Chapter 2

Physicochemical studies on local anaesthetic loaded second generation nanolipid carriers

Abstract: This study is aimed to investigate the effect of hydrocarbon chain length of nonionic surfactants, Tween 40 and Tween 60, on the physicochemical properties of nanostructured lipid carriers (NLCs). Two local anaesthetics, lidocaine (LIDO) and procaine hydrochloride (PRO.HCl), were incorporated in the NLCs. NLC formulations were prepared using sorbitantristearate (Span 65), soy lecithin (SLC) and stearic acid (SA) in 2:2:1 mole ratio employing the hot homogenization technique. Systems were characterized by combined dynamic light scattering (DLS), transmission electron microscopy (TEM), differential scanning calorimetry (DSC) and spectroscopic studies. Formulations were found to be stable upto 60 days when kept at 4 ºC. NLCs stabilized by Tween 60 were superior to the corresponding Tween 40 based formulations. Spherical morphology with smooth surfaces was evidenced by TEM measurements. DSC and polarity studies indicated that LIDO altered the crystallinity of the lipid matrices as it could insert into the core of the NLC. Entrapment efficiency (EE) and loading content (LC) studies revealed that Tween 60 stabilized NLCs have better drug loading capability than the Tween 40 based formulation. Controlled and prolonged drug release was experienced by Tween 60 stabilized drug loaded NLCs as studied by *in vitro* release kinetics. The developed NLCs could thus be considered to have prospects as novel drug carriers for controlled/sustained release to improve the time duration of anaesthesia, especially for topical application.

RSC Adv., 2015, 5, 26061

1. Introduction

For decades, attempts have been made to develop promising drug carriers associated with improved bioavailability, increased therapeutic activities and sustained release using lipid and polymeric nanoparticles[1-5]. However, it has

yet not been possible to develop a single suitable drug delivery system with all the advantages. Different types of drug carriers have been designed using polymers, lipids and/or their composites; each one having its own merits and demerits[4,6-14].[4,6-9,11-15] Attempts towards developing efficient drug delivery systems experienced limited success in the pharmaceutical markets. The present day drug delivery systems include nanoemulsion[13], polymeric nanoparticle[8], liposome[9], solid lipid nanoparticle (SLN)[16] and nanostructured lipid carrier (NLC), a modified form of SLN[5]. Hecht et al. and associates researchers have made a significant contribution in developing different drug delivery systems. These include the polymeric nanoparticles[17], micellar nanoparticles[18], peptides[19], disaccharides moieties of bleomycin[20,21], *etc*. It has been found that some other peptide based drug delivery systems, however, endure poor intracellular delivery and target specific selectivity[19,22]. The major drawbacks associated with nanoemulsions include its physical wavering due to the partitioning of drug into the aqueous phase. Besides, the controlled release cannot be assured due to the high mobility of drug incorporated in the system[12,13,23]. Polymeric nanoparticles suffer from its precincts, *viz.*, cytotoxicity during internalization and degradation inside the cell, non feasibility of large scale production, *etc*[17,18,24]. Also the polymer based drug delivery systems are susceptible to some chemical transformations, *viz.*, hydrolysis during storage and the resulted metabolites may be responsible for some serious toxicity[8,10,25,26]. Although the aforementioned limitations could successfully be overcome with the advent of small molecule based drug delivery systems and liposomes, however, such systems suffer from limitations like the physical unsteadiness, drug leakage, non-specific clearance, cellular penetration efficiency, selectivity and high cost of the excipients, *etc*[9,20,21,26,27].

Since 1990, solid lipid nanoparticles (SLNs) have extensively been explored as potential drug carriers[2,16,28,29]. SLNs are typically spherical particles with an average size between 100 - 1000 nm[30]. The components of SLNs are biocompatible and biodegradable under physiological conditions that minimize the risk of toxicity. The lipids used are solid under physiological condition. It has been reported that the SLNs are capable to act as suitable drug carrier, stable in gastrointestinal fluids and provide improved bioavailability to the drugs[1,2]. SLNs

form highly crystalline lattice due to structurally (hydrocarbon chain) similar lipids which subsequently can afford limited space to drug molecules; this eventually results in the rapid drug expulsion during storage. Such limitation of SLNs could be overcome with the introduction of its modified form, known as nanostructured lipid carriers (NLCs). NLCs usually comprise structurally dissimilar lipidic components; the mismatch in the hydrocarbon chain results in the generation of multicrystalline lipid matrices. Presence of imperfection/void spaces can accommodate significant amount of drug. Subsequently, NLCs can have high entrapment efficiency, loading content, controlled drug release, long term physical stability, preserved chemical degradation of drug during storage, *etc*[5,14,31-34].[5,14,31-34] It has also been reported that NLCs can act as suitable carriers for both hydrophilic and lipophilic drugs[35]. Because of their unique particle size (100 - 1000 nm), internalization of such drug delivery systems into the cells become efficient; this facilitates site specific delivery of therapeutic agents[36,37]. Research on NLCs have thus gained significant importance because of their perspective, yet unexplored application potentials as drug delivery systems for the different routes of administration with an aim to improve the biodistribution and therapeutic efficacy[5,32].

Different types of monoglycerides, diglycerides, triglycerides, waxes, phospholipids and fatty acids have extensively been used to develop NLCs[30]. However, use of sorbitan tristearate (Span 65) as one of the lipidic component in preparing NLCs is not common in literature, in spite of its biocompatibility[38]. It is not unexpected that imperfection/void spaces in the NLC matrices would exist if it is used in combination with soy lecithin and stearic acid. Hydrocarbon chain of the stabilizers also has pronounced effect on creating imperfections. The effect of stabilizers on the solution phase behavior and thermal properties of NLC such as temperature of maximum heat flow (T_m), peak width at half maxima ($\Delta T_{1/2}$), change in enthalpy (ΔH), heat capacity (ΔC_p) and crystallinity index (C.I.) have not meticulously been investigated. The hydrocarbon chain mismatch is expected to make the formulation as a novel carrier for lipophilic, hydrophilic as well as amphiphilic drug molecules. The study related to drug location in NLC is exceptional in literature and more investigations are warranted in this regard. Location of the drugs in the NLC could be predicted with the help of

spectroscopic as well as thermal investigations. In addition, influence of hydrocarbon chain length on the entrapment efficiency, loading content and release kinetics are not well established for this type of small molecules loaded in NLC. Our present study is intended to explore such systems on the basis of detailed physicochemical characterization.

