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PEGylated Lipova E120 liposomes loaded with celecoxib: *in-vitro* characterization and enhanced *in-vivo* anti-inflammatory effects in rat models

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The goal of the current investigation was to prepare PEGylated Lipova E120 liposomes loaded with celecoxib for the effective treatment of rheumatoid arthritis (RA). PEGylated liposomes were prepared and were characterized using techniques such as particle size distribution, polydispersity index (PDI), zeta potential, encapsulation efficiency and *in-vitro* release, *in-vivo* and stability studies. The morphological study was characterized by scanning electron microscopy and transmission electron microscopy. To determine the interaction between drug and polymer Fourier transform infrared, Raman, thermogravimetric analysis and differential scanning calorimetry studies were performed. Results show that formulation F6 was optimized with a particle size of 92.12 ± 1.7 nm, a PDI of 0.278 ± 0.22, a zeta potential of − 40.8 ± 1.7 mV with a maximum encapsulation of 96.6 ± 0.05% of drug in the PEGylated liposomes. The optimized formulation shows a maximum release of drug i.e. 94.45 ± 1.13% in 72 h. Tail immersion assay shows that the optimized formulation F6 significantly increases the reaction time and carrageenan-induced assay shows that the optimized formulation inhibits the increase in paw edema thus providing a pain relief treatment in RA. These results suggest that the PEGylated liposomes provide a sustained release of celecoxib and helps in effective treatment of RA.

Keywords. Celecoxib; PEGylated liposomes; rheumatoid arthritis

1. Introduction

Rheumatoid arthritis (RA) is a type of auto-immune disorder which causes inflammation in joints and thus results in a painful deformity and immobility especially in fingers, wrists, feet and ankles (Siebert *et al.* 2016). In 2015, RA affected 24.5 million people. The annual incidence of RA is ∼40 cases per 10,000 population and the incidence rate was increasing 1% in each year (Gibofsky 2012). The occurrence rate of RA is greater in women as compared to men and mainly affects at the age of 35–50 years (Ahlmen *et al.* 2010). There are various drugs available for the treatment of RA such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease modifying anti-rheumatic drugs, tumor necrosis factor inhibitor, etc., but these drugs suffer from various side effects on oral administration such as weight gain, hepatic cirrhosis and various gastrointestinal disturbances such as irritation, burning, etc. (Kahlenberg and Fox 2011). Nowadays, targeted therapies are generally used for the treatment of various disorders including RA which on parenteral administration cause fewer side effects.

Celecoxib is a NSAID that exhibits anti-inflammatory, analgesic and antipyretic activities. Celecoxib inhibits prostaglandin synthesis, primarily via inhibition of cyclooxygenase-2 but does not inhibit the cyclooxygenase-1 isoenzyme. The inhibition of prostaglandin synthesis helps us to alleviate pain symptoms and thus helps in the treatment of RA (Goldenberg 1999; McCormack 2011).

Liposomes are concentric vesicles in which an aqueous volume is entirely enclosed by one or more membranous lipid bilayers of phospholipids (Li *et al.* 2015). Liposomes can be prepared either from natural phospholipid sources or synthetic phospholipid sources. Liposomes can be used to deliver various types of drugs for the treatment of various disorders such as cancer, neurological disorder, cardiovascular disorder, etc.; liposomes can entrap both hydrophilic and hydrophobic drugs. In addition, liposomes allow target-specific delivery which causes reduction in frequency and intensity of adverse effects. But the major drawback associated with the use of liposomes is that on intravascular administration they are rapidly cleared from the blood circulation after being taken by the mononuclear phagocyte system. To overcome this problem, the surface of the liposomes is treated with polyethylene glycol (PEG) of different molecular weights which helps in shielding of the phospholipid bilayer. Now, these liposomes are known as PEGylated liposomes which release the drug in a controlled manner, and their surface PEGylation (surface engineering)

will provide a long-term circulation in the blood and avoid rapid clearance from the body, improve drug solubility, reduce dosage frequency, potentially reduce toxicity, increase drug stability and enhance protection from proteolytic degradation (Sercombe *et al.* 2015; Wang *et al.* 2016).

In view of these pieces of evidence, in the present study we have prepared PEGylated liposomes of celecoxib which were further characterized using various techniques such as Fourier transform infrared (FTIR), differential scanning calorimetry (DSC), particle size distribution, zeta potential, encapsulation efficiency (EE), *in-vitro* drug release study and *in-vivo* and stability studies.

2. Materials and methods

2.1 *Materials*

Celecoxib was purchased from Cadila Pharmaceutical, Ahmedabad, India. Lipova E120 was provided as a gift sample from VAV Life Sciences, Mumbai, India. Cholesterol and Tween-80 were purchased from Sigma-Aldrich, USA. 1,2-Distearoyl-phosphatidyl ethanolamine-methyl-polyethyleneglycol-2000 (MPEG-$DSPE_{2000}$) was provided as a gift sample from Lipoid Germany, India and trehalose was purchased from Himedia, Mumbai, India. All other chemicals used throughout the experiment were of analytical grade. High-performance liquid chromatography (HPLC) water was used in all experiments.

2.2 *Preparation of celecoxib-loaded PEGylated liposomes*

With the help of a thin film hydration method as described by Bangham in 1965, PEGylated liposomes were prepared. To prepare PEGylated liposomes, first a known amount of drug, Lipova E120 and cholesterol was weighed in different concentrations as shown in table 1. Formulations F1–F4 were prepared by increasing the concentration of lipova along with a constant concentration of cholesterol, whereas formulation F5 was prepared with a low concentration of lipova but with an increased concentration of cholesterol, and formulations F6–F8 were prepared by increasing the concentration of lipova and MPEG-$DSPE_{2000}$ with a constant concentration of cholesterol. Then, the ingredients were taken in a round bottom flask (RBF) and dissolved in 5 mL of chloroform and a thin film was formed using a rotary evaporator at a temperature of 40°C at a speed of 100 rpm. Then, the RBF was placed in a desiccator for the complete hydration of the film. After the complete hydration of the film, it was hydrated with phosphate buffer saline (pH 7.4) with (F5–F8) or without (F1–F4) Tween-80 (3.0% v/v) on the rotary evaporator with a temperature maintained at 60°C at a speed of 100 rpm until the film gets completely hydrated (Shavi *et al.* 2016). Then, the formed liposomal dispersion was sonicated for 15 min in three cycles with 5 min off after each cycle to reduce the particle size of liposomes. Then, the liposomes were freeze dried using trehalose to obtain a dry sample.

2.3 *Characterization of PEGylated liposomes*

2.3.1 *Particle size, polydispersity index and zeta potential analysis:* Average diameter and size distribution of liposome particles were determined using dynamic light scattering principle in a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The liposome suspensions were dispersed in HPLC water and the system was fixed at a 25°C with 90° angle, by considering a refractive index of 1.330 and a viscosity of 0.8872. The surface charge of liposome was determined by measuring the zeta potential using two combined principles laser Doppler velocimetry and phase analysis light scattering in a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The analysis was carried out for all the batches of liposomes at 25°C by suitable dilutions with HPLC water. Particles with the highest positive zeta potential or the lowest zeta potential are generally considered electrophoretically stable (Alavi *et al.* 2014). All measurements were repeated three times.

