CHAPTER 3

Use of ion pair amphiphile as an alternative of natural phospholipids in enhancing the stability and anticancer activity of oleanolic acid loaded nanostructured lipid carriers.

Abstract

In the present study, NLC comprised of soy lecithin (SLC), tristearin (TS) and Palmitic acid (PA) was modified (NLC_{IPA}) by replacing the conventional phospholipid, SLC by synthetic ion pair amphiphile (IPA) prepared by mixing equimolar aqueous solution of SDS and CTAB. Hot homogenization followed by ultrasonication method was employed for the preparation of NLC and NLC_{IPA}. Suitable SLC / IPA ratios for NLC_{IPA} were obtained by studding the interfacial behavior of the lipidic components and IPA using Langmuir monolayer approach at air water interface. Systems with SLC/IPA ratio equal to 40 ; 60, 30 : 70 and 20 : 80 were considered for the NLC_{IPA} from the interfacial behavior. The prepared NLC_{IPA} were characterized by dynamic light scattering (DLS), differential scanning calorimetry (DSC), TEM, FF-TEM and AFM. NLC_{IPA} with SLC/IPA ratio 30 : 70 was found to be the optimum. Oleanolic acid (OLA) was used as drug in the present study. The OLA loaded formulations were also characterized by the above mentioned methods. The interfacial, solution phase and thermal behavior of the OLA loaded systems indicated the accumulation of OLA on the palisade layer. The drug incorporation efficiency (EE%) and the drug loading (DL%) were found to be higher for NLC_{IPA} than conventional NLC. The release of the OLA was also found to get sustained in NLC_{IPA}. The OLA loaded NLC_{IPA} were subjected for the cytotoxicity study using MTT assay on Hepatocellular carcinoma (HepG2), hepatocyte-derived carcinoma (Huh-7) and colorectal carcinoma (HCT-116) cancer cell lines. The activity of OLA was found to enhance in NLC_{IPA} in comparison to the conventional NLC.

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1. Introduction:

Development in the Particulate drug carrier systems put forward a great promise in improving the therapeutic efficacy and performance of the drugs. [1-3] Among several drug carrier systems nano structured lipid carriers (NLC) have taken the spot light in recent time on the field of drug delivery. [4-7] NLCs were found to be very advantageous in comparison to the conventional delivery systems like liposomes, emulsions, polymeric nanoparticles etc. [1, 4-6, 8-10] NLCs are the modified form of solid lipid nano particle having two or more structurally different lipid molecules. They are a relatively new class of drug carrier having submicron size (50 to 1000 nm) prepared from lipids that remain solid or liquid at room and body temperature. [11, 12] NLCs are conveniently prepared using a wide variety of lipids including fatty acids, mono, di or triglycerides, glyceride mixtures or waxes, and stabilized by the biocompatible surfactant(s) mostly non-ionic surfactant. [11, 12] Relatively higher drug incorporation efficacy, sustained drug release capacity make NLCs are superior from previously developed formulations. [7, 8] But stability during storage and ongoing lipid modification make the present situation challenging for the development of physicochemicaly stable NLC formulations,

The stability of the NLC formulations were mainly depends upon the lipid composition and the compatibility among the lipid component. It was previously reported that the stability of the vasicular systems can be improved using synthetic ion pair amphiphile by partially replacing the conventional phospholipid, SLC. [13] In the present study an attempt was taken to improve the stability as well as the biological activity of NLC by extending the previous concept. Till today no systematic attempt has been made in enhancing the stability of the NLC formulations by replacing SLC with IPA to the best of our knowledge. In the present study a well stabilized conventional NLC system comprising SLC, TS and PA having molar ration 2:2:1 has been taken and SL C was progressively replaced by IPA. The IPA was synthesized by mixing equimolar aqueous solution of single chain mixed cationic and anionic surfactants. [13] In the present work SDS and CTAB were used as the anionic amd cationic surfactant respectively.