Lidocaine (LIDO) and procaine hydrochloride (PRO.HCl) are frequently used as local anaesthetics for topical application[25,39,40]. Both LIDO and PRO.HCl are frequently used in order get relieves from pain itching, burn and cutaneous inflammation, *etc*[41]. They induce pain relief by blocking fast voltage-gated sodium channels in the cell membrane of postsynaptic neurons. Thus they prevent depolarization and inhibit the generation and propagation of nerve impulses[42].[42] LIDO and PRO.HCl are marketed as Xylocaine® and Novocaine® respectively[39,43]. While considering the aforementioned dermal applications, it is one of the essential condition that the drug should preferably remain on the skin surface, thus minimizing its side effects[44,45]. Besides, in case of topical formulation, it should be ensured that there should be adequate localization of drug[41], as well as sustained release[39]. In spite of a number of available reports on local anaesthetics loaded drug delivery systems, however, no such single system have been found to be completely prudent in terms of topical applications. The major drawbacks of local anaesthetics in topical applications are characterized by cutaneous lesions, urticaria, edema, *etc*[44,45]. However, the major concern using LIDO or PRO.HCl is its penetration through skin which subsequently increases the plasma level in blood[46]. Studies demonstrated by different researchers[25,39,41] suggest that the severe side effects can drastically be reduced when loaded in suitable drug delivery system. Carafa et al.[41] have made a comparative study on the permeability of LIDO and its protonated form (LIDO.HCl). They have found that for classical liposome formulations permeability of hydrochloride derivative (LIDO.HCl) was less than the corresponding free base. It was rationalized on the basis that the free base, being more lipophilic, could permeate through the hydrophobic membrane bilayer than the hydrochloride derivative. We tried to ensure the validity of this rationalism for NLC formulation using LIDO and PRO.HCl. In order to prolong the anaesthetic effect and reduce dose frequency as well as the skin irritation caused by the high dose of anaesthetics, such formulations are considered worthy to be investigated.

The present study endeavours to investigate the effect of hydrocarbon chain length of the stabilizers (herein Tween 40 and Tween 60) on the formulation and physicochemical properties of NLC. NLCs were prepared by mixing sorbitan tristearate (Span 65), soy lecithin and stearic acid by way of hot homogenization technique in the absence and presence of varying amount of two local anaesthetics, LIDO and PRO.HCl. Dynamic light scattering studies were performed to determine the hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) of the NLCs. Thermal behaviour and the associated parameters, *viz*., temperature of maximum heat flow (T_m), crystallinity index, enthalpy change (ΔH) and heat capacity change (ΔC_p) of the lipid matrices in the absence and presence of the anaesthetics were evaluated by differential scanning calorimetry (DSC). Location and subsequent state of polarity of the drugs were investigated by UV-visible absorption spectroscopy. Furthermore, entrapment efficiency (EE), loading content (LC) and *in vitro* release kinetics of the drugs from the NLCs were studied. It is believed that the limitations of LIDO and PRO.HCl can be circumvented by incorporating them in NLC which are expected to release the anaesthetic in controlled and prolonged fashion at the site of action, even when present in high dose.

2. Materials and methods

2.1. Materials

Sorbitan tristearate (Span 65, 99%) was purchased from S. D. Fine-Chem Ltd., India. [(2R)-2,3-di(tetradecanoyloxy) propyl]-2- (trimethylazaniumyl) ethyl phosphate (soy lecithin, SLC, 98%) was a product from Calbiochem, Germany. Stearic acid (99%) as well as the nonionic surfactants polyoxyethylene (20) sorbitanmonopalmitate (Tween 40) and polyoxyethylene (20) sorbitanmonostearate (Tween 60), all of 98% purity, were purchased from Sisco Research Laboratory, India. Lidocaine (LIDO, 98%), procaine hydrochloride (PRO.HCl, 97%) and the dialysis bag (12 kDa MWCO) were obtained from Sigma-Aldrich Chemicals, USA. All the materials were used as received. HPLC grade solvents from Merck, India were used. Double distilled water with a specific conductance of 2 - 4 µS (at 25 ºC) was used throughout the experiment.

2.1. Methods

2.1.1. Preparation of NLCs

Nanostructured lipid carriers (NLCs) were prepared by hot homogenization method followed by ultrasonication[14,47]. Quantitative amount of Span 65, SLC and SA (2:2:1 M/M/M) was taken in a round bottom flask and was dissolved in chloroform/methanol mixture (3:1, v/v). A thin film was generated in a rotary evaporator. The homogenized thin film was then heated at 70 ºC (5 - 10 ºC above the melting point of all the lipidic components). Aqueous Tween solution (10 mM, preheated was stirred at 1000 rpm using a magnetic stirrer. The coarsely at same temperature) was then added to the molten lipid mixture and emulsified dispersion was then exposed to ultrasonication for an hour (Takashi U250, Tokyo, Japan). The clear medium was then cooled down to room temperature whereby the stable NLC formulation was achieved. The total lipid concentration for all the formulations was kept constant at 5 mM and 10 mM nonionic surfactants (Tween 40 and Tween 60) were used separately as the stabilizers. Different formulations were prepared as either blank-NLC and LIDO or PRO.HCl loaded NLC whereby the drug concentration was varied in the range of 0.5-2.5 mM.