Table 1. Composition of different celecoxib-loaded liposomes with Lipova E120 lipid formulations

	Formulations							
	F1	F2	F3	F4	F5	F6	F7	F8
Lipid molar ratio	4:1	6:1	8:1	9:1	9:2	9:1:0.25	9:1:0.5	9:1:1
Celecoxib (mg)	10	10	10	10	10	10	10	10
Lipova E120 (mg)	80.28	120.4	160.53	180.6	140.46	120.0	180.6	180.6
Cholesterol (mg)	10	10	10	10	20	10	10	10
MPEG-$DSPE_{2000}$ (mg)	–	–	–	–	–	25	50	100
Chloroform (mL)	5	5	5	5	5	5	5	5
Tween-80 (% v/v)	–	–	–	–	3.0	3.0	3.0	3.0
Trehalose (mg)	50	50	50	50	50	50	50	50
Hydration volume (mL)	5	5	5	5	5	5	5	5

2.3.2 *Vesicular shape and surface morphology using scanning electron microscopy and transmission electron microscopy:* The morphology of the liposome vesicles was studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. SEM was executed with the help of a lyophilized sample mounted on carbon tape coated with gold and imaged using SEM (Banasthali) at 20 kV at magnifications of 3300× and 19,000× (Dave *et al.* 2017a, b). The morphology of celecoxib-loaded liposome was examined by using TEM (MNIT, Jaipur) at 200 kV at a magnification of 9900×. TEM was used to evaluate the physical dimensions and structure of particles. A drop of liposome suspension was diluted with HPLC water placed after staining with 2% w/v phosphotungstic acid for 30 s on a grid coated with copper and the sample was allowed to dry before examination under the TEM. Two grids of each sample were prepared and grids opening were selected and viewed randomly (Panwar *et al.* 2010).

2.3.3 *Attenuated total reflection-FTIR analysis:* Infrared spectra of celecoxib, Lipova E120, cholesterol MPEG-$DSPE_{2000}$ and formulation F6 were analyzed using a Bruker EQUINOX 55 spectrophotometer furnished with a liquid nitrogen cooled mercury cadmium telluride detector at a nominal resolution of 2 cm^{-1}. Diamond was used as an internal reflection element placed at an incidence angle of 45°. An innovative attenuated total reflection (ATR) rectification was applied to all spectra, and the spectra were scanned from a region of 4000–400 cm^{-1} and peak fitting was performed using Opus software (Shavi *et al.* 2016; Dave *et al.* 2017a, b).

2.3.4 *Raman spectroscopy analysis:* Raman spectroscopy helps us to provide information on vibrational, rotational and other low-frequency modes in the system. It was performed with the help of thermo-scientific instrument (DxRxi), furnished with OMINICxi analysis software. The 532 nm laser beam was used to assemble the spectra of celecoxib, Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and lyophilized formulation F6 at 5–100 mW laser power. The spectra were scanned in the range of 125–4000 cm^{-1} (Sharma *et al.* 2017).

2.3.5 *DSC analysis:* Celecoxib, Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and lyophilized formulation F6 were analyzed by using DSC-60. The instrument was furnished with a flow controller (FCL 60), calorimeter (DSC 60), thermal analyzer (TA 60) and working TA 60 software from NTZSCH (model: DSC 204 F1 PHOENIK). Samples were placed in an aluminum pan and were crimped with the help of a hydraulic press, monitored by heating under a nitrogen flow (30 mL/min) at a scanning speed of 5°C/min from 30 to 300°C. As a reference, indium was used and placed in another aluminum pan (Atyabi *et al.* 2009; Sant and Nagarsenker 2011). Flow of heat was measured as a function of temperature for all samples. The scanning was recorded and plotted by temperature on the *X*-axis and heat flow on the *Y*-axis.

2.3.6 *Thermogravimetric analysis (TGA) analysis:* TGA of celecoxib, Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and lyophilized formulation F6 was carried out with the help of a TGA-4000 Perkin Elmer (CURIE GRANT) equipped with Pyris Manager software to determine various properties of samples such as physical and chemical properties. TGA was also used to study vaporization, sublimation, weight loss, adsorption, absorption, sublimation, etc. TGA was used to characterize the samples which show either one weight gain or weight loss owing to decomposition. Samples were placed in a crucible and weighed and then placed in the instrument. Weight loss was observed and graphs were obtained (Re *et al.* 2011; Dave *et al.* 2017a, b).

2.3.7 *Percentage EE of celecoxib-loaded PEGylated liposomes:* EE helps to calculate the total quantity of drug loaded in PEGylated liposomes. To determine the amount of celecoxib encapsulated in each liposome, the freshly prepared sample was centrifuged in a cooling centrifuge (Remi) furnished with a rotor TLA-45 at 14,000 rpm at 4°C for 25–30 min. After separation of liposome pellets (encapsulated drug), the supernatant was taken and again centrifuged for 15 min at 14,000 rpm and the amount of unentrapped drug was calculated by using an ultraviolet/visible spectrophotometer at 272 nm (Betageri and Parsons 1992; Rengel *et al.* 2002; Kaiser *et al.* 2003). The experiment was performed in triplicate. The amount of encapsulated drug was calculated using the following equation:

$$(\%)EE = \frac{Amt\ of\ celecoxib\ in\ liposome\ sus(\mu g) - amt\ of\ celecoxib\ in\ supernatant}{Amount\ of\ celecoxib\ in\ liposome\ susp(\mu g)} \times 100$$

2.3.8 *In-vitro drug release studies:* The *in vitro* release of celecoxib from liposome formulations was determined by diffusion cell through a dialysis membrane which works as a semipermeable membrane. The soaked dialysis membrane was fixed cautiously to the hollow glass tube at one end (diffusion tube) of 2 cm diameter (22.7 cm^2), acting as the donor compartment. Into the donor compartment, 2 mL of liposomal formulation was added and spread uniformly. The donor compartment was placed in touching base with the

receptor compartment containing phosphate buffer saline (pH 7.4) with the temperature maintained at 27 ± 0.5°C and with a constant stirring at 500 rpm using a magnetic stirrer. At a predetermined time intervals (1, 2, 4, …, 18, 24, 48 and 72 h), 5 mL sample from the receptor compartment was withdrawn and the same volume (5 mL) of phosphate buffer saline was immediately added to the receptor compartment to maintain the sink conditions. The concentrations of drug were determined at 248 nm against a suitable blank sample (Er *et al.* 2009; Rathode and Deshpande 2010; Yalcin *et al.* 2017). The experiment was performed three times and expressed as mean ± SD. From the drug concentration, percent cumulative drug release was calculated.

