	Y (2)	1 (6000)	H2 (120)
CBY2	B (1:3)	Y (2)	2 (10000)	H2 (120)
CBY4	B (1:3)	Y (2)	4 (16000)	H2 (120)
CBY3H1	B (1:3)	Y (2)	3 (14000)	H1 (60)
CBY3H3	B (1:3)	Y (2)	3 (14000)	H3 (180)
CBY3H4	B (1:3)	Y (2)	3 (14000)	H4 (240)

C- PCL polymer, B- 1:3 Drug-polymer ratio, Y- 2% w/v PVA, 1 – 6000 RPM, 2 – 10000 RPM, 3 – 14,000 RPM, 4-16,000 RPM, H1 – 60 min, H2-120 min, H3-180 min, H4-240 min. Nos: Noscapine, NPs: Nanoparticle, RPM: Revolutions per minute, PCL: Polycaprolactone

Instruments, Malvern, UK). The particle size was measured using dynamic light scattering technique, which measures the diffusion of particles moving under Brownian motion. These measurements are converted to size and a size distribution by applying the Stokes–Einstein relationship. Simultaneously, PDI which is a measure of particle homogeneity in the dispersion was also measured. The zeta potential of a particle is the total charge acquired by it in a specific medium. This technique measures the electrophoretic mobility exhibited by a charged particle as it moves with a velocity, in a dispersion medium connected with a pair of electrodes. When a voltage is applied, this particle moves with a velocity toward the oppositely charged electrodes. Samples were diluted to 5 mL volume with Milli-Q water (Merck Millipore), and the analysis was carried out in triplicates.[17]

Encapsulation efficiency

The entrapment efficiency of NPs was determined by separating the NPs from the aqueous medium by centrifugation at 20,000 RPM (minimum 10,000 g) for 30 min. The amount of free Nos in the supernatant was measured by UV/visible spectrophotometry at 312 nm. Entrapment efficiency (%) was calculated using this formula: [18]

% Entrapment efficiency = (Total amount of drug−free drug in supernatant)/Total amount of drug×100

Scanning electron microscopy

Scanning electron microscopy is performed at high magnifications, generates high-resolution images, and precisely measures very small features and objects. A drop of the NPs formed was mounted on metal (aluminum) stubs, using double-sided adhesive carbon tape, and fractured with a razor blade, whereas unprocessed Nos powder was simply spread on double-sided tape. The samples were sputter coated with gold/palladium for 120 s at 14 mA under argon atmosphere for secondary electron emissive SEM (Hitachi-S 3400N) and observed for morphology at an acceleration voltage of 15.0 kV.[19]

In vitro drug release study

In vitro drug release from Nos-loaded PCL NPs was performed by dialysis tube method. The dispersion of Nos-loaded PCL NPs in 30 ml of phosphate buffer saline (pH 7.4) equivalent to 2 mg of Nos was placed in dialysis tube (cutoff 12,000 Da; HiMedia Laboratories Pvt. Ltd., India). The dialysis tube was immersed in the receptor compartment containing 100 ml of phosphate buffer pH 7.4. The receptor compartment solution was stirred at 100 RPM, and temperature was maintained at 37±2°C. 1 mL of samples were collected at regular intervals and replaced with an equal volume of fresh release medium. The samples withdrawn were centrifuged at 5000 RPM for 2 min; supernatant was passed through 0.45-µm membrane

filter; and the filtrate was analyzed by UV-visible spectroscopy at 312 nm. The amount of Nos released in each sample was estimated from a calibration curve. Results of *in vitro* drug release studies obtained are represented graphically as cumulative percentage drug release versus time.[20]

Statistical Analysis

The results are reported as mean ± standard deviation of triplicate measurements in terms of particle size, zeta potential, PDI, and entrapment efficiency of Nos-loaded PCL-NPs.