Oleanolic acid (3bhydroxy- olea-12-en-28-oic acid) is a naturally occurring pentacyclic triterpenoid compound. [14-16] It is mainly extracted from leaves and roots of *Olea europaea, Viscum album L., Aralia chinensis L. etc*., 120 different plant species. [14-16] OLA shows many biological activities such as anti-inflammatory, antitumor, antiviral, hepatoprotective, anti-hyperlipidemic effects, anti cancer activity and has been worn in traditional Chinese medicine in

the treatment of liver disorders. [14-19] However poor aqueous solubility and low bioavailability of OLA make it necessary to develop suitable formulations for its pharmaceutical applications. Several attempts has been made in formulating OLA loaded SLN to improve biodistribution and activity. [14-16, 18, 19] But no comprehensive attempts has been made to develop OLA loaded NLC in presence of IPA as the substitute of conventional phospholipid to the best of our knowledge.

In the present set of work, NLC comprised of SLC, TS and PA with molar ratio 2 : 2 : 1 has been taken and the conventional phospholipid SLC, was progressively replaced by IPA and IPA modified NLC (NLC_{IPA}) were prepared. Before preparing the NLC_{IPA}, the lipid composition was tuned by considering miscibility and mutual interaction among the lipid components in the presence of IPA at the air water interface by Langmuir monolayer approach. By studying the mutual miscibility the lipid composition of the NLC_{IPA} formulations were fixed. The prepared conventional NLC and NLC_{IPA} were characterized using DLS and DSC to get idea regarding the solution behavior like hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) and the thermal behavior respectively. The solution behavior and the thermal properties of the OLA loaded conventional NLC and NLC_{IPA} were also studied like the base formulations. The morphology of the conventional NLC and NLC_{IPA} in the absence and presence of OLA were also evaluated using conventional TEM and FF-TEM. Surface roughness of the studied formulations was also analyzed by AFM and corresponding surface roughness were also evaluated. In addition to this the drug incorporation efficiency (EE%), drug loading capacity (DL%) and the drug release kinetics of the OLA loaded conventional NLC and NLC_{IPA} formulations were evaluated and compared to obtain idea regarding the effect of IPA on the performance of NLC_{IPA} as delivery system for OLA. The OLA loaded NLC formulations were also subjected for anti cancer activity studies using human GIT cancer cell lines. Hepatocellular carcinoma (HepG2), hepatocyte-derived carcinoma (Huh-7) and colorectal carcinoma (HCT-116) cell lines were used. The cytotoxic effect on GIT cancer cells of OLA loaded conventional NLC and NLC_{IPA} were compared to get idea on the efficiency of NLC_{IPA} over conventional NLC

2. Materials and method

2.1. Materials

Soybean lecithin (SLC), tristearine (TS) palmatic acid (PA) and oleionic acid (OLA) were purchased from Sigma-Aldrich Chemicals (USA). Tween 60, SDS and CTAB were purchased from Sisco Research Laboratory (SRL), India.. All other chemicals and solvents of

analytical grade were used. Hepatocellular carcinoma (HepG2), hepatocyte-derived carcinoma (Huh-7) and colorectal carcinoma (HCT-116) cell lines were collected from National Facility for Animal Tissue and Cell Culture, Pune, India. Double distilled water having specific conductance of 2 mS at 25 ºC was used throughout the study.

2.2. Preparation of IPA

IPA was prepared by mixing equal volume of 0.1 M aqueous solution of HTMAB and SDS. The equimolar amount of HTMAB and SDS neutralized their charge and a semisolid white precipitate was found to appear (IPA). The mixture was then stirred and stored for overnight to ensure complete precipitation. IPA was then extracted from aqueous medium using a separating fulel using chloroform as organic phase. After that chloroform was removed by evapouration under vacuam and the obtained white solid powdery IPA was subjected for characterization by means of XRD, 1H NMR and FT-IR studies. The results were found in accordance with our previously published results. Hence, the detailed characterizations were not mentioned here. [13, 20]

