Research Article

Preparation, process optimization and cytotoxicity evaluation of lyophilized Mitomycin C loaded nanoparticles

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Abstract

Objective: The purpose of this study was to develop freeze dried Polylactide (PLA) nanoparticles loaded with Mitomycin C intended to be administered intravenously with improved therapeutic efficiency of the drug and thereby reduced dose related toxicity associated with oncology drugs. **Materials and methods**: The Mitomycin C loaded nanoparticles were prepared using modified spontaneous emulsification solvent diffusion method (SESD) and nanoprecipitation method. The in Vitro Release study of Mitomycin C loaded Polylactide (PLA) –NP was carried by dialysis bag diffusion technique. **Results and discussion**: The incorporation efficiency was found to be higher in Modified SESD technique than emulsification solvent evaporation method. The release behavior of Mitomycin C exhibited a biphasic pattern characterized by an initial burst release followed by a slower and continuous release. The nanoparticles were characterized by particle size, zeta potential, polydispersity index, DSC and FTIR. The long term stability was achieved by lyophilisation technique. The cell line study using XTT assay on LNCaP prostate tumor cell lines revealed that Mitomycin C loaded nanoparticles showed greater cancer cell inhibition compared to plain nanoparticles and marketed conventional formulation.

***Keyword*:** PLA, Mitomycin C, Lyophilization, Cryoprotectant, cell line studies, cytotoxicity studies

Introduction

Submicronic colloidal vectors have gained a considerable interest in the last few years because of their ability to ensure a specific drug targeting by both the oral route and the parenteral route. Such particulate systems have been widely investigated for gene delivery to cells and tissues as in the delivery of anti-sense oligonucleotides and also in cancer therapy and diagnosis. Among these vectors, liposomes and nanoparticles have special advantages with regards to the modulation of an active ingredient distribution within the human body.

In the development process of a nanoparticulate drug delivery system for *in vivo* application, biodegradability without toxic by-products is one of the major claims, a potential matrix molecule has to fulfill. Within the past decades, a multitude of protocols described in literature used synthetic or natural base products for the preparation of biodegradable nanoparticles. Instead of a complete listing of all approaches, only a selection of the most significant biodegradable nanoparticle types will be reviewed here. With regards to nanoparticles based on synthetic polymers, polylactide (PLA), polyglycolide (PLG), and poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles represent the most extensively investigated ones (Panyam and Labhasetwar, 2003).

Poorly water soluble drugs pose a significant challenge in their delivery. A large number of drugs are discarded from consideration in their early stages of development owing to poor bioavailability. Such drugs are an excellent candidate for nanoparticulate drug delivery, which can avoid the allergic side effects due to the use of cremophors (e.g. polyethyoxylated caster oil) in conventional formulations used for effective solubilization of drugs. However, for drugs with crystal forming habits, there is always a hazard of the formation of large micro particles from aggregation/bonding of nanoparticles; this can lead to infarction or blockage of the capillaries, resulting in ischemia or oxygen deprivation and

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possible tissue death. Hence, the nanoparticles need to be stabilized using biocompatible polymers (e.g. Polylactide, Poly (lactide-co-glycolide) (Murakami et al., 1999).

Materials and Methods

Mitomycin C was a kind gift sample from Sun Pharma Advanced Research Centre, India. Poly (L-Lactide), Inherent viscosity: 0.8 to 1.2 dl/g was purchased from Boegher Intellgam, Germany. LNCaP Cell line was purchased from National Cancer Research Institute, Pune. Various media like RPMI-1640, Bovine Serum Albumin, streptomycin and other antibiotic were purchased from M.P. Biogen, USA.

Quantification of Mitomycin C by HPLC method

The reversed Phase HPLC method for determination of content of Mitomycin C in various studies was developed. The mobile Phase was optimized and selected for methanol/water (35/65).The reversed Phase column was Phenomanax, cromacil 4.6 ID, 25 cm length C-18. The flow rate was set to 1 ml/ min and detection wavelength was set to 354 nm. The sample injection volume was set to 1 ml/min. The HPLC was calibrated with standard solution of 2.5ug/ml to 20ug/ml by dissolving Miomycin-C in Methanol/water solution. A standard solution was prepared 100ug/ml and from that a various stock solution of 2.5 ug/ml, 5ug/ml, 10 ug/ml, 15 ug/ml and 20 ug/ml. A calibration plot was developed finally (Jung-sun park et al., 2008).

Preparations of Mitomycin C loaded Polylactide based nanoparticles

Here Mitomycin C loaded Polylactide based Nanoparticles were prepared by Modified Spontaneous Emulsification Solvent Diffusion (SESD) and Nanoprecipitaiton techniques.PLA nanoparticles were prepared by the modified –SESD method using various solvent systems consisting of water miscible organic solvents. First in emulsication solvent evaporation, dichloromethane and acetone, a less water miscible and water miscible solvent were selected in a ratio of 1:1, 1:2 and 2:1 and 4:1 with different drug: polymer concentration. 1%w/w aqueous Poloxamer 188 solution is used as a surfactant in for stabilization of nanoparticles. In modification; a water miscible organic solvent like DMF was taken with acetone. In another method, nanoprecipitation, various organic solvent of increasing dielectric constant like THF, Acetonitrile and Methanol were selected .Both drug and polymer were dissolved in a organic solvent and this mixture was poured in to a aqueous phase containing water under mechanical stirring for 4 to 5 hours. The resultant nanoparticles were separated by ultracentrifugation at 15000 rpm for 30 min. The resultant nanoparticles were resuspended to desire system for lyophilization (Baert, 2005; Yoo, 1999).