In vitro cell line study - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The human glioblastoma cell line (U87 MG) was plated separately using 96 well plates with the concentration of 1×10^4 cells/well in DMEM media with 1X Antibiotic, Antimycotic Solution and 10% fetal bovine serum (HiMedia, India) in CO_2 incubator at 37°C with 5% CO_2. The cells were washed with 200 µL of 1X PBS, then the cells were treated with various concentrations of Nos pure drug (20 µg to 100 µg with increment of 20 µg for each sample), Nos-loaded PCL NPs in serum-free media and incubated for 24 h, 48 h, and 96 h in CO_2 incubator. The medium was aspirated from cells at the end of the treatment period. 0.5 mg/mL MTT prepared in 1X PBS was added and incubated at 37°C for 4 h using CO_2 incubator. After incubation period, the medium containing MTT was discarded from the cells and washed using 200 µL of PBS. The formed crystals were dissolved with 100 µL of DMSO and thoroughly mixed. The development of color intensity was evaluated at 570 nm. The formazan dye turns to purple-blue color. The absorbance was measured at 570 nm using microplate reader.[21]

Table 3: Linearity of Nos hydrochloride using UV–visible spectroscopy

Sample ID	Concentration (µg/mL)	Absorbance at wavelength 312 nm
Blank	0.0	0.000
STD 1	20.0	0.139
STD 2	40.0	0.277
STD 3	60.0	0.414
STD 4	80.0	0.543
STD 5	100.0	0.670

Nos: Noscapine

RESULTS

UV–Visible Spectroscopy: Analytical Method for Determination of Nos Hydrochloride

Analytical method development of Nos, absorption scans were performed in UV/visible region. Nos stock solution was diluted (1:10) and scanned in UV/visible region to get its absorption peaks; wavelength of maximum absorption (λ max) was found to be at 312 nm (Absorbance: 0.647).

Linearity

Calibration curves were generated using five concentrations (in triplicates) in the range of 20–100 µg/ml. The linearity was calculated using the coefficient of determination (R^2) of the calibration curves. The good linearity with correlation coefficient of >0.999 was obtained, data presented in Table 3 and Figure 1.

FTIR Spectroscopy

FTIR spectroscopy [Figure 2a] of Nos shows the sharp characteristics peak at 1766 cm^{-1}, 1108 cm^{-1}, 1616 cm^{-1}, 2940 cm^{-1}, 1396 cm^{-1} of C=O ester, C-O, C=C (aromatic), C-H (Alkane), C-N (amine) function groups, respectively. FTIR spectroscopy of PCL polymers [Figure 2b] shows sharp characteristic peak at 1767 cm^{-1} of C=O ester functional group. The prominent peaks representing Nos and PCL appear in the spectra of Nos-loaded PCL NPs and did not show any significant shifting in the position of the absorption speak [Figure 2c].