2.1.2. Instrumentation

The mean particle size, polydispersity index (PDI) and zeta potential (Z.P.) of the NLCs in the absence and presence of the two drugs were investigated by dynamic light scattering (DLS) studies (Zetasizer Nano ZS90, ZEN 3690, Malvern Instrument Ltd., U.K.). Data were recorded at 90º using a He-Ne laser (632.8 nm). Prior to measurement all the samples were filtered using 0.45 µm cellulose acetate membrane. Considering apparent spherical geometry, surface area of the NLC was calculated. Taking into account of the average molecular cross sectional area of the individual lipidic components, the NLC concentration was found to be of the order of 25 nM for an overall 5 mM lipid concentration. It is also assumed that at this fairly dilute concentration the inter particle interaction was insignificant. Shape and morphology of the NLCs were investigated by transmission electron microscopy (TEM, Hitachi, Japan). UV-visible absorption spectra of drug loaded NLCs were recorded by a UV-visible spectrophotometer (UVD-2950, Labomed Inc., USA) in the range of 200 - 400 nm; corresponding

NLC without the drug was used as reference. Thermal analyses were carried out using a differential scanning calorimeter, DSC 1 STAR^e system (Mettler Toledo, Switzerland). Samples were sealed in 40 µL standard aluminum pans and were quickly equilibrated in the temperature range of 15 - 80 ºC within 15 min. DSC scan was performed with a scan rate of 2 ºC/min in the temperature range 15 - 80 ºC under nitrogen purge for both heating and cooling cycles. Corresponding surfactant, used as stabilizer for NLC preparation, was used in the reference pan. Thermal parameters, *viz.*, temperature of maximum heat flow (T_m), peak width at half maximum ($\Delta T_{1/2}$), changes in enthalpy (ΔH) and specific heat capacity (ΔC_p) were evaluated using DSC-STAR^e software. Entrapment efficiency (E.E.) and loading content (L.C.) of both LIDO and PRO.HCl loaded NLCs were evaluated by measuring the free drug concentration in the continuous phase of the NLC dispersion[48]. Briefly, 10 mL of drug loaded NLC dispersion was centrifuged at 10000 rpm for 1 hr at 4 ºC (REMI, India). The drug loaded NLC thus got precipitated. The amount of free drug in the supernatant was quantified by measuring the absorbance at 264 nm and 293 nm for LIDO and PRO.HCl respectively (absorbance maxima of the drug in the Tweens). Entrapment efficiency (E.E.) and loading content (L.C.) were subsequently calculated by the following equation:[48,49]

$$\% \text{ E.E.} = \frac{Weight_{intotal} - Weight_{freedrug}}{Weight_{intotal}} \times 100 \qquad (1)$$

$$\text{Loading Content } \% (LC) = \left(\frac{W_a - W_s}{W_a + W_s - W_l}\right) \times 100\% \qquad (2)$$

where, W_a, W_s and W_L were the weight of drug added in the NLC, analyzed weight of drug in supernatant and weight of lipid added in NLC, respectively. *In vitro* release kinetics was monitored by conventional dialysis bag method using aqueous surfactant solution as the release medium[14]. Dialysis bag (MWCO12 KDa) was soaked in the corresponding release medium overnight. Freshly prepared 10 mL drug loaded NLC dispersion was placed in the dialysis bag sealed and immersed in a beaker containing 50 mL of release medium with constant stirring. 2 mL of the aliquot was withdrawn at different time interval and was replaced by 2 mL of fresh release medium to maintain the sink condition.

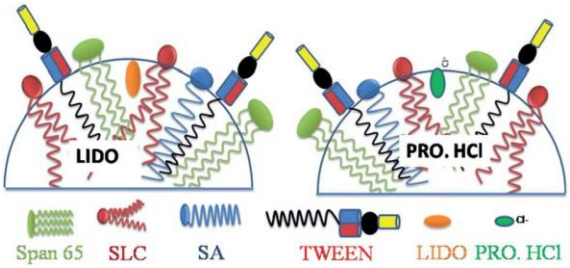
Quantification of LIDO and PRO.HCl was made colorimetrically at 264 and 293 nm respectively.

All the experiments, except the calorimetric studies, were carried out at controlled room temperature (25 ºC). An average of three measurements has been reported for each set of studies.

3. Results and discussions

3.1. DLS studies

Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) values are some of the stability indicators of NLC formulation[50]. Effect of hydrocarbon chain length of the Tweens and drug payload on d_h, PDI and Z.P. values of different NLC formulations were studied by DLS technique. d_h - time profiles for different NLC formulations in the absence and presence of the drugs have been presented in Figure 1. d_h values were found to be dependent on the type of Tween surfactant as well as on the nature and concentration of the local anesthetics. NLC formulations were stable upto 60 days, after which phase separation of the components was noticed. Size of the blank NLCs depended on the hydrocarbon chain length of Tween. In case of Tween 40 stabilized NLC, size varied in the range of 250 - 475 nm; for Tween 60 the values were in the range of 43 - 75 nm. In both the cases d_h values increased with time.



Scheme 1. Proposed model for the organization of lipids and drugs in the NLC formulations stabilized by Tweens.

Increase in hydrodynamic diameter was due to the structural reorganization of the lipidic components as well as the Ostwald ripening process, common for the colloidal dispersions[51,52]. Size constriction, in case of Tween 60 stabilized systems, compared to Tween 40, can be rationalized by considering the insertion of the hydrocarbon chain of Tweens into the NLC matrices, as proposed in Scheme 1. Because of its similarity in the hydrocarbon chain length with the

other lipidic components (stearic acid and Span 65, both having C_{18} hydrocarbon chains), Tween 60 could get inserted in a better way than Tween 40.

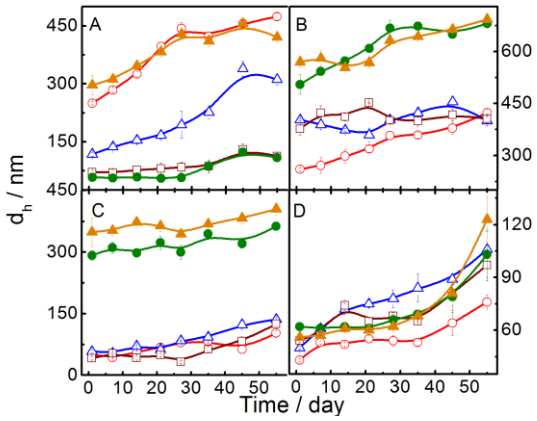


Figure 1. Hydrodynamic diameter (d_h) – time profile of NLCs (Span 65 + SLC + SA , 2:2:1 M/M/M) dispersed in Tweens in presence of varying concentration of drugs. Panel A: LIDO loaded NLC in Tween 40; panel B: PRO.HCl loaded NLC in Tween 40; panel C: LIDO loaded NLC in Tween 60 and panel D: PRO.HCl loaded NLC in Tween 60. 5 mM NLC was dispersed in 10 mM Tweens in each case. Drug concentration (mM) : O, 0; Δ , 0.5; □, 1; ●, 2 and ▲, 2.5 . Temp. 25 ºC.