***In vitro* release study**

The in vitro release profiles were studied by dialysis tube diffusion method using phosphate buffer saline at pH: 7.4, as a release medium. The in vitro release experiment was carried out as follows:20 mg of lyophilized Mitomycin C loaded PLA nanoparticles and 5 ml PBS(Phosphate Buffer Saline) (pH:7.4) was placed into dialysis bag that immersed into 100 ml PBS solution and the system was placed in a orbital shaker bath, which was maintained at 37±0.1^{0}C. and shaken horizontically at 100 min.At predetermined intervals ,aliquots of the release medium (5 ml) was taken out and assayed for dug release and replaced by 5 ml of fresh buffer at each sampling point and agitation was continued. Mitomycin C release was quantified at 354 nm by reversed phase HPLC method (Yoo, 1999; Jiao, 2002).

FTIR (Fourior Transform Infrared) Spectroscopy

A Shimatzu Fourior Tranform Infrared Spetrophotometer (FTIR) was used for IR analysis of samples. About 1–2 mg of sample were mixed with dry potassium dicromate and the samples were examined at transmission mode over wave number range of 4,000 to 400 cm^{-1} (Brigger Iet al., 1999).

DSC study of Mitomycin C

Mixtures were obtained as dry lyophilized powders prior to DSC analysis. DSC instrument of Shimatzu (Japan) were used in which within the temperature range of 25–300 ^{0}C under oxygen atmosphere, Samples weighing approximately 3mg were heated in a hermetically sealed aluminum pan at a rate of 10 ^{0}C/min (Sheng et al., 2009).

Size distribution and zeta potential

Different batches of Nanoparticles were monitored for their morphological attributes with the help of scanning electron microscope (SEM) (JSM, JEOL, Tokyo, Japan). Size distribution and Zeta potential of nanoparticle were measured by dynamic light scattering method using Zetasizer nano ZS (Malvern Instruments Ltd., UK) (Dong et al., 2009).

Lyophilization and *in vitro* stability study of Mitomycin C Nanoparticles

A common limitation of using polymeric nanoparticles in aqueous suspension was due to their poor chemical and physical stability when conserved for a long time. So freeze drying of these colloidal systems was an alternative method to achieve long-term stability. Here various parameter like cooling rate, cryoprotectant ratio were optimized and the particle size was determined before freezing and after freezing and final to initial size (S_F/S_I) was calculated (Sahoo et al., 2002; Fayam et al., 2003).

PBS stability assays for long –term stability were carried out where the NPs were dialyzed in PBS over 120 hrs at 37°C and withdrawn at 24 hour intervals. Particle size measurement demonstrated that the NPs remained stable over 6 days for the batches manufactured thru Modified spontaneous emulsification solvent diffusion method with no significant change in size and polydispersities for Mitomycin C where as for the batches from nanoprecipitation shown relatively less stability of 4 days with no significant change in size and polydispersities. The Mitomycin C based lyophilized nanoparticles were subjected to accelerated stability for 6 months at ICH temperature (4°C to 8°C) and (25°C ±2). After 6 months period, the freeze dried nanocarrier were redispersed again with PBS and sterile water for injection (SWFI) and evaluated for various parameters (Reddy et al., 2005; Sergio et al., 2005; Ehninger et al., 1991).

In vitro antitumor effect of Mitomycin C loaded Nanoparticle on LNCaP cell lines

The cytotoxicity of PLA Plain Nanoparticles, Mitomycin C loaded nanoparticles and plain Mitomycin C as a control were investigated using prostate tumor carcinoma cell lines (LNCaP) by XTT assay. LNCaP cell line (primary culture) seeded and sub cultured in T flask in bio safety cabinet hood. Here growth media, 100 RPMI -1640, with L-Glutamine, Na Carbonate and glucose were added. The media was supplemented with 10 ml fetal bovine serum for the purpose of generating monolayer. Addition of 1 ml of penicillin and Amphotericin B prevented the fungal growth. Now T flask were incubated for 15 days in Co_2 incubator with every 3 to 4 days for media change. Now cell viability checked by trypsinization , then LNCaP cells were seeded in 96 well plate sat at density of 10,000 cells per well in 100µl RPMI supplemented with 10% fetal bovine serum along with 50µl of cell culture. Cytotoxicity was carried out at three fold dilution with concentration range of 100mMolar. Twenty-four hours after plating, incubation at 37°C, 50µl of XTT die was added into each well, after 4 hours, the well plates were read in micro plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm. The experiments were repeated in triplicate. Cytotoxicity was expressed as % reduction cell viability (Jianjun et al., 2007).