2.3.9 *In-vivo studies:* All animal experimental protocols were approved by Institutional Animal Ethical Committee (Ref. No. BU/3769/18-19) and conducted strictly in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Male wistar rats (weighing 250–300 g) were employed for the study. The rats were procured from Lala Lajpat Rai Veterinary and Animal Sciences, Hisar, India. They were kept in polyacrylic cages and maintained under standard housing conditions of temperature (25 ± 2°C) and humidity (60–65%) with 12 h light–dark cycles. They were acclimatized for 1 month and given *ad libitum* access to food (dry pellets) and water.

2.3.9.1 Tail immersion assay Rats were divided into different groups ($n = 6$) in which group I was considered as a control group, group II received pure celecoxib, group III received optimized formulation at a dose of 10 mg/kg (according to the weight of the test animal) and group IV received optimized formulation F6 at a dose of 20 mg/kg. An antinociceptive response was evaluated by quantifying tail-withdrawal latency of rats against radiant heat, as described by D'Armour and Smith and revised by Kulkarni. Baseline latencies to tail withdrawal from radiant heat (7–8 s) were recognized. A cut-off time of 15 s was taken to avoid any harm to the rat tail. To estimate mean basal reaction time four readings of each animal were recorded (Jain and Kulkarni 1999; Sofidiyaa *et al.* 2014).

2.3.9.2 Carrageenan-induced paw edema assay Rats were divided into three groups with six animals in each group. Acute edema was induced by injecting 0.1 mL of freshly prepared 1% solution of carrageenan into sub-plantar tissue of right hind paw of rat. Group I was considered as the control group. After 30 min of induction, group II received pure celecoxib, group III received optimized formulation at a dose of 10 mg/kg and group IV received optimized formulation F6 at a dose of 20 mg/kg. Using plethysmometer, the swelling index was measured at 15 min intervals for 2 h after each administration. The % change in the paw volume was determined and conveyed as the swelling index (Cheow *et al.* 2011; Naveed *et al.* 2014).

2.3.10 *Stability study:* The loss of drug from the liposomal formulation was determined using the accelerated stability study of lyophilized formulation. An accelerated stability study of the optimized formulation was performed according to the ICH guidelines (ICH guidelines 'Q1A (R2) 2003, Stability Testing of New Drug Substances and Products'). Lyophilized samples were stored at room temperature (25°C) and at 4°C for 3 months in glass vials. After the storage duration of 7, 15, 30, 60 and 90 days, lyophilized liposome samples were redispersed in HPLC water and further particle size, zeta potential and EE were calculated to observe the changes in the formulation with respect to time (Bhalerao and Harshal 2003; Begum *et al.* 2012).

3. Results

3.1 *Particle size, PDI and zeta potential analysis*

The particle size of vesicles and their size distribution are the most important parameters which express the quality of liposome dispersion. The particle size and PDI were determined by using a Malvern Zetasizer Nano ZS (Malvern Instrument, UK). The results of the particle size and PDI are shown in table 2. Formulations F1–F8 showed particle sizes ranging from 92.13 ± 1.7 to 163 ± 3.8 nm with PDIs ranging between 0.68 ± 0.26 and 0.354 ± 0.21, respectively. Formulations F1–F4 with an increased Lipova E120 concentration showed particle sizes ranging from 125 ± 2.7 to 151 ± 1.8 nm with PDIs ranging from 0.287 ± 0.12 to 0.68 ± 0.26. Formulation F5 with a low concentration of Lipova E120 but with a high concentration of cholesterol showed a particle size of 163 ± 2.7 nm and a PDI of 0.285 ± 0.18, whereas formulation F6 with a low concentration of Lipova E120 and cholesterol along with the addition of Tween-80 and MPEG-$DSPE_{2000}$ (25 mg) in low concentrations showed a mean particle size of 92.12 ± 1.7 nm with a PDI of 0.278 ± 0.22. Formulations F7 and F8 with a high concentration of Lipova E120 and MPEG-$DSPE_{2000}$ showed a particle size of 101 ± 3.4 and 110 ± 2.7 nm with PDIs of 0.239 ± 0.19 and 0.354 ± 0.21, respectively.

Liposomes' stability was defined by zeta potential which was determined using a Malvern Zetasizer Nano ZS (Malvern Instrument, UK). In table 2, results of zeta potential values are shown. Formulations F1–F4 with an increased concentration of Lipova E120 showed zeta potential ranging from 18.8 ± 2.7 to 28.3 ± 2.1 mV, whereas formulation F5 with a high concentration of cholesterol showed a zeta potential of − 29.8 ± 3.1 mV. Formulation F6 with a low concentration of Lipova E120, cholesterol and MPEG-$DSPE_{2000}$ with Tween-80 showed a zeta potential of − 40.8 ± 1.7 mV. Formulations F7 and F8 with a high concentration of Lipova E120 and MPEG-$DSPE_{2000}$ showed zeta potentials of − 35.8 ± 3.1 and − 33.7 ± 1.7 mV. The study was further supported by Alavi *et al.* (2014) who found the same result. The study was performed in triplicate and results were shown as mean ± SD.

Table 2. Characterization of PEGylated liposomes containing celecoxib

Characterization	F1	F2	F3	F4	F5	F6	F7	F8
Particle size (nm)	125 ± 2.7	134 ± 3.8	150 ± 1.7	151 ± 1.8	163 ± 2.7	92.12 ± 1.7	101 ± 3.4	110 ± 2.7
PDI	0.68 ± 0.26	0.471 ± 0.29	0.345 ± 0.19	0.287 ± 0.12	0.285 ± 0.18	0.278 ± 0.22	0.239 ± 0.19	0.354 ± 0.21
Zeta potential (mV)	18.8 ± 2.7	23.71 ± 2.9	28.3 ± 2.1	22.7 ± 1.7	− 29.8 ± 3.1	− 40.8 ± 1.7	− 35.8 ± 3.1	− 33.7 ± 1.7
% EE	64.3 ± 0.2	76.24 ± 0.53	80.28 ± 0.4	82.26 ± 0.56	88.35 ± 0.15	96.6 ± 0.05	90.4 ± 0.3	92.3 ± 0.23
% Cumulative drug release	54.08 ± 0.35	62.13 ± 0.13	68.08 ± 0.55	64.33 ± 0.64	72.01 ± 0.83	94.45 ± 1.13	86.09 ± 1.43	84.34 ± 0.34

3.2 *Vesicular shape and surface morphology using SEM and TEM*

The surface morphology and three-dimensional nature of liposomes were observed by SEM which was executed with the help of lyophilized sample F6 coated with gold. For imaging of liposome SEM was used at 15 kV at magnifications of 1000× and 2000×. An SEM image of PEGylated liposome formulation F6 is shown in figure 1A which indicates that liposome was spherical in shape with clearly defined boundaries and with the size range of 50–100 nm. The result was further supported by TEM analysis. It was concluded that the liposomes formulated with Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and drug have stable particle size.