Size of the drug loaded NLC formulations depended on type of the Tweens used as well as the anesthetics and its concentration. While LIDO led to an overall increase in the size of the NLC formulations, for PRO.HCl loaded systems, the effects were less significant. PRO.HCl, being ionic in nature, is expected to reside on the palisade layer of the NLCs. On the contrary, LIDO, being more lipophilic, is expected to get inserted into the lipidic core to higher extent, which subsequently results in the size enhancement of the NLCs.

However, insertion of LIDO into the NLC core resulted in an increase in polydispersity as well. In fact, similar observations were experienced while considering the PDI values of the formulations. Results are shown in Figure 2. It was observed that for PRO.HCl loaded NLCs PDI values were lower than the LIDO loaded systems. Results clearly suggest that PRO.HCl comprising systems were more homogeneous than the other. It was also observed that in case of PRO.HCl the PDI values did not appreciably change with time, however for LIDO loaded systems significant increase in PDI value with time was noticed. Size of the NLCs decreased with increasing LIDO concentration for Tween 40 stabilized systems. On the contrary increasing concentration of LIDO resulted in

the size enhancement for Tween 60. This can further be rationalized on the basis of hydrocarbon packing of the Tweens in the NLC core.

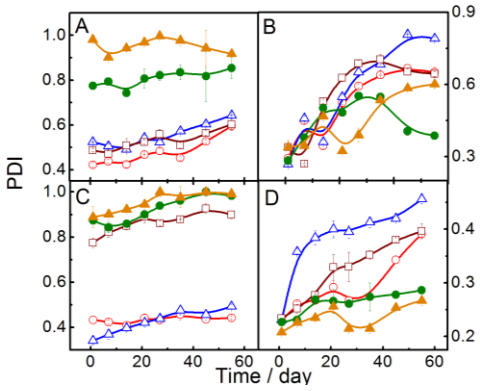


Figure 2. Variation in the polydispersity index (PDI) of NLCs (Span 65 + SLC + SA, 2:2:1 M/M/M) with time in presence of varying concentration of drugs. Panel A: LIDO loaded NLC in Tween 40; panel B: PRO.HCl loaded NLC in Tween 40; panel C: LIDO loaded NLC in Tween 60 and panel D: PRO.HCl loaded NLC in Tween 60. 5 mM NLC was dispersed in 10 mM Tween in each case. Drug concentration (mM) : O, 0; Δ , 0.5; □, 1; ●, 2 and ▲, 2.5 . Temp. 25 ºC.

The drug LIDO, being amphiphilic in nature, enhances the adsolubilization of Tween 40 over the NLC surface. However, in case of Tween 60, addition of LIDO, which prefers to get deeply inserted into the NLC, will result in the swelling of the NLC core; subsequently sizes of the formulations were enhanced. Almost similar effect was noticed in case of PRO.HCl.

Like other colloidal dispersions, NLC formulations are also charged which impart the kinetic stability[53]. Effects of drug concentration on the magnitude of zeta potential are graphically shown in Figure 3. In all the cases zeta potential values were negative, due to the presence of the dissociated form of stearic acid. Magnitude of negative zeta potential values were higher in case of Tween 60 stabilized systems, suggesting higher dissociation of the fatty acid. In case of Tween 60, NLC surface is less masked by the hydrophobic environment because of its better insertion capability (as proposed earlier). Subsequently dissociation of the fatty acid becomes easier compared to Tween 40. Magnitude of the negative zeta potential increased with increasing LIDO concentration. LIDO being amphiphilic in nature results in the adsolubilization of the lipidic components for which the dissociation of the fatty acid becomes easier. In case of PRO.HCl, as it is positively charged, it is not unexpected that it will mask the

zeta potential through interfacial adsorption and charge neutralization. However such systems were found to be equally stable as the LIDO loaded systems. Steric stabilization by the Tweens could prevent the agglomeration of such NLC formulations.

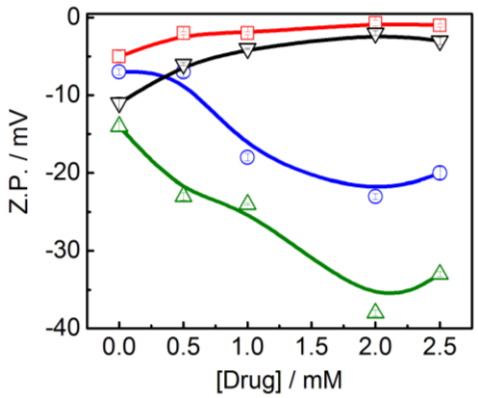


Figure 3. Influence of LIDO and PRO.HCl on the (Z.P.) of NLCs (Span 65 + SLC + SA , 2:2:1 M/M/M) dispersed in Tweens. Systems: O, LIDO -Tween 40; Δ, LIDO -Tween 60; □, PRO.HCl-Tween 40 and ▽, PRO. HCl -Tween 60. Temp. 25 ºC. 5 mM lipid in the absence and presence of the drug was dispersed in 10 mM aqueous Tween solution.

3.2. Transmission electron microscopy (TEM) study

Shape and morphology of the NLCs were checked by TEM in order to get more information about particle size and shape. Spherical and smooth surface morphology of the NLCs were observed (Figure 4). NLCs were almost spherical in shape within 100 - 500 nm range, which were reflected with the size data determined by DLS. Smaller size, as obtained by the TEM studies, was probably due to the drying phenomena during sample preparation.

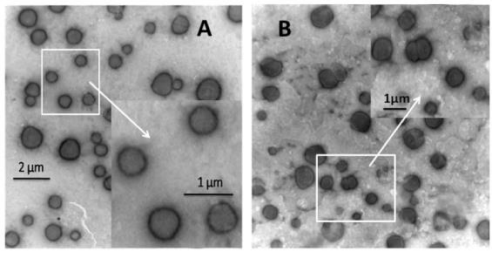


Figure 4. RepresentativeTEM images of LIDO loaded NLC dispersion in Tween 40 (A) and PRO.HCl loaded NLC dispersion in Tween 40 (B).