Results and discussion

Particle size and size distribution

In Modified SESD method, the optimized batch has an uniform particle size of 246 nm with zeta potential value of -3.9mV, polydispersity index of 0.096 and Entrapment efficiency of 58.44±3.2. Here Drug: Polymer ratio was 1:1 and DMF: Acetone ratio was 2:1. Upon modified SESD process, two water miscible organic solvents such as DMF/acetone employed this alteration help to achieve lower sized nanoparticles with less chances of aggregation and avoidance of toxic solvents like dichloromethane.

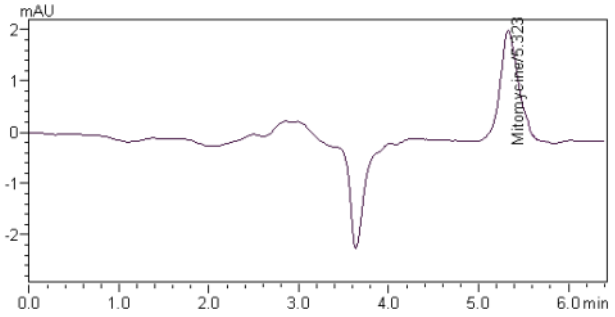

Figure 1. Chromatogram of Mitomycin C (1µg/ml) standard with corresponding retention time at 354 nm by RP-HPLC method

Table 1. Process optimization of Mitomycin C loaded PLA nanoparticles by Emulsification solvent evaporation

CH_2Cl_2/ Acetone Ratio	CODE	Drug: Polymer ratio	Particle size (nm)	Zeta potential (mV)	(PDI)	Entrapment efficiency± S.D
1:1	MT1	1:1	578	-4.5	0.191	48.56±3.4
	MT2	1:2	587	-4.1	0.178	49.45±2.1
	MT3	1:4	611	-5.1	0.188	51.34±4.1
	MT4	1:1	498	-4.1	0.211	45.76±2.3
1:2	MT5	1:2	523	-3.9	0.201	44.87±3.4
	MT6	1:4	554	-4.8	0.198	47.23±3.3
	MT7	1:1	566	-5.2	0.119	54.45±2.1
2:1	MT8	1:2	576	-5.4	0.098	52.18±4.4
	MT9	1:4	588	-4.9	0.119	49.98±3.4
	MT10	1:1	643	-6.9	0.109	45.78±2.5
4:1	MT11	1:2	678	-5.2	0.115	47.56±3.2
	MT12	1:4	745	-5.5	0.105	42.32±4.5

Table 2. Process optimization of Mitomycin C loaded PLA nanoparticles by modified spontaneous emulsification solvent diffusion

DMF/ Acetone Ratio	CODE	Drug: Polymer ratio	Particle Size (nm)	Zeta potential (mV)	(PDI)	Entrapment efficiency± S.D
1:1	MT13	1:1	432	-3.9	0.178	51.13±3.4
	MT14	1:2	467	-4.1	0.156	55.56±3.4
	MT15	1:4	465	-4.4	0.342	52.87±2.5
	MT16	1:1	427	-4.2	0.301	55.43±3.5
1:2	MT17	1:2	425	-4.4	0.335	54.65±3.1
	MT18	1:4	447	-4.0	0.343	57.34±4.5
	MT19	1:1	246	-3.9	0.096	58.44±3.2
2:1	MT20	1:2	374	-4.0	0.212	56.76±3.4
	MT21	1:4	389	-4.4	0.122	53.98±3.2
	MT22	1:1	362	-4.9	.219	42.91±4.5
4:1	MT23	1:2	382	-4.2	0.171	41.21±2.7
	MT24	1:4	401	-3.2	0.201	44.33±4.3

Table 3. Process optimization of Mitomycin C loaded PLA nanoparticles by Nanoprecipitation

THF/ Water Ratio	CODE	Drug: Polymer ratio	Particle Size (nm)	Zeta potential (mV)	(PDI)	Entrapment efficiency± S.D
1:1	MT25		556	-5.9	0.119	48.45±3.3
1:2	MT26	1:2	546	-4.9	0.098	45.11±3.5
1:5	MT27		541	-4.8	0.105	49.32±2.1
Acetonitrile/Water Ratio						
1:1	MT28		543	-4.8	0.232	51.33±3.1
1:2	MT29	1:2	532	-4.4	0.201	49.35±2.5
1:5	MT30		535	-3.9	0.190	48.54±2.5
Methanol/ Water Ratio						
1:1	MT31	1:2	456	-4.4	0.211	42.21±2.1
1:2	MT32		474	-4.0	0.201	40.55±3.5
1:5	MT33		465	-4.2	0.194	42.65±1.5