TEM analysis gives information on the surface morphology of liposomes. The surface morphology of the prepared celecoxib-loaded liposome of formulation F6 is shown in figure 1B. The inner areas of the liposome appeared dark which indicates the dense packing of phospholipid bilayer vesicles. The results of the TEM analysis showed discrete, homogenous, round, particles which show that the encapsulation of celecoxib in the liposomes does not affect the surface morphology of the vesicles.

3.3 *ATR-FTIR analysis*

The ATR-FTIR results of celecoxib, Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and liposomal formulation F6 are shown in figure 2. ATR-FTIR of celecoxib shows major characteristic peaks at 3332.89 and 3228.47 cm^{-1} due to primary and secondary amines, 3097 cm^{-1} due to the aromatic CH_2 stretching, 1590.43 and 1560.94 cm^{-1} due to amine bending, 1473.31 cm^{-1} due to aromaticity C=C, 1445 cm^{-1} due to CH_2 bending, 1372.47 cm^{-1} due to CH_3 bending, 1346 cm^{-1} due to the S=O symmetric stretching, 1160 cm^{-1} due to the S=O asymmetric stretching, 1098 cm^{-1} due to SO_2, 1015.47 cm^{-1} due to C=C and 1226 cm^{-1} due to CF_3. Formulation F6 showed peaks at 3278, 3009, 1736, 1463, 1374, 1360, 1237, 1083, 1016 and 966 cm^{-1}.

3.4 *Raman spectroscopy*

The results of Raman spectroscopy of celecoxib, Lipova E120, cholesterol, MPEG-$DSPE_{2000}$ and liposomal formulation F6 is shown in figure 3. The spectra of celecoxib (sample drug) show the major peaks at 3288 cm^{-1} due to the –NH stretching, 3071 cm^{-1} due to the aromatic CH_2 stretching, 1615 cm^{-1} due to the –OH bending, 1590 cm^{-1} due to the amine bending, 1378 cm^{-1} due to the CH_3 bending, 1229 cm^{-1} due to CF_3, 1068 cm^{-1} due to SO_2 whereas formulation F6 showed major peaks at 3291.22, 2961.47, 2908.04, 2738, 1462.20 and 1131.45 cm^{-1}.

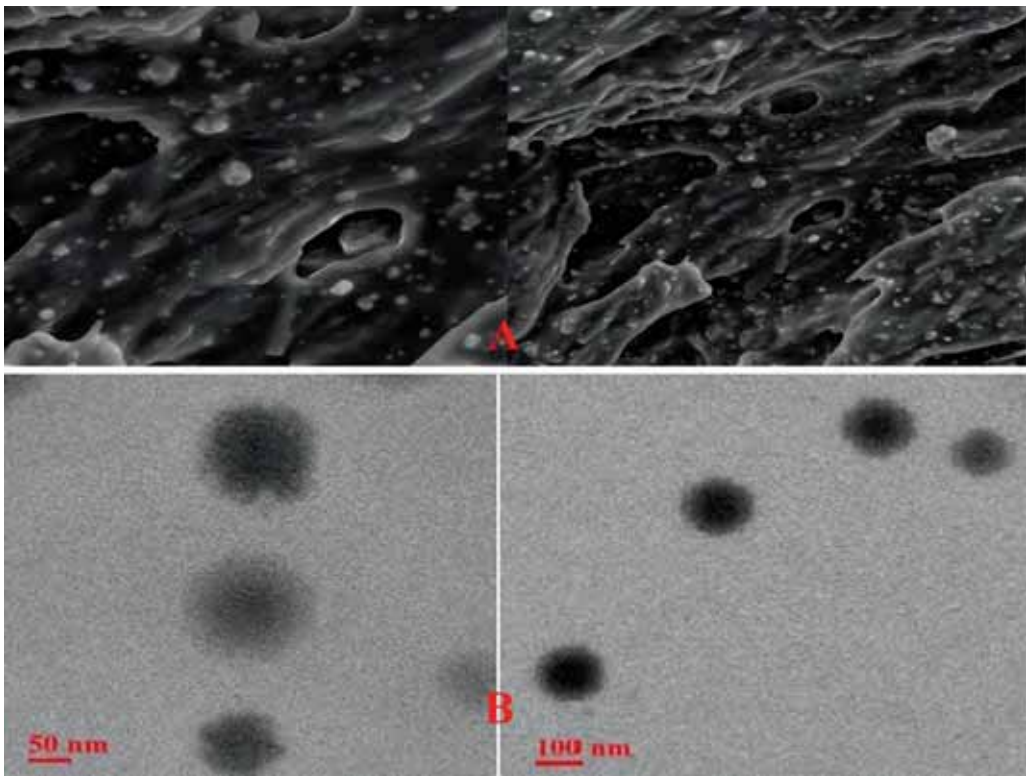

Figure 1. (**A**) SEM image of optimized formulated liposome (F6) and (**B**) TEM image of optimized formulated liposome (F6).

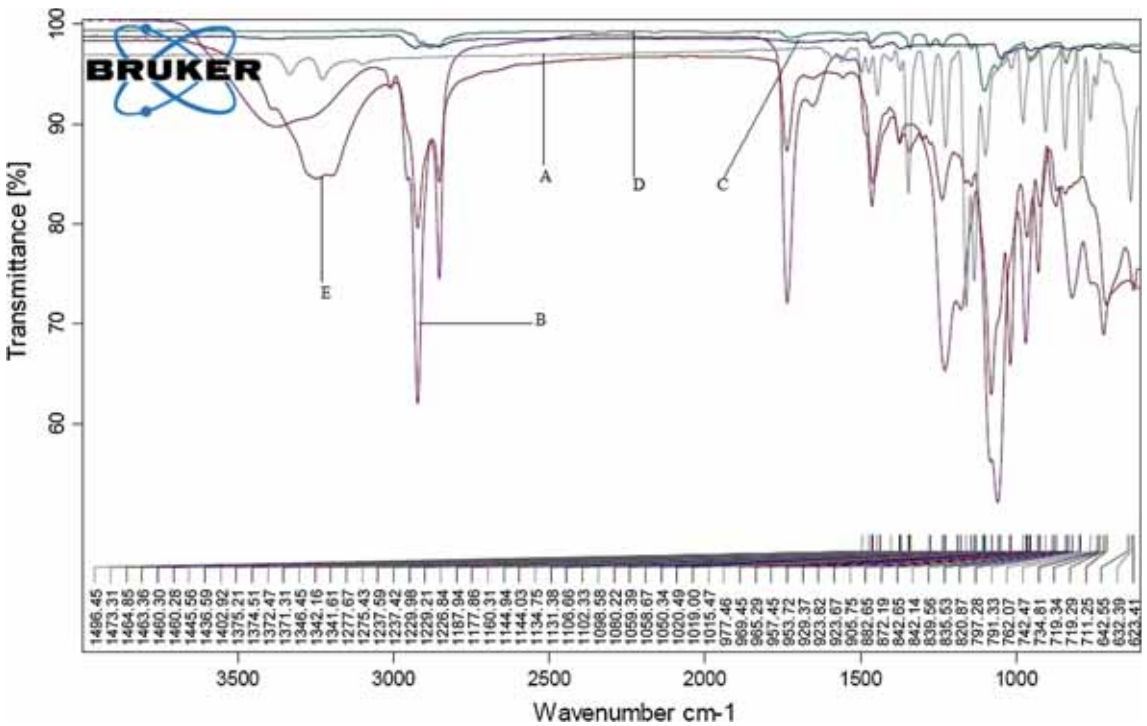

Figure 2. Overlay of FTIR spectra of (**A**) celecoxib, (**B**) Lipova E120, (**C**) cholesterol, (**D**) MPEG-DSPE$_{2000}$ and (**E**) liposomal formulation F6.