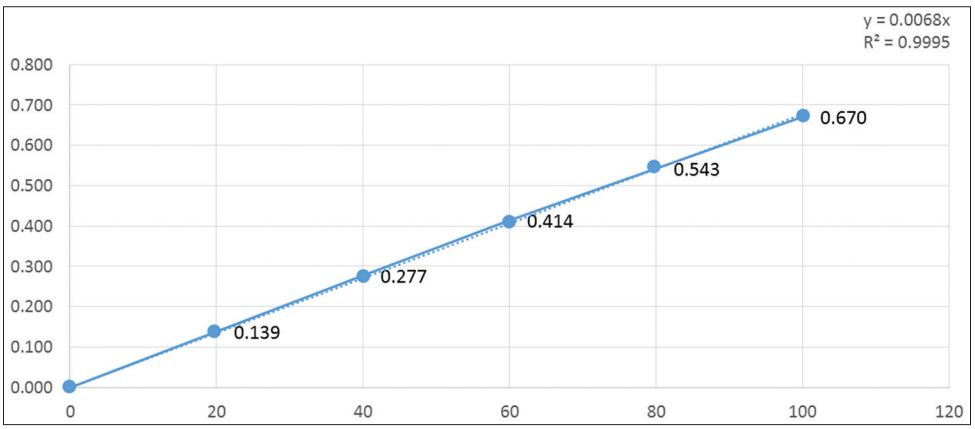

Figure 1: Calibration curve of noscapine hydrochloride

DSC

The thermogram of Nos-loaded NPs [Figure 3] exhibited endotherm at 64°C, which corresponds to melting endotherm of PCL polymer. This thermogram also showed a broad endotherm between 197°C and 207°C corresponding to the melting endotherm of Nos. This is may be due to the conversion of drug from crystalline to amorphous form. Therefore, it may be concluded that the Nos in PCL-NPs existed in the amorphous form of a molecular dispersion or solid solution state in the polymer matrix.

Preparation of Nos Hydrochloride-Loaded PCL NPs

Method of Preparation: Double emulsion solvent evaporation method: (Optimization of product Parameters (drug-to-polymer ratio, surfactant concentration (%)).

Effect of polymer concentration and surfactant concentration on particle size, PDI, zeta potential, EE (%)

The particle size increases from 148 nm to 652 nm, as increase in concentration of polymer from 1:1 to 1:5 in the all three different surfactant concentrations (1%, 2%, and 3%). Decrease in PDI ranges from 0.838 to 0.384 as surfactant concentration increases in all three different polymer concentrations shows good size distribution with increase in surfactant concentration.

Increase in particle size may be due to the increase in viscosity leading to increased emulsion droplet ultimately leading to an increase in particle size of the NPs. High concentration of the polymer results in diminished shearing efficiency that may also be explained for increased size. The higher concentrations of surfactant facilitated the lowering of particle size, whereas the lower concentrations of surfactant considerably increased the particle size.

Zeta potential ranges from −10.5 mV to −15.8 mV for polymer concentration of 1:1, 1:3, and 1:5 shows better stability for polymer concentration of 1:3 than 1:1 and 1:5. Entrapment efficiency increases from 38% to 72.5% as polymer concentration increases from 1: 1 to 1:3 and decreases from 1:3 to 1:5 ranges from 72.5% to 52.8% due to increased particle size and leaching during emulsification. The data of particle size, PDI, zeta potential, and entrapment efficiency shown in Table 4.

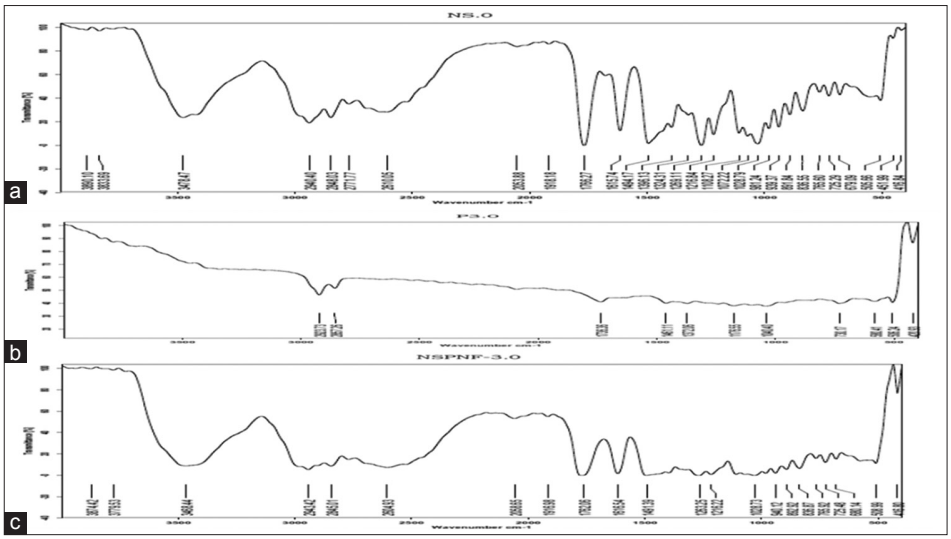

Figure 2: (a-c) Fourier transform infrared spectrum of noscapine, polycaprolactone (PCL), and noscapine-PCL nanoparticles