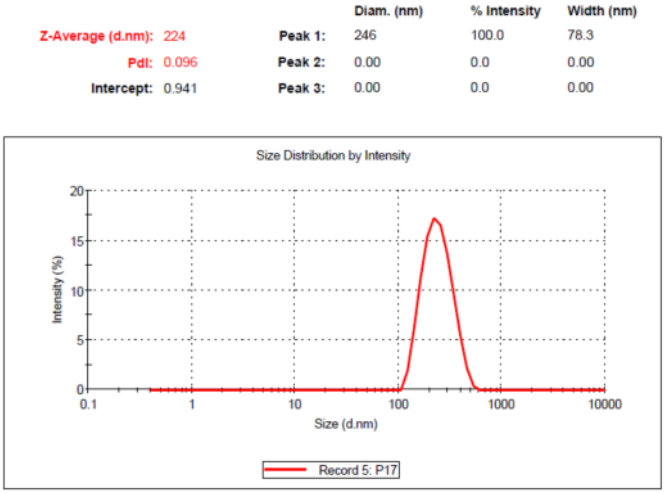

Figure 2. Particle size distribution of optimized batch

Upon improving the polymer concentration, the particle size and zeta potential also improved with no significant polydispersity index change. In nanoprecipitation technique, the optimized batch has drug: polymer ratio of 1:2 in Methanol: Water ratio of 1:1. The mean particle size was 456 nm with zeta potential value of -4.4 mV, polydispersity index of 0.211 and entrapment efficiency of 42.21±2.1. Here organic solvents of increasing dielectric constants (THF, Acetonitrile, Methanol) exhibit increasing polarity and water miscibility which leads reduction in particle size of nanoparticles. The trend toward larger NPs was observed as the solvent used became less water miscible and the polymer concentration was raised. No significant chance of zeta potential was observed.

Scanning electron microscopy

Scanning electron microscopy image of Mitomycin C loaded freeze dried Polylactide based nanoparticle revealed the surface texture of freeze dried power which shows a amorphous nature of the nanoparticles.

In vitro release study

The in vitro release profiles were studied by dialysis tube

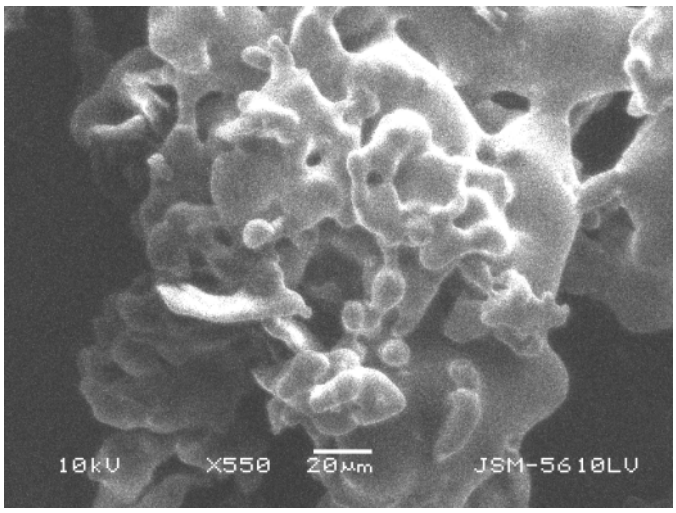

Figure 3. Scanning electron micrograph of freeze dried Mitomycin C nanoparticles.

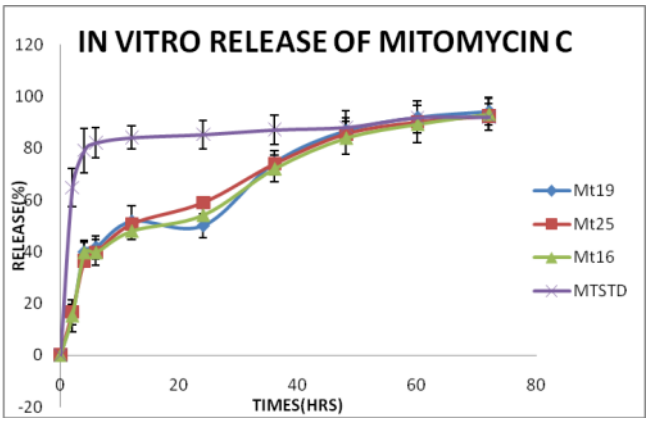

Figure 4. In vitro release pattern of Freeze dried nanoparticle of Mitomycin C

diffusion method using phosphate buffer saline at pH: 7.4, as a release medium. The in vitro release experiment was carried out as follows:15 mg of lyophilized Mitomycin C loaded PLA nanoparticles and 5 ml PBS (pH:7.4) was placed into dialysis bag that immersed into 100 ml PBS solution and the system was placed in a orbital shaker bath, which was maintained at 37±0.1°C and shaken horizontically at 100 min. At predetermined intervals, aliquots of the release medium (5 ml) was taken out and assayed for drug release and replaced by 5 ml of fresh buffer at each sampling point and agitation was continued. Mitomycin C release was quantified at 354 nm by reversed phase HPLC method. While at the same wavelength there was no interference of PLA was observed. The release behavior of Mitomycin C exhibited a biphasic pattern characterized by an initial burst release followed by a slower and continuous release. All release experiments were performed in triplicate. The release pattern follow a Higuchi (R^2=0.945) and nearby zero order of release (R^2=0.965).