2.3. Preparation of NLC

The NLC formulations were prepared using hot homogenization followed by ultrasonication technique. The detailed preparative procedure can be found in our recent publications. [11, 12, 21] In the present study conventional NLC was prepared by SLC, TS and PA and they were used in the molar ratio 2 : 2 : 1. NLC_{IPA} was prepared by partially replacing SLC by IPA. In other word, the conventional phospholipid SLC was used in combination with IPA in different ratio along with other lipid components, keeping the overall molar ratio fixed. The concentration of the conventional NLC and NLC_{IPA} formulations were kept fixed at 1 mM.. In case of drug loaded formulation OLA was added with the lipid physical mixture during the lipid melting process during the preparation of NLC. In this present study, the concentration of OLA was 10 µM.

2.4. Analytical instrumentation

A langmuir trough (Micro Trough X, Kibron, Finland) was used to find the optimum SLC/IPA ratio fot the NLC_{IPA} composition and to get idea regarding their molecular interaction. Size, zeta potential and PDI of the NLC formulations were determined using a dynamic light

scattering instrument (Zetasizer Nano ZS90 ZEN3690, Malvern Instruments Ltd., U.K.). Conventional TEM (Hitachi H-600, Japan), FFTEM (FR-7000A, Hitachi High Technologies Ltd., Japan) and AFM (Bruker Nanoscope V Multimode SPM) were used for the morphological analysis. Differential scanning calorimeter (Mettler Toledo, Switzerland) was used for the thermal analysis of NLC formulation. DSC1 STAR^e software was used for the analysis of the thermal data.

2.5. Drug loading and drug incorporation efficiency

The incorporation efficiency and the loading capacity OA in the conventional NLC and NLC_{IPA} were studied using the method of centrifugation. [11, 12, 21] The OLA loaded formulations were centrifuge at 15000 rpm for 15 min at 4 ºC temperature. The collected supernatant of the samples were analyzed colorimetricaly to determine the amount of the incorporated OLA. The EE% and DL% of the studied conventional NLC and NLC_{IPA} were determine using the following equations[11, 12, 21]

$$EE\% = \frac{W_{loaded\ OLA}}{W_{total\ OA}} \times 100\ \%$$

$$DL\% = \frac{W_{loaded\ OLA}}{W_{lipid}} \times 100\ \%$$

$W_{total\ OLA}$, W_{lipid} and $W_{loaded\ OLA}$ were the weight of total OLA added in NLC, weight of lipids used in NLC and weight of OLA entrapped into NLC, respectively.

2.6. *In vitro* release of OLA

The *in-vitro* release of OLA was examined by the dialysis bag method. [11, 20] In brief, OLA loaded NLC was taken in the dialysis bag and the dialysis bag was placed into 20 mL of dispersion medium with 15% ethanol and stirred at 100 rpm on a magnetic stirrer at 25 °C. 1 mL of dispersion medium was withdrawn and fresh release medium of equal volume was added immediately to maintain the volume (20 mL) constant at the predetermined intervals. The released OLA was assayed colorimetricaly. The experiment was performed in triplicate.

2.7. *In vitro* cytotoxicity study

Studied GIT cancer cells ($1x10^5$) were taken in 96-well plates and kept under CO_2 atmosphere for 24 hr before experiment. The cells were treated with conventional NLC and NLC_{IPA} having different OLA concentrations (2.5, 5, 7.5, and 10 µM) for 24, 48 or 72 hr in a humidified atmosphere having 5% CO_2 at 37 °C. In the present set of work untreated cells were used as control. MTT assay was used to measure the percentage of cell inhibition. The measurements were performed by recording the absorbance of the experimental solution at a specific wavelength (HepG2, Huh-7 and HCT-116 at 540 nm) using a microplate manager (Reader type: Model 680 XR Bio-Rad laboratories lnc.). IC_{50} values for all the cell lines were also calculated for 24, 48 and 72 hrs.