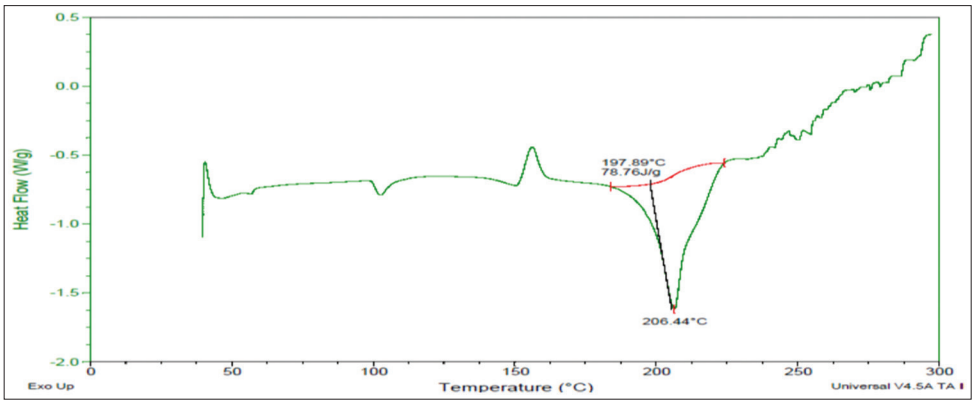

Figure 3: Differential scanning calorimetry curves of noscapine-loaded polycaprolactone nanoparticles

Optimized drug-polymer ratio (1:3) and surfactant concentration (2%) are selected for further optimization of process parameters (stirring speed and stirring time).

Effect of stirring speed on particle size, PDI, zeta potential, EE (%)

The particle size decreases from 815 nm to 280 nm, as increase in stirring speed from 6000 RPM to 16000 RPM, PDI ranges from 0.523 to 0.68, zeta potential ranges from −9.6 mV to −16.8 mV, entrapment efficiency increases from 55.4% to 78.9% as increase in stirring speed from 6000 RPM to 16000 RPM.

Optimized formulation for drug-to-polymer ratio (1:3), surfactant concentration (2%), and stirring speed (14000 RPM) selected for further optimization of stirring time shows better particle size, PDI, zeta potential, and entrapment efficiency.

Effect of stirring time on particle size, PDI, zeta potential, EE (%)

The particle size decreases from 390 nm to 282 nm, as increase in stirring time from 60 min to 240 min, PDI increases from 0.48 to 0.72, zeta potential ranges from −8.8 mV to −16.8 mV, entrapment efficiency increases from 44.2% to 64.5% as increase in stirring time from 60 min to 240 min.

Optimized formulation code (CBY3) for drug-to-polymer ratio (1:3), surfactant concentration (2%), stirring speed (14000 RPM), and stirring time (120 min) is selected for further characterization studies. Data shown in Table 5 and Figures in 4-6.

The Figures 4-6 shows the particle size, PDI, zeta potential, and entrapment efficiency of Nos-loaded PCL NPs.

Scanning Electron Microscopy

The scanning electron microscopy images of the NPs are shown in Figure 7. The images depicted that the NPs were roughly spherical in shape. Images also display the smooth surface without any perceptible pinholes or cracks.

In vitro Drug Release Study

The drug release from the Nos-loaded PCL NPs is presented in Figure 8.

The percentage of cumulative release of Nos-loaded PCL NPs was plotted against time in Figure 8. *In vitro* release profile showed biphasic behavior consisting of initial burst release followed by a sustained release phase over a period of 96 h. The initial burst release of drug may be due to the release of

Table 4: Effect of polymer concentration and surfactant concentration on particle size, PDI, zeta potential, and entrapment efficiency of Nos-loaded PCL-NPs