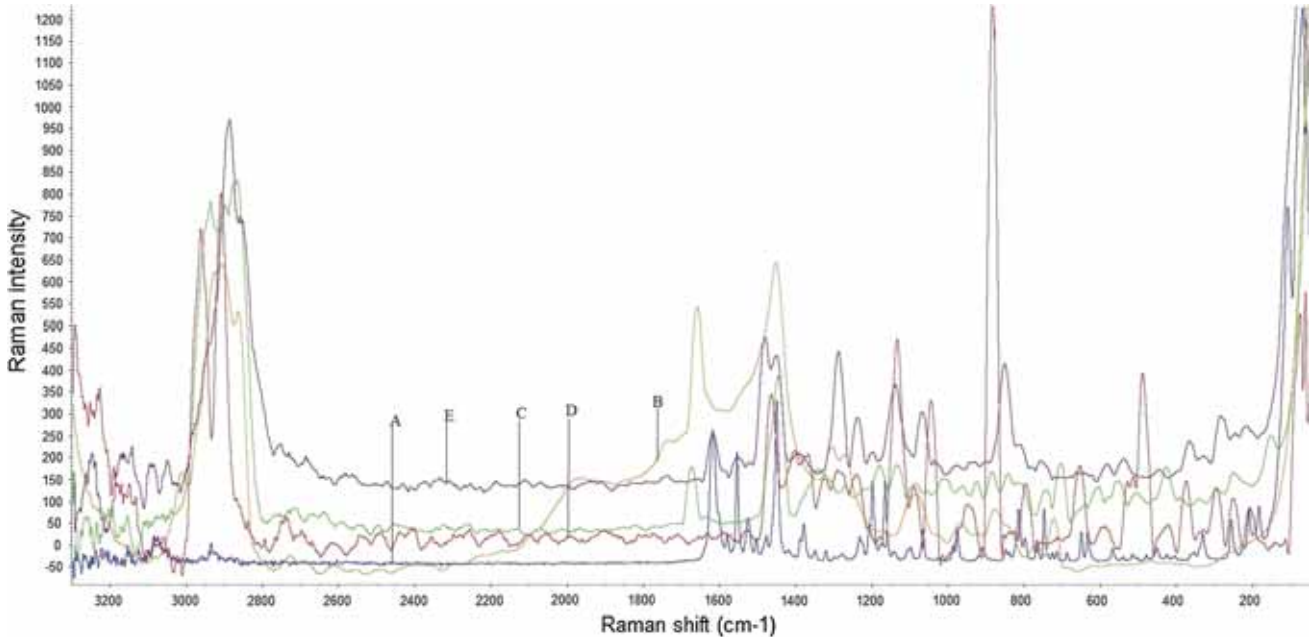

Figure 3. Overlay of Raman spectra of (**A**) celecoxib, (**B**) Lipova E120, (**C**) cholesterol, (**D**) MPEG-DSPE$_{2000}$ and (**E**) liposomal formulation F6.

3.5 *DSC analysis*

The DSC study was carried out to study the physical state of drug and lipid in the liposome and also used to study the interaction between drug and excipient. The DSC thermograms of celecoxib, Lipova E120, cholesterol, MPEG-DSPE$_{2000}$ and liposomal formulation F6 are shown in figure 4. Celecoxib showed a sharp endothermic peak at 166.77°C. Melting points of lipid (Lipova E120, cholesterol and MPEG-DSPE$_{2000}$) were observed at 134, 144 and 63.89°C. The melting point for celecoxib-loaded liposome F6 was observed at 145.74°C. The shift in the peak of the formulation toward lower temperature suggested that all lipid components present in the liposomal dispersion interact with each other and also with the drug to a great extent to form a bilayer of lipids and lead to the development of a new

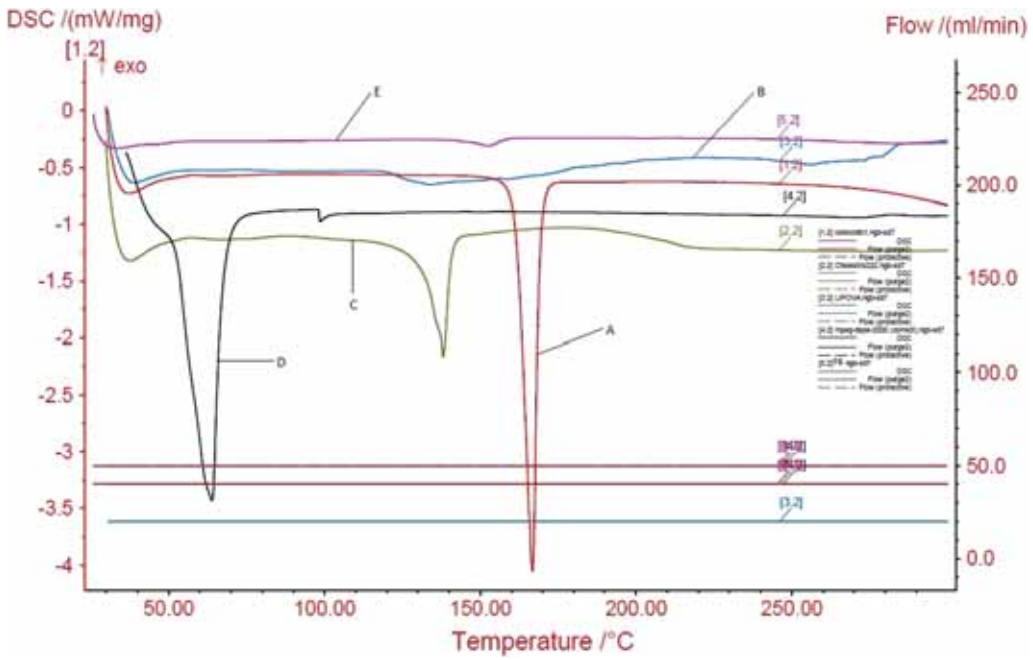

Figure 4. Overlaying DSC thermograms of (**A**) celecoxib, (**B**) Lipova E120, (**C**) cholesterol, (**D**) MPEG-DSPE$_{2000}$ and (**E**) liposomal formulation F6.