3.3. Differential scanning calorimetric (DSC) studies

Results on the DSC studies are presented in Figures 5 - 8, and Table 1 respectively. Figure 5 demonstrates the general pattern of the endothermic and exothermic peaks during heating and cooling scans respectively for LIDO loaded NLC dispersed in Tween 60 aqueous solution. While the heating curve was broader and shallow, the cooling curve was well defined, more pronounced, narrow and sharp. This kind of observation is not uncommon in the literature[54]. As the exothermic peaks were more prominent, hence the cooling curves were taken into account for further data analyses. The exothermic peaks of the drug free as well as drug loaded NLC formulations were comparatively broader compared to the previously reported systems[40]. DSC cooling curves for different NLC formulations in the absence and presence of drugs are shown in Figure 6.

In case of drug free systems, significant difference in the T_m values were noted between Tween 40 and Tween 60 stabilized NLCs. T_m values were 35.61 ºC and 32.58 ºC for Tween 40 stabilized system, and Tween 60 stabilized systems respectively. Decrease in the T_m value with decreasing size could be explained by Thomson proposition[48,55-57]. It has already been observed from the DLS studies that the NLCs stabilized by Tween 40 were larger than the Tween 60 stabilized systems. Therefore it is not unexpected that the smaller entities would have lower melting temperature than the larger particles.

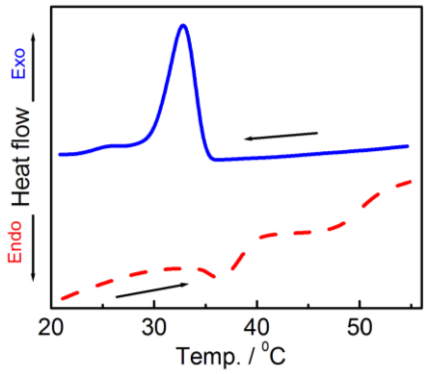


Figure 5. DSC heating (- - -) and cooling (——) thermogram of 2.5 mM LIDO loaded NLC (5 mM, Span 65+SLC+SA, 2:2:1 M/M/M) dispersed in Tween 60 (10mM). Scan rate: 2 ºC/min.

T_m values progressively decreased with increasing LIDO concentration (Figure 7 panel A). The effect was less pronounced for Tween 40 comprising systems than the Tween 60. Blank NLC formulations with Tween 60 exhibited lower T_m

values and it continued for the LIDO loaded systems. In case of PRO.HCl, the T_m value did not change appreciably with varying drug.

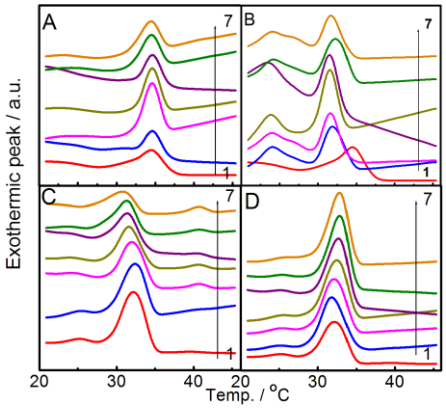


Figure 6. DSC cooling curves of LIDO (panel A) and PRO.HCl (panel B) loaded NLC dispersed in Tween 40; LIDO (panel C) and PRO.HCl (panel D) loaded NLC dispersed in Tween 60. 5 mM NLC with Span 65 + SLC + SA , 2:2:1 M/M/M ,was dispersed in 10 mM aqueous Tween solution. Concentration of drug (mM): 1, 0; 2,0.2; 3,0.5; 4,1.0; 5,1.5; 6,2.0 and 7,2.5. Scan rate 2 °C/min.

Difference in the T_m values among LIDO and PRO.HCl loaded systems could be rationalized by the same proposition as mentioned in the DLS studies. LIDO, being amphiphilic in nature, can have higher penetration into the NLC core than PRO.HCl. Thus the melting point is expected to decrease due to the added drug.

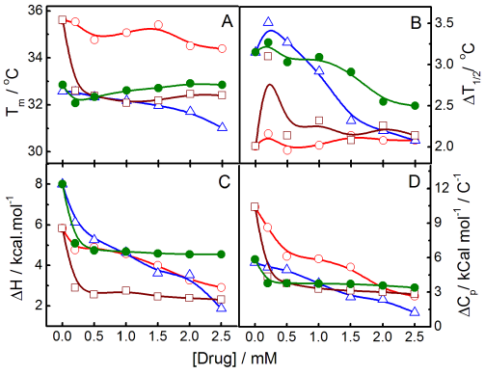


Figure 7. Effects of drugs on the temperature of maximum heat flow (T_m, panel A), width of half peak height ($\Delta T_{1/2}$, panel B), change in enthalpy (ΔH, panel C) and heat capacity (ΔC_p, panel D) of NLCs (Span65 + SLC + SA , 2:2:1 M/M/M). 5 mM lipid was dispersed in 10 mM aquoues Tween solution. System O; LIDO loaded in Tween 40: Δ; LIDO loaded Tween 60: □; PRO.HCl loaded Tween 40: and ●; PRO.HCl loaded Tween 60.

For PRO.HCl, as the drug resides preferably on the NLC surface, hence it could not significantly perturb the packing of hydrocarbon chains in the NLCs. Studies on the thermal behaviour of the drug loaded NLC formulations thus could shed light on the location of the drugs.

Wide peaks indicate the presence of multicrystalline entities in the NLC formulation. Effect of drug on the thermal behaviour of NLCs were further scrutinized through the $\Delta T_{1/2}$–drug concentration profile (Figure 7 panel B). Herein $\Delta T_{1/2}$ represents the peak width (thermal scan) at half maximum. Higher $\Delta T_{1/2}$ indicates multicrystallinity or crystal imperfection as well as the mismatch / adsolubilization of the lipidic components, especially induced by drugs. Panel B of Figure 7 implies that the $\Delta T_{1/2}$ values were higher for Tween 60 stabilized systems. Also for this surfactant, the $\Delta T_{1/2}$ value for both the drugs decreased with the increasing drug concentration. For Tween 40 based formulations, variation in the $\Delta T_{1/2}$ values with drug concentration was less significant. Results further support our proposition as already mentioned previously. Adsolubilization of the lipidic core by LIDO resulted in the lowering of $\Delta T_{1/2}$ values. In case of PRO.HCl loaded systems, as the drug was only adsorbed onto the NLC surface, addition of this drug could not significantly alter the $\Delta T_{1/2}$ values.