Formulation code	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)
CAX3	194±12	0.838±0.012	−11.6±1.3	37.8±9.8
CAY3	163±14	0.590±0.010	−12.7±1.6	45.6±7.2
CAZ3	148±13	0.536±0.011	−10.5±1.2	48.4±10.5
CBX3	258±13	0.585±0.006	−14.3±1.1	66.7±7.8
CBY3	252±18	0.447±0.012	−15.8±0.9	68.9±6.6
CBZ3	233±12	0.417±0.013	−13.8±1.6	72.5±9.9
CcX3	652±41	0.501±0.011	−12.7±2.1	45.6±10.8
CCY3	590±20	0.600±0.011	−13.6±2.8	48.9±11.2
CCZ3	370±19	0.384±0.010	−11.8±1.8	52.8±12.2

Nos: Noscapine, PCL: Polycaprolactone, NPs: Nanoparticle

Table 5: Effect of stirring speed and stirring time on particle size, PDI, zeta potential, and entrapment efficiency of Nos-loaded PCL NPs

Formulation code	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)
CBY1	815±93	0.680±0.014	−9.6±3.2	55.4±11.1
CBY2	581±17	0.523±0.013	−10.5±2.9	64.7±12.8
CBY4	281±13	0.568±0.017	−16.8±1.4	78.9±18.4
CBY3H1	391±21	0.558±0.024	−8.8±3.5	44.2±9.8
CBY3H3	270±11	0.480±0.014	−16.8±2.3	63.7±11.5
CBY3H4	283±14	0.720±0.014	−16.6±1.8	64.5±10.5