3. Results and discussion

3.1. Langmuir monolayer studies

To investigate the interfacial behavior of the monomolecular film of the mixed lipidic systems (SLC/IPA+TS+PA, 2:2:1, M/M/M), л -A isotherms were constructed using Langmuir monolayer approach at the air water interface. The main aim of the study was to tune the lipidic composition in terms of SLC/IPA ratio for the preparation of IPA modified NLC (NLC_{IPA}). In the present study conventional phospholipid (SLC), of the mixed lipidic system was progressively substituted by synthetic ion pair amphiphile (IPA) and л -A/isotherms of the mixed lipid systems having different SLC/IPA ratio were constructed. In addition to this, the interactions of OLA with the mixed lipidic system were also analyzed at the air water interface. Some representative isotherms of the mixed lipidic systems in the absence and presence of IPA have been presented in the Fig. 2. Whereas, л -A isotherms of the pure components were presented graphically in the Fig. 1. Lift off area of SLC, TS, PA, IPA and OLA were found at 0.1, 0.6, 0.23, 0.11 and 0.57 nm^2 respectively.

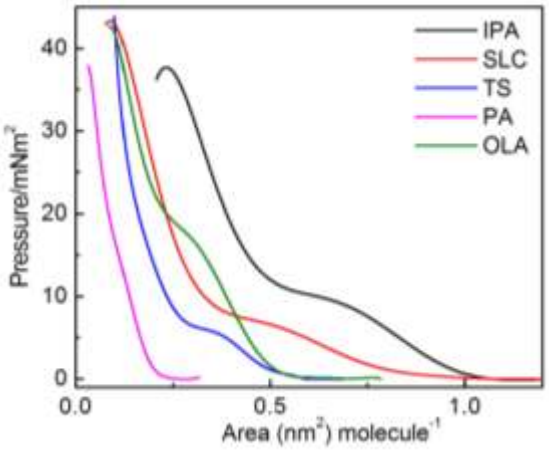

Figure 1. Surface pressure (π) – area (A) isotherm of pure lipidic components and OA using water as subphase at 25 °C. The different systems were mentioned in the figure.

The obtained data were found to be in good agreement with the previous reports. [12, 13] In case of the mixed lipidic systems, a smooth progression of the isotherms were observed with the surface compression.

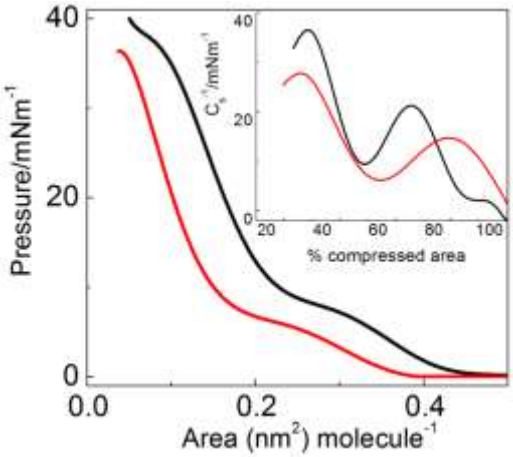

Figure 2. Surface pressure (π) – area (A) isotherm of mixed lipidic system (SLC/IPA+TS+PA, 2:2:1, M/M/M) in the absence (red) and presence (black) of IPA using water as subphase. Corresponding C_s^{-1} vs. % compressed area has been presented in the inset of the Figure. Temperature 25 ºC

The observed smooth progression indicated the compatibility of the lipid components and IPA in the mixed lipidic system. [13] The lift off area for the mixed lipidic systems in the absence of IPA was found at 0.52 nm^2. Significant down shift in the lift off area was noted when IPA was used in combination with the conventional phospholipid SLC in the mixed lipidic system keeping the overall molar ratio intake. System having SLC/IPA ratio equal to 30:70 was found to show the lift off area at 0.38 nm^2. Reduction in the lift off area indicated the condensing effect of IPA over the other lipid components of the mixed lipidic systems. The condensing effect of IPA was found to be analogous to the condensing effect of cholesterol. The enhanced van der waal force of attraction and increased hydrophobic interaction in the presence of IPA was mainly responsible for the observed behavior of the monolayer in the presence of IPA.