FTIR study of Mitomycin C Nanoparticles

A Shimatzu Fourior Tranform Infrared Spetrophotometer (FTIR) was used for IR analysis of samples. In plain Mitomycin C spectra, characteristic bands were NH_2 stretch vibration of the aziridine at 3460, 3310 cm^{-1} and the C=O

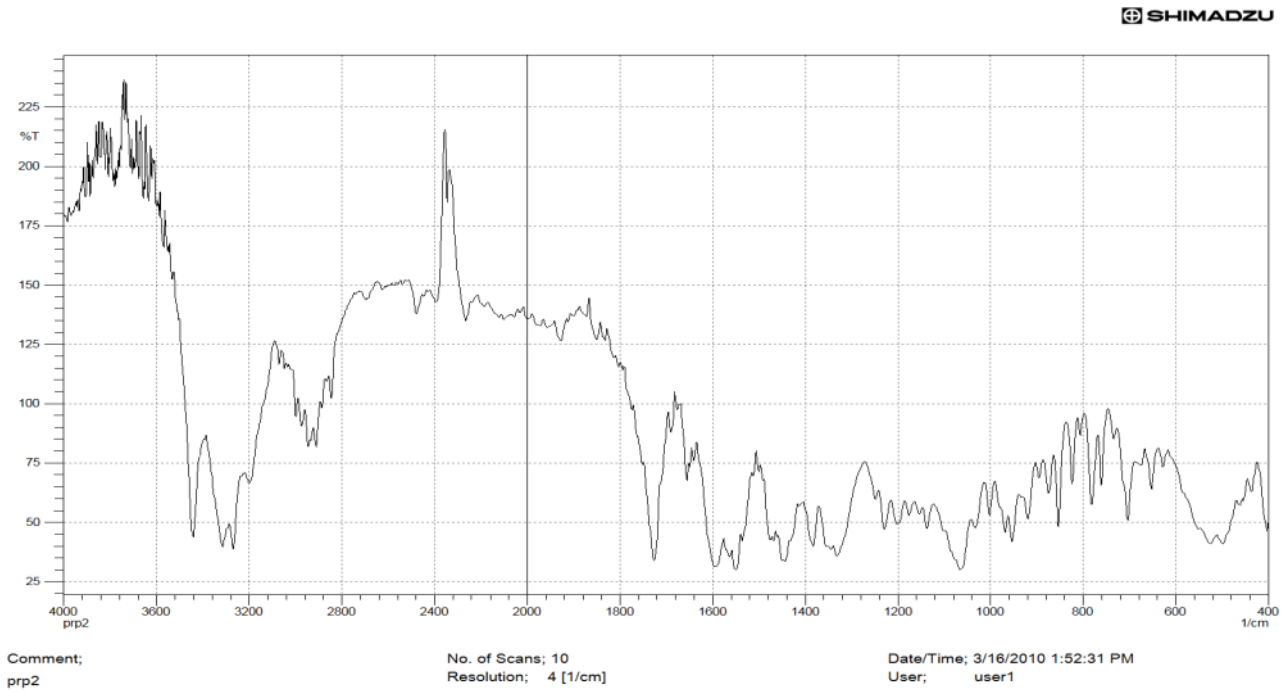

Figure 5a. FTIR spectra of Plain Mitomycin C Drug

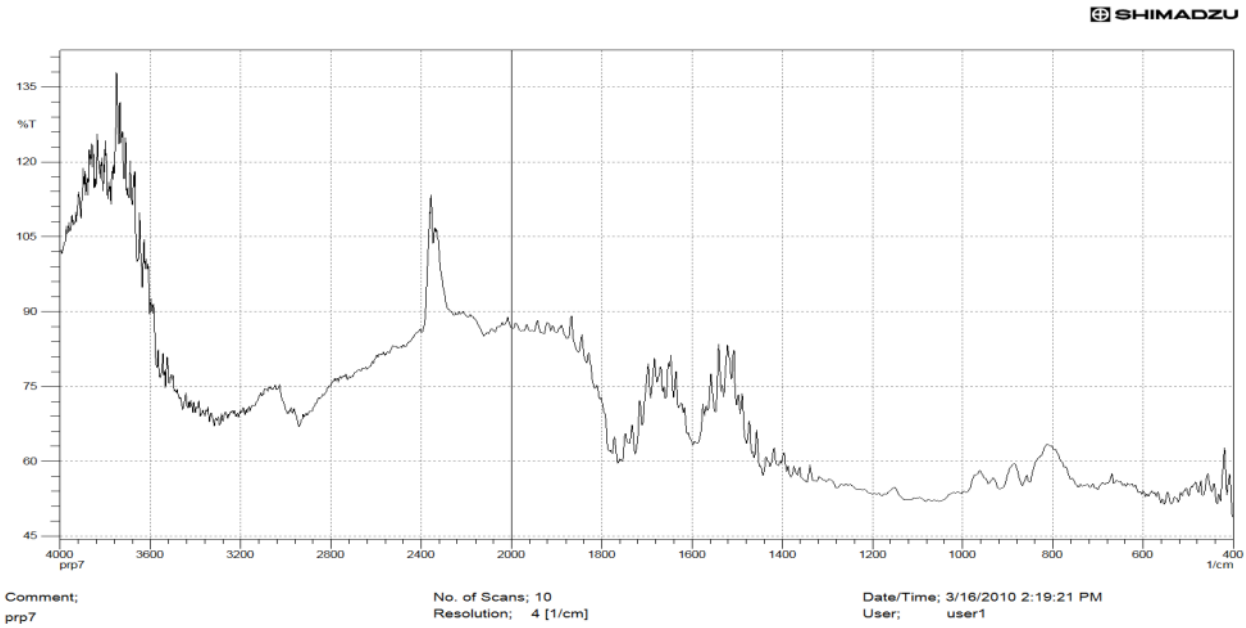

Figure 5b. FTIR spectra of Mitomycin C loaded nanoparitcles

stretch vibration at1600 cm^{-1} of quinone group, C=O functional group of carbamate at 1735cm^{-}1 observed. In FTIR of Mitomycin encapsulated nanoparticles, the corresponding peaks