Nos: Noscapine, PCL: Polycaprolactone, NPs: Nanoparticle

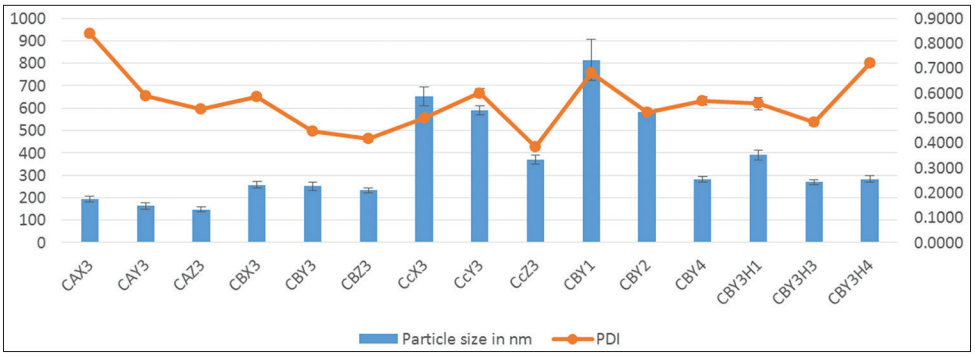

Figure 4: Noscapine-loaded PCL nanoparticles

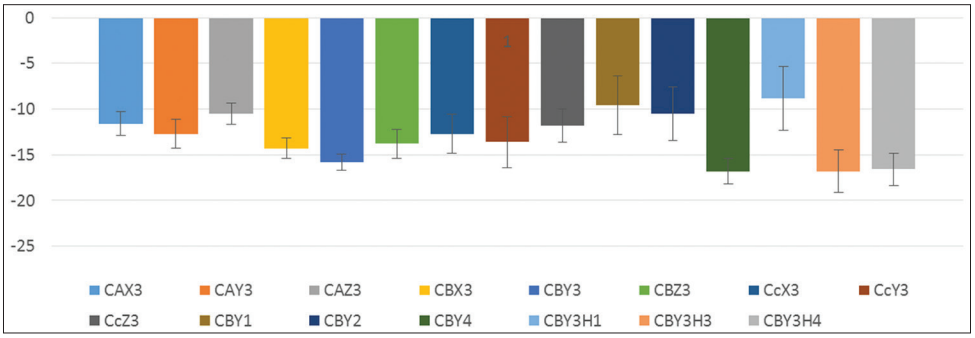

Figure 5: Zeta pontential (mV) of noscapine-loaded PCL nanoparticles

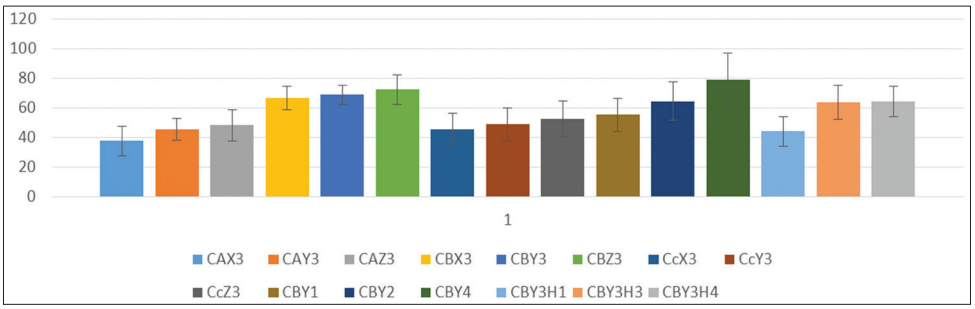

Figure 6: Encapsulation efficiency of noscapine-loaded PCL nanoparticles

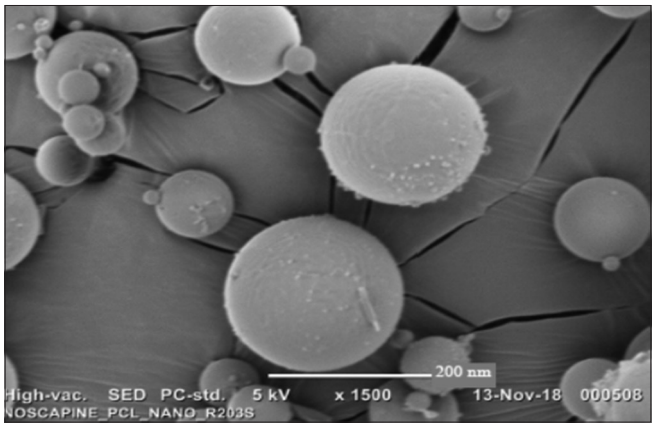

Figure 7: Scanning electron microscopy image of noscapine-loaded polycaprolactone nanoparticles

Nos which loosely bound or adsorbed on the surface of the NPs.

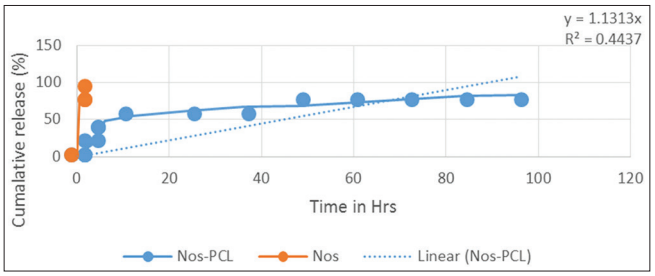

Figure 8: Noscapine-loaded –PCL nanoparticles *in vitro* release kinetics

In vitro Cell Line Study - MTT Assay

Nos pure drug has been shown to inhibit cell proliferation in glioma cells (U87MG). Results showed that Nos (120 µg) shown a significant decrease in the number of colonies of glioma cells (U87MG), with IC50 of 257 µM concentration. The different