Further information regarding the rigidity / fluidity and the monolayer mechanical property were also investigated by calculating the compressibility moduli (C_s^{-1}) of the mixed lipidic monolayer. C_s^{-1} of the studied isotherms were calculated using the following equation. [12, 13]

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)$$

The calculated C_s^{-1} were plotted against % of compressed area and some representative profiles have been given in the inset of Fig 1. In the absence of IPA, maximum C_s^{-1} was found at 28.2 mNm^{-1}. With the addition of IPA, reduction in C_s^{-1} also confirmed the enhanced association among the lipidic components in the presence of IPA. [13] The lowest value of C_s^{-1} (26.3 mNm^{-1}) was observed for the lipid system having SLC/IPA ratio of 30:70 indicated maximum association and considered as the optimum SLC/IPA ratio for the mixed lipidic systems.

To gather further quantitative information regarding the interaction among lipidic components and the stability of the mixed lipidic system, excess surface area and the excess free energy of the lipid monolayer were calculated at different surface pressures. The excess surface area was calculated using the following equation[12, 13]

$$A_{ex} = A_{12} - A_{id}$$

Where A_{12} is the experimentally obtained surface area and A_{id} is the ideal surface area. The ideal surface area was calculated using the following equation[12, 13]

$$A_{id} = A_1 x_1 - A_2 x_2$$

Where x_1, x_2 and A_1, A_2 represented the mole fraction and surface area of the lipid component 1 and 2 respectively. In the present study SLC was considered as the component 1 and IPA was taken as the component 2 keeping the overall molar ratio of all the lipidic component unchanged (SPC?IPA+TS+PA, 2:2:1, M/M/M). The calculated A_{ex} values were plotted against the mole fraction of IPA and presented in the panel A of Fig. 3.