Changes in enthalpy values for the drug free systems were higher than the corresponding drug loaded NLC formulations (shown in the panel C of Figure 7). However the ΔH – drug concentration profiles were different for LIDO and PRO.HCl. While for LIDO loaded NLCs, the ΔH values decreased monotonously, however in case of PRO.HCl the ΔH values did not change appreciably with added drug. Increase in multicrystallinity with increasing drug concentration would effectively result in decreased ΔH value. As LIDO is capable of perturbing the lipid core structure, its progressive addition would result in the decrease of ΔH values. On the other hand, PRO.HCl predominantly resides on the NLC surface; hence its impact on the hydrocarbon chain packing was less prominent. Similar trend in the ΔC_p *vs.* drug concentration profile (panel D, Figure 7) further supports this proposition[59].

Table 1. Temperature for maximum heat flow (T_m), the width at half peak height ($\Delta T_{1/2}$), change in enthalpy (ΔH), heat capacity (ΔC_p) and percentage of crystallinity (C.I.) of blank as well as LIDO and PRO.HCl loaded NLC (5mM; Span 65+SLC+SA, 2:2:1 M/M/M).

[Drug]/ mM	T_m/ ºC	$\Delta T_{1/2}$/ ºC	ΔH/kcal.mol^{-1}	ΔC_p/kcal.mol^{-1}C^{-1}	CI (%)
Lidocaine loaded NLC in Tween 40					
0.0	35.61	2.01	5.83	10.40	100
0.2	35.53	2.16	4.77	8.63	81
0.5	34.77	1.96	4.89	6.12	83
1.0	35.07	2.02	4.57	5.95	78
1.5	35.40	2.14	4.00	5.18	68
2.0	34.51	2.08	3.27	3.18	56
2.5	34.39	2.08	2.91	2.65	49
Lidocaine loaded NLC in Tween 60					
0.0	32.58	3.15	8.00	5.85	100
0.2	32.55	3.51	6.13	5.18	76
0.5	32.33	3.27	5.26	4.95	65
1.0	32.18	2.92	4.64	3.78	58
1.5	31.97	2.32	3.62	2.58	45
2.0	31.71	2.20	3.53	2.38	44
2.5	31.02	2.08	1.87	1.23	23
Procaine hydrocloride loaded NLC in Tween 40					
0.0	35.61	2.01	5.83	10.40	100
0.2	32.60	2.02	2.89	4.36	49
0.5	32.38	2.00	2.56	3.76	43
1.0	32.08	2.34	2.75	3.28	47
1.5	32.18	2.10	2.46	3.12	42
2.0	32.45	2.02	2.38	3.01	40
2.5	32.40	2.01	2.31	2.83	39
Procaine hydrocloride loaded NLC in Tween 60					
0.0	32.58	3.15	8.99	5.85	100
0.2	32.07	4.09	5.09	3.79	63
0.5	32.34	3.68	4.74	3.79	59
1.0	32.61	3.49	4.68	3.74	58
1.5	32.71	3.29	4.59	3.72	57
2.0	32.91	3.24	4.54	3.58	56
2.5	32.85	3.29	4.55	3.40	56

DSC measurements were performed on day one of sample preparation.

Crystallinity index (CI) is another DSC derived thermal parameter which can highlight the effect of drug on the molecular packing of the NLC components. Usually percentage of crystallinity index is compared with respect to the drug free system (blank NLCs are considered to be 100% crystalline).

CI value can be derived from the DSC data using the following equation:[57,60,61].

$$\text{CI (\%)} = \frac{Enthalpy_{(NLC)} \times 100}{Enthalpy_{(BlankNLC)} \times Concentration Lipid phase\ (\%)} \times 100 \qquad (3)$$

Effects of LIDO and PRO.HCl on different formulations are shown in Figure 8. NLC crystallinity (with respect to the corresponding blank formulation) progressively decreased with increasing LIDO concentration.

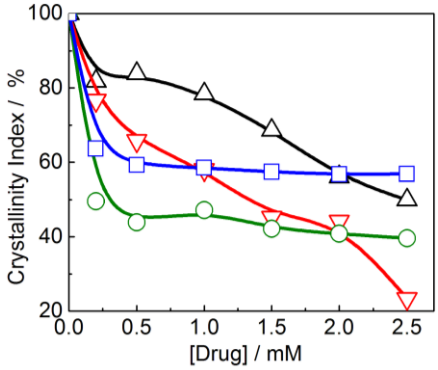


Figure 8. Effects of drug concentration on the degree of crystallinity of NLC (5 mM, Span 65+SLC+SA, 2:2:1 M/M/M). Systems: Δ, LIDO -Tween 40; ▽, LIDO -Tween 60; O, PRO.HCl-Tween 40 and □, PRO.HCl-Tween 60.

This was due to the adsolubilization of the lipidic components by LIDO; which resulted in the softening of the packed lipidic components. In case of PRO.HCl although the CI values decreased initially, however as the drug predominantly resides on the NLC surface, it hardly could alter the molecular packing of the lipidic components.

3.4. UV-visible absorption spectral studies

UV-visible spectral study is a simple yet informative approach to understand the localization of the drug loaded in NLCs. State of polarity of the drugs and the local environment of the NLCs were evaluated by spectroscopic analysis whereby the drug molecules themselves were used as molecular probes. Absorption spectra of drug loaded NLCs were recorded in the wavelength range 200 - 400 nm. Polarity of the local environment of a drug incorporated in NLC can greatly affect its spectral behavior;[62,63] spectra of the drugs were also

recorded in solvents of different polarities to make comparisons. UV-visible spectra of the drugs loaded in NLC as well as in different solvents are shown in Figure 9. Absorption maxima of the drugs were found to be dependent on the polarity of the medium, as shown in Figure 10.