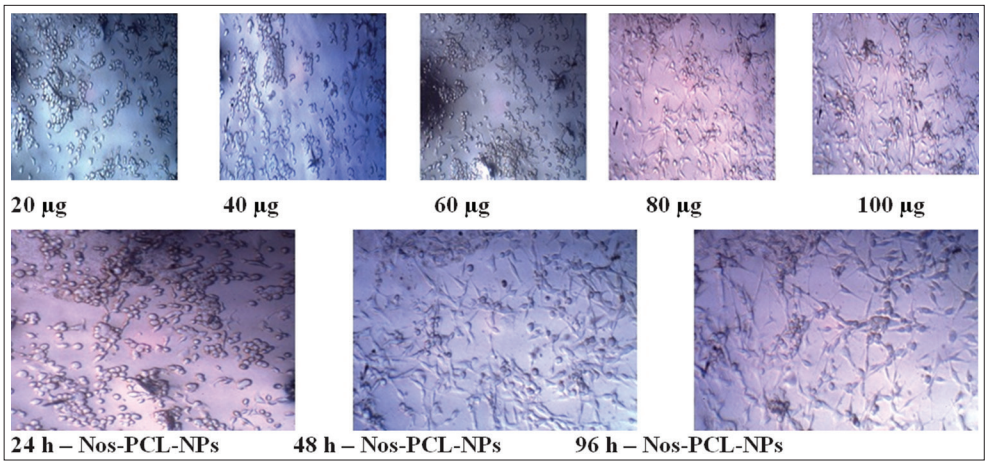

Figure 9: Images of U87 MG cell line

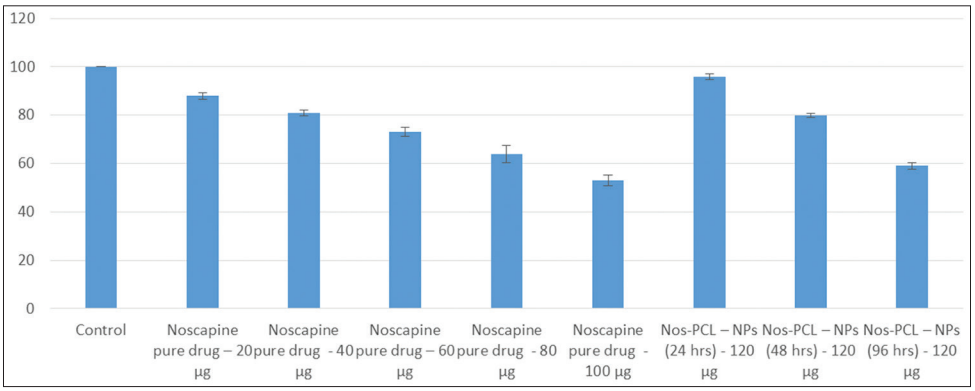

Figure 10: MTT assay percentage viability

Table 6: Effect of noscapine and noscapine loaded PCL nanoparticles on inhibition of cell proliferation

Sample concentration	% of cell viability	IC_{50}
Control	100±0.00	257 µM
Nos pure drug – 20 µg	088±1.37	
Nos pure drug – 40 µg	081±1.24	
Nos pure drug – 60 µg	073±1.90	
Nos pure drug – 80 µg	064±3.64	
Nos pure drug – 100 µg	053±2.14	
Nos-PCL – NPs (24 h) – 120 µg	096±1.12	270 µM
Nos-PCL – NPs (48 h) – 120 µg	080±0.86	228 µM
Nos-PCL – NPs (96 h) – 120 µg	059±1.24	176 µM

Nos: Noscapine, PCL: Polycaprolactone, NPs: Nanoparticle

concentration (20 µg–100 µg) of Nos and Nos-loaded PCL NPs at different time points and its IC_{50} and percentage cell viability shown in Table 6 and Figures 9 and 10. The Nos-loaded PCL-NPs (120 µg) showed IC_{50} of 176 µM.

The Nos-loaded PCL NPs showed 31% less dose required produce the same effect of Nos. The 96 h MTT assay of Nos-loaded PCL-NPs correlates with *in vitro* drug release studies.

CONCLUSION

In our study, Nos-loaded PCL NPs are optimized for polymer concentration (1:3), surfactant concentration (2%), stirring speed (14000 RPM), and stirring time (120 min). There was no drug-polymer interaction as revealed from the FTIR and DSC studies. The optimized particle size for Nos-PCL NPs (CBY3) was particle size of 252 nm, PDI of 0.447, and zeta potential of −14.8 mV. Entrapment efficiency was optimized for 68% with biphase release pattern. The scanning electron microscopy images show smooth and spherical shape NPs. *In vitro* cytotoxicity assay (MTT) showed 31% reduced IC_{50} of Nos-loaded PCL NPs when compared with Nos.

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