of aziridine ring and C=0 group peaks were disappear or buried which indicating the drug entrapment in polymer matrix.

DSC study of Mitomycin C nanoparticles

Mixtures were obtained as dry lyophilized powders prior to DSC analysis. The change in endothermic peak attributed to dissolution of Mitomycin C in PLA polymer matrix and decrease in melting temperature of nanoparticulated

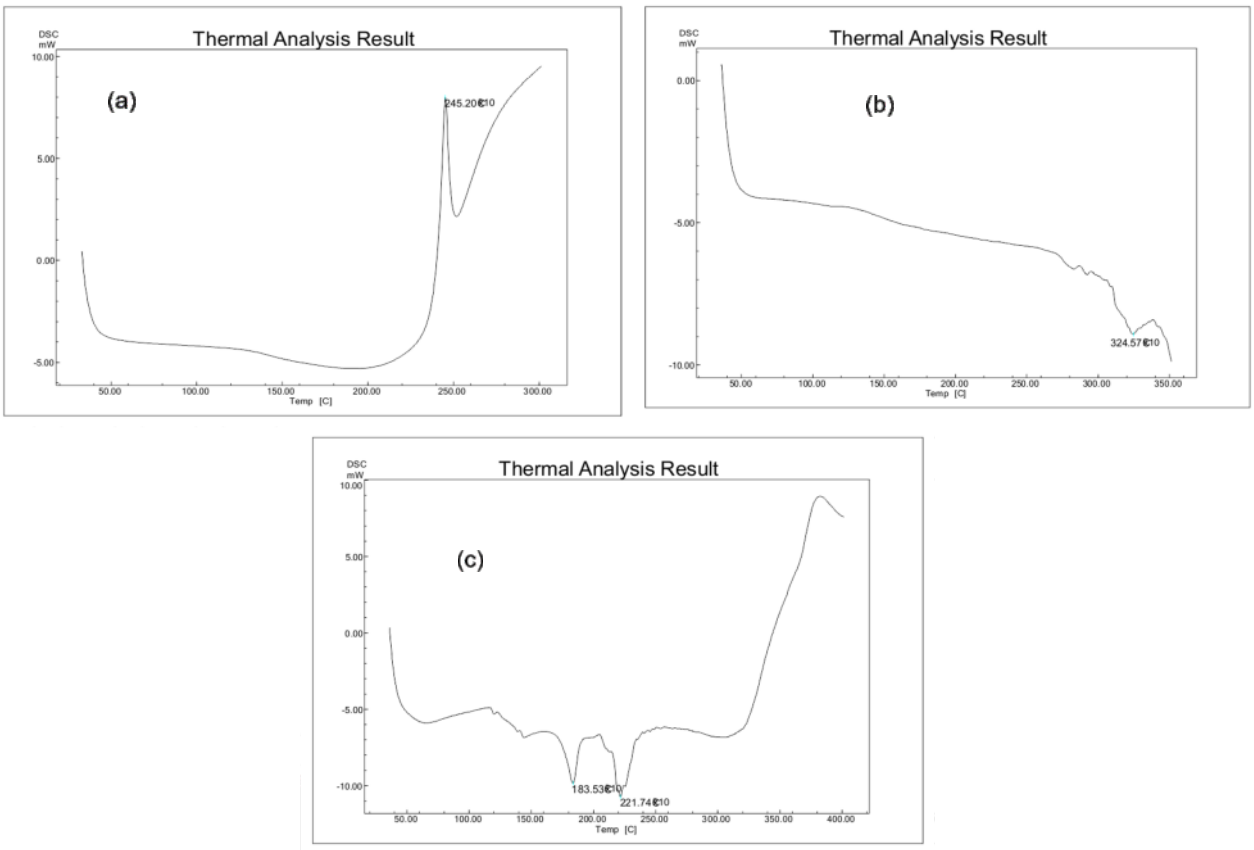

Figure 6. DSC thermogram of **(a)** Plain Mitomycin C (b) Poly L-(lactide) (c) Mitomycin C nanoaparticles

formulation of Mitomycin C compared to bulk has been indicating to their small size and presence of surfactant.