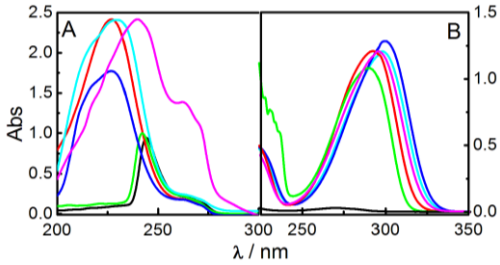


Figure 9. UV-visible absorption spectra of 1 mM LIDO (panel A) and PRO.HCl (panel B) loaded in NLC as well as solvents of different polarity; at 25 ºC. Systems : ——, n-hexane; ——, acetonitrile; ——, chloroform; ——, ethanol; ——, methanol and——, drug loaded NLC respectivly.

Spectra were suitably processed to determine the E_T (30) value[64] of the continuous medium (both the different solvents and drug loaded NLCs). There occurred a blue shift in the absorption maxima of both the drugs with decreasing solvent polarity. In case of LIDO loaded NLCs, the absorption maximum (λ_{max}) appeared at 239 nm, very close to that of LIDO in n-hexane. It suggests that LIDO being more lipophilic in nature could reside in the NLC core.

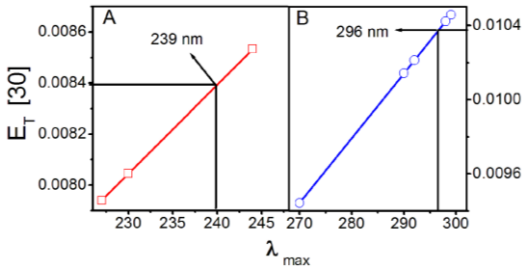


Figure 10. Dependence of absorption maxima (λ_{max}) of LIDO (panel A) and PRO.HCl (panel B) on the E_T30 scale of medium at 25 ºC.

In case of PRO.HCl the λ_{max} value appeared at 291 nm, which was in between the λ_{max} of PRO.HCl in methanol and ethanol. This confirms the localization of the drug on a more hydrophilic environment, herein on the surface of the NLCs.

3.5. Entrapment efficiency (EE) and loading content studies

Efficiency of an NLC formulation in terms of its use as a drug vehicle depends on its ability to incorporate/ load/ entrap the quantity of a drug. Results are summarized in Figure 11. Tween 60 stabilized NLCs exhibited superior entrapment efficiency and loading content compared to the Tween 40 stabilized systems. Such a phenomenon could be explained on the proposition as mentioned earlier[48,55,56]. Because of ionicity, efficiency in entrapping PRO.HCl was less than LIDO in both the NLC formulations (Tween 40 and Tween 60). While considering the effect of drug concentration it was observed that the entrapment efficiency increased upto 0.5 mM, beyond which it did not change appreciably. This was due to the saturation of NLCs with respect to the drugs. There was no significant difference in the drug loading content among the different formulations. The drug loading content increased almost linearly with the added drug concentration (panel B). However, to address this issue in a better way, further studies are warranted using other drugs which is considered to be one of the future perspectives.

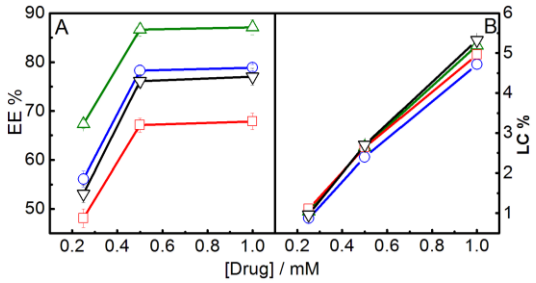


Figure 11. Dependence of entrapment efficiency (E.E.) (panel A) and loading content (L.C.) (panel B) of NLC (5mM, Span 65 + SLC + SA, 2:2:1 M/M/M) on the concentration of drugs. LIDO loaded NLCs dispersed in: O, Tween 40; Δ, Tween 60; and PRO.HCl dispersed in □, Tween 40 and ▽, Tween 60. Each value represents the mean ± (S.D.) (n=3). Temp. 4 ºC.

3.6. *In vitro* release kinetics

Release kinetics of an entrapped drug from the NLC matrix need to be investigated in order to assess the efficacy of the formulation. Drug release kinetics studies were performed by the conventional dialysis bag method, where the membrane retained the drug bound to the NLC and allowed the free drug to diffuse out into the release medium[1]. *In vitro* release behavior of the drugs loaded

the NLC matrices have been demonstrated as the percentage of cumulative release in Figures 12 -13. LIDO and PRO.HCl showed 95.63% and 100% of release within 144 and 5 hr respectively in Tween 60 solution. Parallel measurements were carried out using the anaesthetics separately in the absence of NLC. It has been proposed by Carafa et al.[41] that the diffusion of drug across the dialysis membrane was not the limiting step of the overall diffusion process. Release of both the drugs in presence of NLC was retarded compared to the same in absence of NLC as also reported by others[65,66].

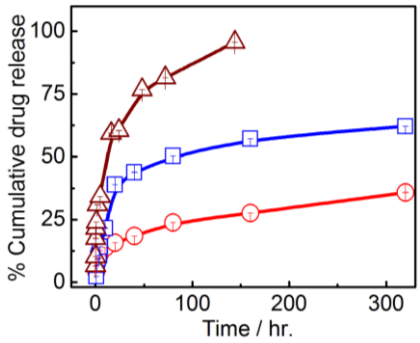


Figure 12. *In vitro* cumulative LIDO release from NLC (5mM, Span 65 + SLC + SA, 2:2:1 M/M/M). System: LIDO loaded NLC dispersion in Tween 40 (O), Tween 60 (□) and free LIDO in Tween 60(Δ). Each point represents the mean ± (S.D.) (n=3).Drug concentration was kept constant at 0.5 mM in each case. Temp. 25 ºC.

The Initial burst release, followed by a sustained/prolonged for both the drugs loaded in NLCs are not uncommon[25].