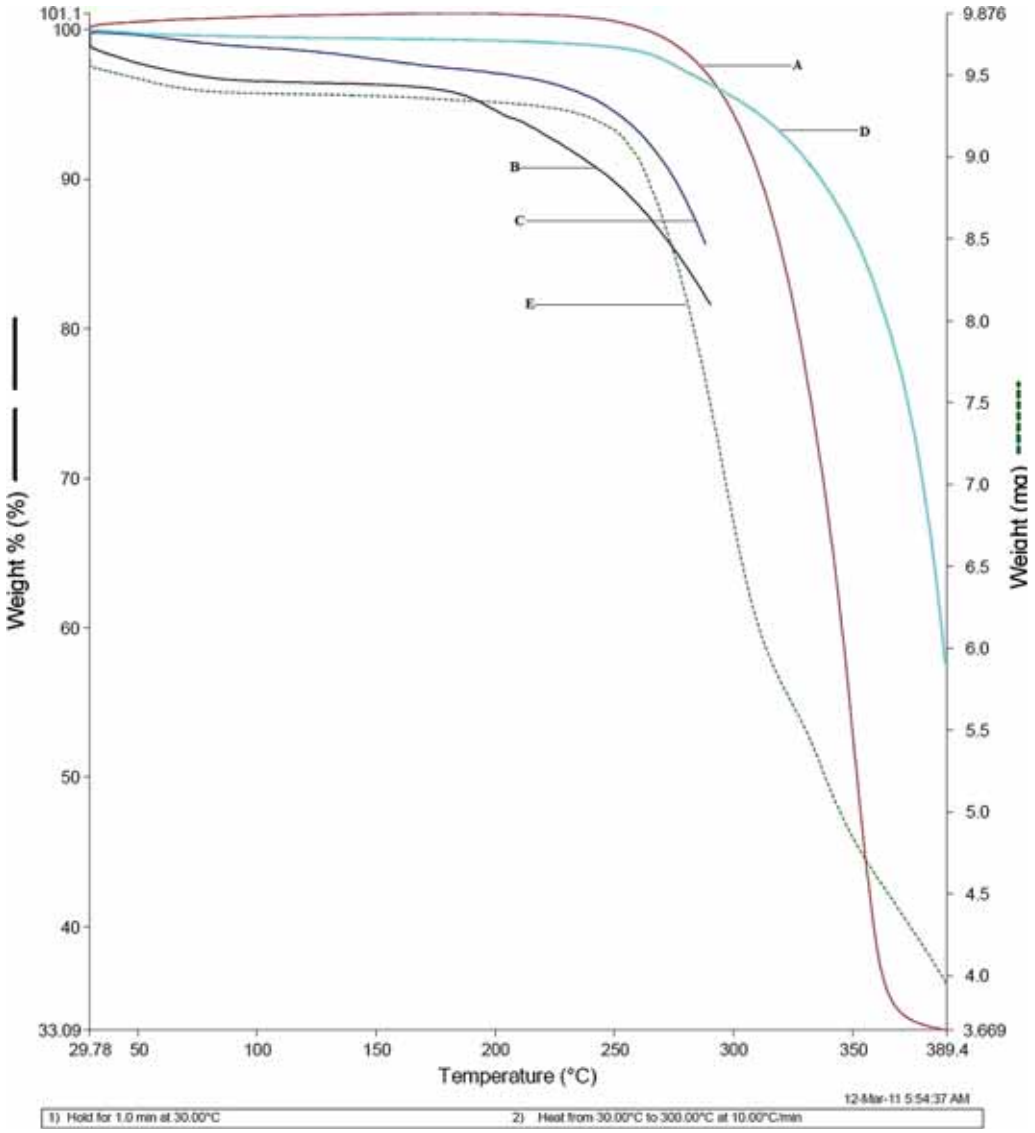

Figure 5. Overlay of TGA thermograms of (**A**) celecoxib, (**B**) Lipova E120, (**C**) cholesterol, (**D**) MPEG-DSPE$_{2000}$ and (**E**) liposomal formulation F6.

phase which decreases the crystallinity of the drug and became amorphous and thus increases the stability of the formulation.

3.6 *TGA analysis*

With the help of TGA, the thermal properties of celecoxib, Lipova E120, cholesterol, MPEG-DSPE$_{2000}$ and liposomal formulation F6 were studied and are shown in figure 5. The TGA thermograms of celecoxib, Lipova E120, cholesterol, MPEG-DSPE$_{2000}$ and optimized formulation F6 showed single curves due to the combustion of only component that depicted marked difference between the drug and optimized formulation; that means the drug is highly entrapped in vesicles. The % weight loss of celecoxib, Lipova E120, cholesterol, MPEG-DSPE$_{2000}$ and optimized formulation F6 started at 98, 97, 96, 98 and 96% at 270–380, 250–380, 220–280, 280–380 and 250–380°C. Therefore, TGA was a suitable tool for assessing the weight loss of various lipids and drug.

3.7 *Percentage EE of celecoxib-loaded PEGylated liposomes*

Encapsulation of celecoxib in the PEGylated liposomes depends on the amount of lipid used in the preparation and the ratio of drug to lipids. The results of EE are shown in table 2 and figure 6. For formulations F1–F4 with an increased Lipova E120 concentration, EE ranged from 64.3 ± 0.48 to 82.26 ± 0.56% and for formulation F5 with

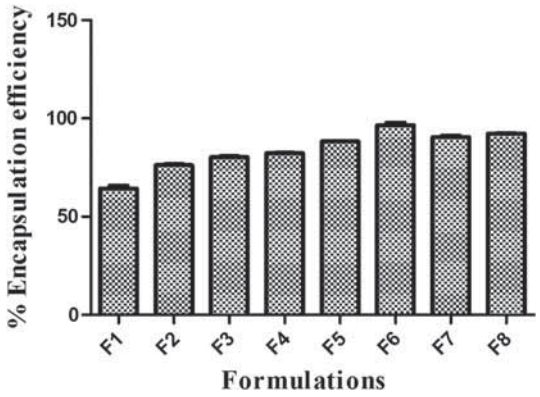

Figure 6. EE of celecoxib-loaded PEGylated liposomes.