Lyophilization of Mitomycin C nanoparticles

Freezing drying of these colloidal systems is an alternative method to achieve long-term stability. Freezing is considered to be the most aggressive and critical step during the lyophilization. Here various parameter like cooling rate and cryoprotectant ratio were optimized and the particle size was determined before freezing and after freezing and final to initial size (S_F/S_I) was calculated.

Here Mitomycin C nanoparticles prepared by Modified SESD method were subjected to various cooling rate and different cryoprotectant ratios. The results in table 4 and 5 shown clearly that Mitomycin C nanoparticles with 10 %w/v cryoprotectant (Sucrose) and cooling procedure in liquid Nitrogen seem very stable among the three procedures of freezing as the ratio S_F/S_I was very near from 1 confirming a high stability. So it can be said that cooling procedure and cryoprotectant ratio has an influence on the size and polydispersity of nanoparticles. It was observed an increase in the size and polydispersity when a slow cooling procedure, and low cryoprotectant ratio applied, whereas a flash cooling (-150^{0}C) using liquid nitrogen reduces the ratio S_F/S_Iabout 1.08.

Table 4. Stability study during freezing at -150°C in liquid nitrogen with ramp shelf temperature of -50°C for 24 hrs for sublimation (MT19)

Sucrose (%w/v)	Before freeze drying particle size(nm)	After freeze drying size(nm)	S_F/S_I
0	356	956	2.68
5	356	498	1.39
10	356	387	1.08

Table 5. Stability study during freezing at -80°C in deep freezer for 45 minutes with ramp shelf temperature -50°C for 24 hrs for sublimation (MT19)

Sucrose (%w/v)	Before freeze drying particle size (nm)	After freeze drying size(nm)	S_F/S_I
0	356	1021	2.86
5	356	501	1.40
10	356	400	1.12

Table 6. Stability study during freezing at 1°C to -50°C in freezer condenser ramp for 45 minutes with shelf temperature ramp -50°C for 24 hrs for sublimation (MT19)

Sucrose (%w/v)	Before freeze drying particle size(nm)	After freeze drying size(nm)	S_F/S_I
0	356	1010	2.83
5	356	512	1.43
10	356	398	1.10

***In vitro* stability studies**

PBS stability assays for long term stability were carried out where the NPs were dialyzed in PBS over 120 hrs at 37^{0}C and withdrawn at 24 hour intervals. Particle size measurement demonstrate that the NPs remain stable over 6 days for the batches manufactured thru emulsification solvent diffusion with no significant change in size and polydispersities for Mitomycin C where as for the batches from nanoprecipitation shows relatively less stability of 4 days with no significant change in size and polydispersities. These results suggest the steric stabilization of surfactant concentration in the formulation play important role.

The Mitomycin C based lyophilized nanoparticles were subjected to accelerated stability for 6 months at ICH temperature (4°C to 8°C) and (25°C ±2). After 6 months period, the freeze dried nanocarrier were redispersed again with PBS and SWFI and evaluated for various parameters. lyophilized nanoparticles were showed better stability in terms of mean particle size diameter , polydispersity index and redispersibility.

***In vitro* antitumor effect on LNCaP cell lines**

The in vitro anticancer cytotoxic activity of free Mitomycin C and Mitomycin C -loaded PLA nanoparticle and plain nanoparticles on LNCaP cells, expressed as % reduction of cell viability. The 50% growth inhibitory concentration (IC_{50}) values for free Mitomycin C and Mitomycin C loaded nanoparticles were estimated from the available cytotoxicity Data .The nanoparticles loaded with Mitomycin C exhibited

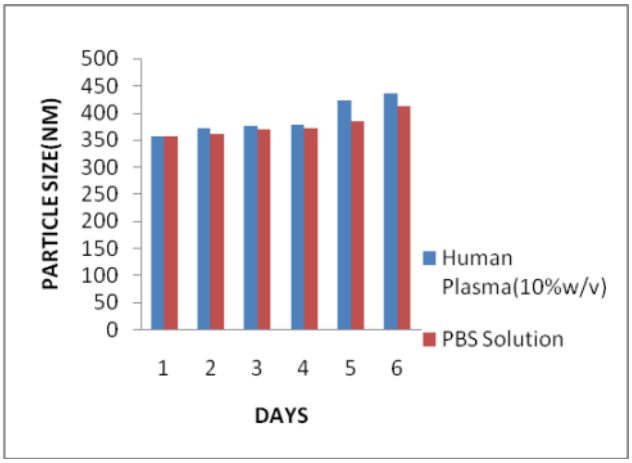

Figure 7. *In vitro* long term stability assays of Mitomycin C nanoparticles

Table 7. IC_{50} values (mMolar/ml) for free mitomycin C and Mitomycin C loaded PLA nanoparticles