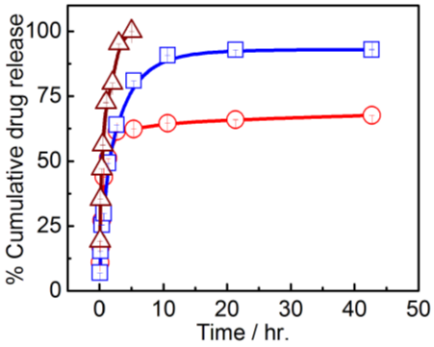


Figure 13. *In vitro* cumulative PRO.HCl release from NLC (5mM, Span 65 + SLC + SA , 2:2:1 M/M/M). Systems: PRO.HCl loaded dispersion in Tween 40 (O), Tween 60 (□) and free PRO.HCL in Tween 60(Δ). Each point represents the mean ± (S.D.) (n=3).Drug concentration was kept constantat 0.5 mM in each case. Temp. 25 ºC.

Thus, NLC systems could be considered to have potential application for sustaining the release of both the anaesthetics. Results revealed that the release rate of LIDO was slower as compared to that of PRO.HCl. This could be accountable to the fact that LIDO, being more lipophilic in nature, is inserted into the lipid core. On the contrary, PRO.HCl is interfacially adsorbed on the NLC surface owing to its ionic nature. While considering the initial burst release, it was observed that the process was complete when about 43% and 30% of PRO.HCl was released from the NLC in Tween 40 and Tween 60 respectively. The corresponding time was found to be 40 min. In case of LIDO, the values were 9% in Tween 40 and 6% in Tween 60 comprising systems respectively. In both Tween 40 and Tween 60, the initial burst release was complete within 2hr. Results further support better stabilization of LIDO in the NLC than the PRO.HCl which may be considered on the basis of its higher lipophilicity than PRO. HCl. Amount of the released drugs depended on the type of Tween surfactant. In case of Tween 40 stabilized system, 35 - 67% of the entrapped drug was released compared to 62 - 93% release for Tween 60 stabilized system. Higher loading capacity and larger imperfections for Tween 60 stabilized NLCs (in terms of hydrocarbon chain packing) compared to Tween 40, were responsible for this phenomenon[48,55,57].

Data of the drug release profile were suitably processed and scrutinized for different release kinetics models such as First order, Higuchi, Korsemeyer-Peppas, Hixson-Crowell models. No formulations followed pseudo first order kinetics as reflected through the non-linear variation in the release profiles (Figures 12 - 13). The obtained data were fitted into following drug release models by using DDSolver 1.0, and Add-In program for modelling and comparison of drug dissolution profiles:[67]

Higuchi model: $F = k_H . t^{0.5}$ (4)

Korsemeyer–Peppas model: $F = k_k\, t^n$ (5)

Hixson-Crowell model: $F = 100.[1 - (1 - k_{HC} . t)^3]$ (6)

First order model: $F = 100.\left(1 - e^{-k_1 . t}\right)$ (7)

where, F is the percentage of the drug released, k_H, k_k, k_{HC} and k_1 are the release rate constants of Higuchi, Korsemeyer-Peppas, Hixson-Crowell and First order model respectively, t represents the time lag of the dissolution process, and n is the release exponent obtained from Korsemeyer-Peppas model. Among all the four models, the best-fit model was decided based on the highest regression values (r^2) for all the formulations. The release rate constant was calculated from the slope of the appropriate plots and regression co-efficient were accordingly determined. The results are summarized in Table 2. Release kinetics for both the drugs followed Korsemeyer-Peppas model. The regression coefficient (r^2) values of Korsemeyer-Peppas model for LIDO loaded in Tween 40 and Tween 60 were 0.99 and 0.95 respectively. In case of PRO.HCl, the (r^2) values were 0.99 and 0.94 respectively.

Table 2. Drug release kinetics profiles of LIDO and PRO.HCl loaded NLCs.

Formulation	Korsemeyer-Peppas			Higuchi		Hixson-Crowell		First order	
	r^2	k	n	r^2	k	r^2	k	r^2	k
LIDO-T40	0.99	6.84	0.283	0.98	2.35	0.93	0.001	0.93	0.002
LIDO-T60	0.95	10.25	0.333	0.91	4.47	0.87	0.002	0.90	0.007
PRO.HCL-T40	0.99	40.03	0.178	0.75	15.51	0.76	0.031	0.93	0.393
PRO.HCl-T60	0.94	41.93	0.256	0.86	0.271	0.90	0.036	0.92	0.446

*r^2=regression coefficient, k= release rate constant, n=diffusional exponent.

The release exponent (n) determined from Korsemeyer-Peppas model for LIDO and PRO.HCl in both Tween 40 and Tween 60 stabilized NLCs were found to be less than 0.5 indicating release mechanisms being controlled by Fickian diffusion in all the systems. Values of release rate constants of LIDO loaded in NLCs for the Korsemeyer-Peppas model were 6.84 hr^{-1} and 10.25 hr^{-1} for Tween 40 and Tween 60 respectively. However in case of PRO.HCl, release rate constants were found to be 40.03 hr^{-1} and 41.93 hr^{-1}in NLCs stabilized with Tween 40 and Tween 60 respectively.

4. Summary and conclusions

Nanostructured lipid carriers (NLCs) were formulated by using Span 65, soy lecithin and stearic acid dispersed in aqueous Tween 40 or Tween 60 solution. Tween 60 provided better stabilization than Tween 40 because of its longer hydrocarbon chain. Hydrocarbon chains of Tween 60 could penetrate to greater extent than Tween 40 into the NLC matrices. TEM study confirmed the spherical morphology of the NLCs with smooth surface. Higher amount of LIDO could be encapsulated into the NLC than PRO.HCl. LIDO resides in the core of the NLC for its relatively higher hydrophobic nature. PRO.HCl, being ionic, preferentially adsorbs over the NLC surface. Apart from the DLS studies, DSC and spectroscopic investigations on the drug loaded NLC further supported such proposition. Because of its larger lipophilicity LIDO could be entrapped to greater extent compared to PRO.HCl. *In vitro* drug release study revealed that the lipidic matrices could act as promising vehicles for two most widely used local anaesthetics with controlled and prolonged release. Biphasic release behaviour was experienced by all the combinations. In order to further explore the viability, the formulations may be subjected to *in vitro* studies under biological condition. Besides, the *in vivo* studies as well as some clinical trials are warranted which are considered as the future perspectives.

References

References are given in BIBLIOGRAPHY under Chapter II (pp. 176–179).