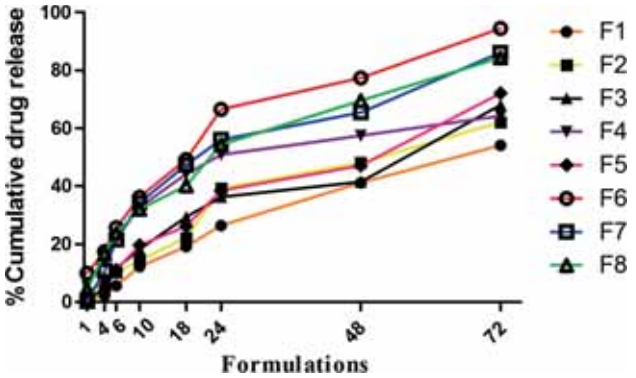

Figure 7. % Cumulative drug release of celecoxib-loaded PEGylated liposomes.

a high concentration of cholesterol along with Tween-80 EE was 88.35 ± 0.15%. Formulation F6 with an optimum concentration of Lipova E120 and cholesterol with MPEG-$DSPE_{2000}$ (in low concentration) having Tween-80 showed an EE of 96.6 ± 0.05%. Formulations F7 and F8 with an increased Lipova E120 and MPEG-$DSPE_{2000}$ concentration with Tween showed an EE of 90.4 ± 0.3 and 92.3 ± 0.23%, respectively. From the results, it was observed that formulation F6 showed the highest encapsulation of drug with an optimum concentration of lipids along with Tween-80. The study was performed in triplicate and results were expressed as mean ± SD.

3.8 *In-vitro drug release studies*

The release of celecoxib from the PEGylated liposomes was controlled with a change in concentration of lipid like Lipova E120, cholesterol and MPEG-$DSPE_{2000}$. The results of *in-vitro* release are shown in figure 7 and table 2. Formulations F1–F3 show a drug release of 54.43 ± 0.55, 62.13 ± 0.62 and 68.08 ± 0.35% in 72 h, whereas formulations F4 and F5 show a higher release of drug i.e. 64.83 ± 0.64 and 72.01 ± 0.83% in 72 h. Formulation F6 shows the maximum release i.e. 94.45 ± 1.13% due to the optimum concentration of lipids and high encapsulation of drug in the formulation. Thereafter, formulations F7 and F8 show a release of 86.09 ± 1.45 and 84.34 ± 0.34% in 72 h. The experiment was performed in triplicate and results were expressed as mean ± SD.

3.9 *Tail immersion assay*

Optimized formulation showed a dose-dependent significant increase in the reaction time as compared to pure celecoxib. Pure celecoxib shows a reaction time from 7.95 ± 0.27 to 9.14 ± 0.46 s. Formulation F6 at a dose of 10 mg/kg shows an increase in the reaction time i.e. from 8.69 ± 0.67 to 10.96 ± 0.58 s and at a dose of 20 mg/kg the reaction time increased from 8.99 ± 0.25 to 14.23 ± 0.75 s. To assess the central analgesic activity of the formulation, tail immersion assay was carried out. It is known that centrally acting analgesic drugs increase the pain threshold of rat associated with pressure and heat. The results of tail immersion assay are shown in table 3 and figure 8.

3.10 *Carrageenan-induced paw edema assay*

Carrageenan-induced paw edema is a widely used model to assess anti-inflammatory capacity of the formulation. A biphasic response is usually achieved after parentarally administered carrageenan, i.e. the first phase of 15–45 min includes the release of mediators such as bradykinin, serotonin and histamine, while the second phase of 60–90 min implicates the release of prostaglandins only. The % change in the paw volume was determined and conveyed as the swelling index (Cheow *et al.* 2011; Sofidiyaa *et al.* 2014). The assay shows that the optimized formulation at a dose of 10 mg/kg inhibits the increase in paw edema, i.e. 3.84 ± 0.02 to 3.30 ± 0.06 from 15 to 90 min and at a dose of 20 mg/kg inhibits the increase in paw edema from 3.65 ± 0.05 to 3.08 ± 0.06 as compared to pure celecoxib, i.e. 3.90 ± 0.05 to 3.68 ± 0.02 (Naveed *et al.* 2014). The results are shown in table 4 and figure 9. The study was further supported by Naveed *et al.* (2014).

3.11 *Stability study*

To identify the changes in the shape of liposomal vesicles and loss of drug after the storage of liposomes at different temperatures, a stability study was carried out as per the ICH guidelines. The optimized lyophilized formulation F6 was selected to perform the stability study and the results of the study are shown in table 5. The stability study was conducted at a refrigerator temperature (4 ± 2°C/60 ± 5% RH) and at room temperature (25 ± 2°C/60 ± 5% RH) for 90 days. At a predetermined intervals (7, 15, 30, 60 and 90), samples were taken and observed for particle size, PDI, zeta potential and EE. It was observed that at room temperature formulation shows a

Table 3. Screening of analgesic activity of optimized formulation using a tail immersion test in rats

Sl. no.	Treatment	Reaction time (s)			
		15 min	30 min	45 min	60 min
I	Control	6.81 ± 0.45	6.97 ± 0.68	7.28 ± 0.28	7.79 ± 0.12
II	Pure celecoxib	7.95 ± 0.27	8.28 ± 0.54	8.79 ± 0.85	9.14 ± 0.46
III	F6 (10 mg/kg)	8.69 ± 0.67	9.19 ± 0.49	10.14 ± 0.68	10.96 ± 0.58
IV	F6 (20 mg/kg)	8.99 ± 0.25	9.76 ± 0.54	12.46 ± 1.03	14.23 ± 0.75

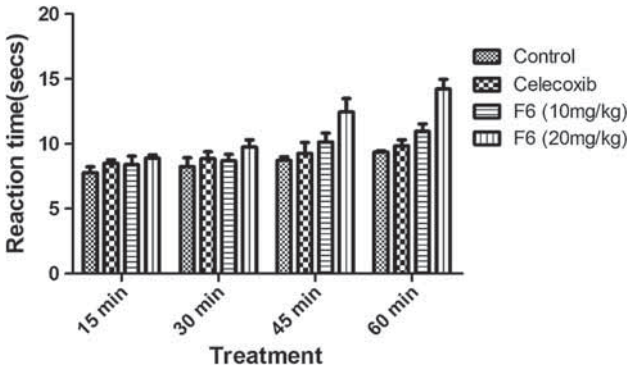

Figure 8. Effect of formulation F6 on reaction time in the tail immersion test.

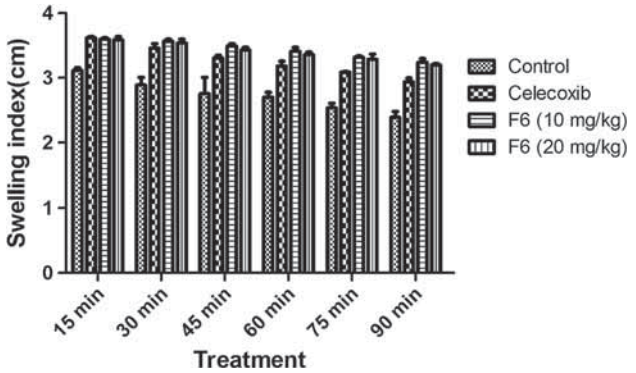

Figure 9. Effect of formulation F6 on the swelling index in carrageenan-induced inflammation.

more decrease in the % EE and the particle size much increased much as compared to formulation stored at 4 ± 2°C/60 ± 5% RH. The study was performed in triplicate and results were expressed as mean ± SD.