Formulation s	IC_{50} values at different time		
	24 hrs	48 hrs	72 hrs
Free mitomycin C formulation	112.88	96.51	94.55
Mitomycin C based nanoparticles	77.29	64.48	56.22

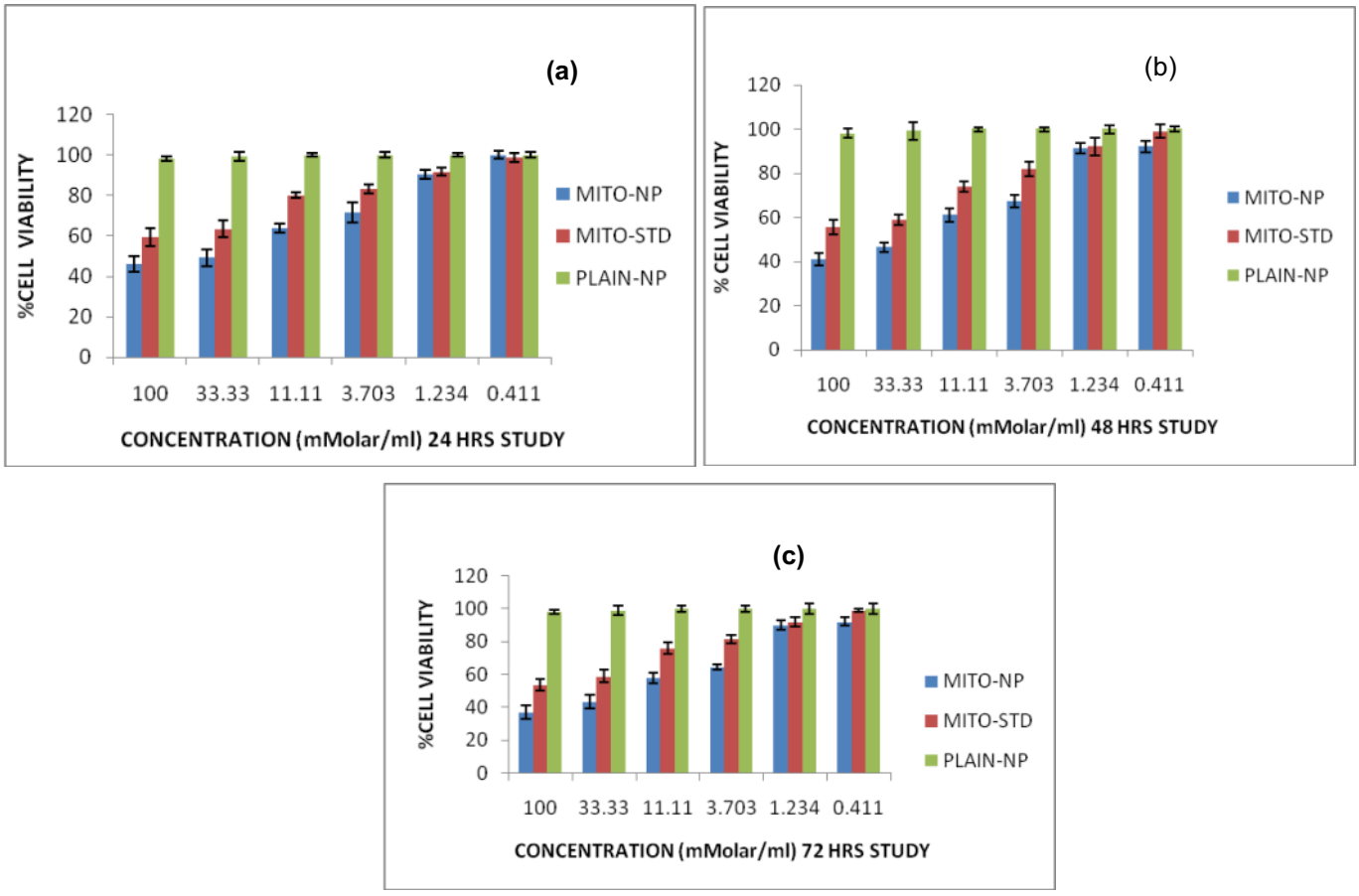

Figure 8. Cytotoxicity study on LNCaP cell line in **(a)** 24 hrs **(b)** 48 hrs **(c)** 72 hrs

more in vitro anticancer activity comparable to that of free Mitomycin C. The activity of both free and nanoparticle-entrapped Mitomycin C increased with increasing drug concentration and incubation time, whereas plain nanoparticles showed no change in viability of cancer cells.

Conclusion

The Present study demonstrates that both modified SESD and Nanoprecipitation method was feasible, viable and advantageous method to get nanoparticles of desired property. The size and polydispersity index of nanoparticles can be controlled by various parameters like drug: polymer ratio, binary mixture of organic solvents, and organic to aq. Phase ratio. The lyophilization technique provides us an excellent source of getting a stable nanoparticle formulation for longer period of time. In vitro antitumor activity indicated a nanoparticulate formulation was therapeutically more effective compare to conventional system.

Conflict of interest

There is no conflict of interest between the authors of the article.

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