4. Discussion

From the results of the particle size and PDI, it was concluded that on using a higher concentration of Lipova E120, cholesterol and MPEG-DSPE$_{2000}$ the particle size of vesicle was increased and the addition of Tween-80 into the formulations (F5–F8) results in the decrease of the particle size as compared to the formulations (F1–F4) which does not contain Tween-80. Thus, Tween-80 helps us to provide stability to the liposomes by providing a stearic barrier on the surface of liposomes. From the results of the particle size, formulation F6 was optimized. Zeta potential assay shows that formulation which contains Tween-80 in the formulation possesses negative charge which helps in providing more stability to the liposomes. Negative charge provides the repulsive force to the particles and thus decreases the aggregation of the particles and increases their stability. From the result formulation F6 was optimized.

From the results of SEM and TEM, it was determined that the PEGylated liposomes were smooth and spherical in shape and no visible cracks and pinholes were visualized in the formulation.

The ATR-FTIR study shows that when the peak of celecoxib was compared with the peak of the formulation it was observed that there was a shift in the peak of the formulation as compared to the peak of pure celecoxib which shows that an interaction occurred between the drug and lipid, indicating that the drug gets entrapped in the lipid bilayer vesicles leading to the formation of a new phase. The Raman study shows that the peak of celecoxib and peak of formulation were overlapping indicating the presence of celecoxib in the PEGylated liposome. The DSC study shows that the

Table 4. Screening of anti-inflammatory activity of optimized formulation in carrageenan-induced paw edema in rats

Sl. no.	Treatment	Swelling index (cm)					
		15 min	30 min	45 min	60 min	75 min	90 min
I	Control	3.99 ± 0.05	3.95 ± 0.06	3.90 ± 0.04	3.85 ± 0.04	3.80 ± 0.08	3.76 ± 0.02
II	Pure celecoxib	3.90 ± 0.02	3.82 ± 0.03	3.75 ± 0.04	3.69 ± 0.06	3.58 ± 0.02	3.50 ± 0.06
III	F6 (10 mg/kg)	3.84 ± 0.02	3.66 ± 0.07	3.59 ± 0.04	3.50 ± 0.08	3.44 ± 0.18	3.30 ± 0.06
IV	F6 (20 mg/kg)	3.83 ± 0.05	3.59 ± 0.06	3.47 ± 0.04	3.30 ± 0.04	3.20 ± 0.08	3.08 ± 0.02

Table 5. Stability study of lyophilized formulation F6

Time (day)	Microscopic observation	Particle size (nm)	Zeta potential (mV)	% Encapsulation efficiency
At 25°C/60 ± 5% RH (n = 3)				
Initial	Smooth spherical vesicles	92.12 ± 1.7	− 40.8 ± 1.7	96.6 ± 0.05
7	Smooth spherical vesicles	95 ± 0.65	− 38.2 ± 0.76	91.18 ± 0.75
15	Smooth spherical vesicles	103 ± 0.98	− 34.09 ± 1.98	88 ± 0.95
30	Smooth spherical vesicles	117 ± 1.98	− 30.76 ± 0.87	81.09 ± 0.64
60	Rough spherical vesicles	129.87 ± 0.95	− 27.03 ± 2.8	74.07 ± 0.54
90	Rough spherical vesicles	135.98 ± 0.75	− 23.95 ± 0.65	66.54 ± 0.09
At 4°C/60 ± 5% RH (n = 3)				
Initial	Smooth spherical vesicles	92.12 ± 1.7	− 40.8 ± 1.7	96.6 ± 0.05
7	Smooth spherical vesicles	93 ± 1.05	− 39 ± 1.2	95.5 ± 0.75
15	Smooth spherical vesicles	95 ± 0.65	− 37.69 ± 0.8	91.3 ± 0.95
30	Smooth spherical vesicles	98 ± 0.73	− 36.83 ± 0.67	89.04 ± 0.64
60	Rough spherical vesicles	111.63 ± 1.87	− 34.03 ± 0.23	85.07 ± 0.54
90	Rough spherical vesicles	120.98 ± 0.23	− 34.95 ± 0.61	81.54 ± 0.09

formulation peak shifts toward lower temperature indicating a thermodynamically stabilizing interaction between different polymers used in the formulation and it can be concluded that encapsulation of drug could retard the release of drug from the liposome.

EE shows that formulations F1–F4 show a decrease in the encapsulation of drug because they do not contain Tween-80 as it is a type of non-ionic surfactant which increases the solubility of the drug because it gets permeate between the junction of drug and lipid and thus increases the encapsulation of drug or decreases the leakage of drug from liposomes. With a further increase in the MPEG-$DSPE_{2000}$ concentration in formulations F7 and F8, it was observed that there was no major difference in EE which could be due to the increased binding of celecoxib to the lipid. Cholesterol was used in the liposomal formulation to increase the packing of the lipid bilayer but at higher concentration it decreases the EE of drug (Betageri and Parsons 1992). Hence the main optimization parameter for the PEGylated liposome preparation was the molar ratio of lipids as it affects the encapsulation of drug in the PEGylated liposomes. The results were found according to Shavi *et al.* (2016).

From the results of the *in vitro* study, it was observed that formulations F1–F4 initially show a rapid release of drug due to the absence of MPEG-$DSPE_{2000}$ and Tween-80 as lipid bilayers are not stabilized and the drug is leeched out very fast and also due to the low encapsulation of drug in the formulation. Formulations F6–F8 show the maximum release of drug in 72 h which indicates that the PEGylated liposomes show a sustained release of drug and will help to retain the drug in the systemic circulation for longer duration. PEGylated liposomes will help to provide a depot effect because the lipid bilayers are stabilized with the help of Tween-80 and cholesterol due to which the drug takes time to release out. From the overall study, it was concluded that a small variation in the lipid molar ratio may show a drastic variation in the release of drug. The results were further supported by Er *et al.* (2009). According to the obtained results formulation F6 was optimized and shows a sustained release of drug.

Formulation F6 was used for the *in-vivo* study because it exhibits minimum particle size, high zeta potential, high EE and good drug release profile as compared to all other formulations.

The results obtained from the tail immersion assay indicate that the antinociceptive activity of the liposomal formulation may expressively include the central mechanism of action and the study was further supported by Sofidiyaa *et al.* (2014). From the study, it was concluded that formulation F6 at a dose of 20 mg/kg show a much increase in the reaction time as compared to the dose at 10 mg/kg. From the carrageenan-induced paw edema assay, it was concluded that formulation F6 at a dose of 20 mg/kg shows a much decrease in the swelling index as compared to the dose of 10 mg/kg. From the overall stability study, it was concluded that the formulation was more stable when it was stored at refrigerator temperature.

Overall, it can be concluded that due to the nanosize of the liposomes they can be easily given by parenteral administration and thus the PEGylated liposomes will provide a sustained release of drug and helps in achieving the depot effect. However, further studies are still required to confirm the efficacy of formulation in varied pathological and physiological conditions of recipients.

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