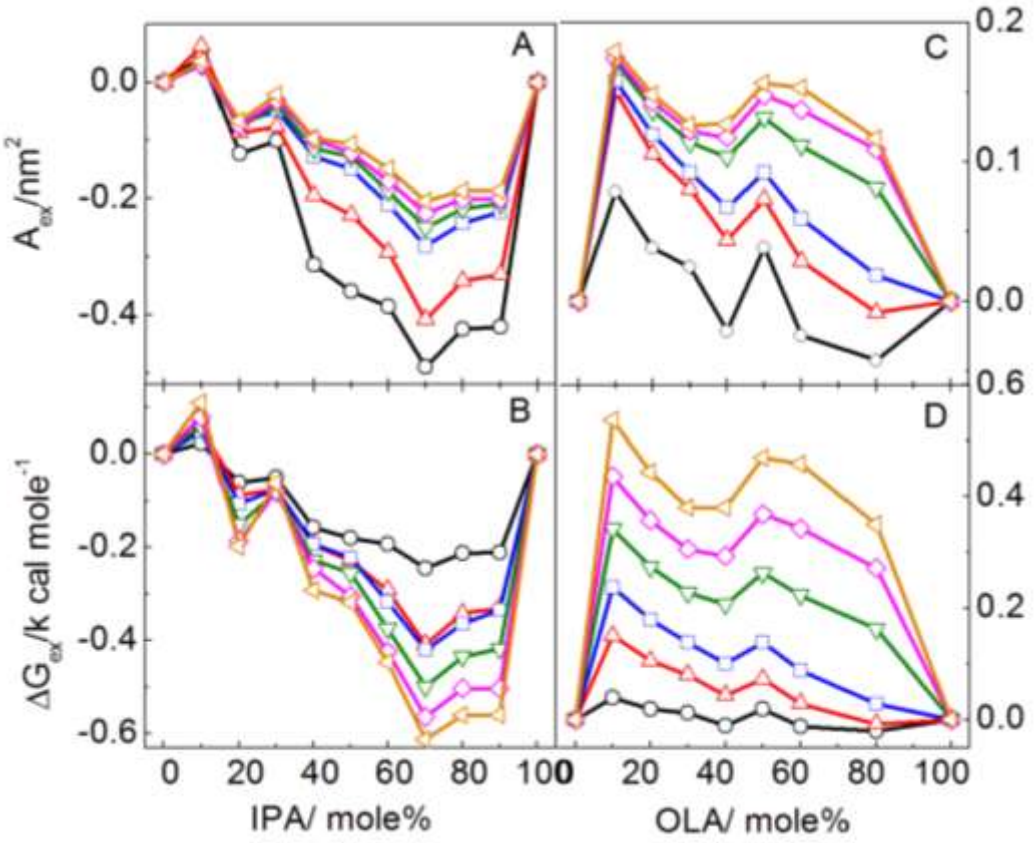

Figure 3. Variation in A_{ex} (panel A & C) and ΔG^0_{ex} (panel B & D) of the mixed lipidic system with mole % of IPA (panel A, B) and OLA (panel C, D). For panel A, B: Component 1: SLC & Component 2: IPA. For panel C, D: Component 1: SLC/IPA (30:70)+TS+PA (2:2:1, M/M/M) & Component 2: OLA. Surface pressures (mNm^{-1}): O, 5; Δ, 10; □, 15; ▽, 20;◊, 25 and ◁, 30. Temperature: 25 °C.

In case of the studied mixed lipidic systems, negative deviations in A_{ex} value was observed with the increasing proportion of IPA. The maximum negative deviation from ideality was observed for the mixed lipidic system having the SLC/IPA ratio equal to 30:70. The observed negative deviation indicated the enhanced associative interaction among the lipidic components. The observed maximum negative deviation at the SLC/IPA ratio 30:70 indicated the optimum SLC/IPA ratio for the preparation of the stable composition.

The stability of the mixed lipidic systems were also investigated by calculating the excess free energy of the mixed lipidic systems. The excess free energy of the monomolecular layer were calculated using the following equation[12, 13]

$$\Delta G_{ex}^{0} = \int_{0}^{\pi} [A - (A_1 x_1 + A_2 x_2)] d\pi$$

The ΔG_{ex} values were also plotted against the mole fraction of IPA and presented graphically in the panel B of Fig.3. Similar to A_{ex}, negative deviations were also observed for the ΔG_{ex} with the increasing proportion of IPA in the mixed lipidic system. The negative deviation also indicated greater stability of the mixed lipidic system in the presence of IPA. The observed maximum negative deviation was observed for the mixed lipidic system having the SLC/IPA ratio equal to 30:70. The obtained maximum negative deviation indicated the additional stability of the mixed lipidic system witb SLC/IPA ratio equal to 30: 70. [12]

Being optimum, the mixed lipidic system (SLC/IPA+TS+PA, 2:2:1, M/M/M) having SLC/ IPA ratio 30: 70 was considered for further study in evaluating the extent of miscibility of OLA with this lipid system. In this case the base lipidic system was taken as the component 1 and OLA was considered as component 2. No significant variation in the nature of π – A isotherms from the base lipidic system were observed in the presence of OLA. No significant variations in the lift off area were observed for the mixed lipidic systems in the presence of OLA. The observations signified that OLA was not interacting with the hydrophobic part of base lipid system and convey a preliminary idea regarding the location of OLA in the palisade layer of the mixed lipidic system. The calculated C_s^{-1} values of the mixed lipidic systems in the presence of OLA were found very close to the base lipidic system. No significant variation in C_s^{-1}indicated the insignificant interaction of OLA with the hydrophobic part and confirmed the accumulation

of OLA in the palisade layer of the mixed lipidic system. To collect further information regarding the nature of drug lipid interaction, A_{ex} and ΔG_{ex}values were also calculated and plotted against mole % of OLA (panel C and D of Fig. 3 respectively). Observed positive deviation in both A_{ex} and ΔG_{ex} indicated the repulsive type interaction of OLA with the lipid system. [12] The amphiphilic nature of OLA was mainly responsible for the observed repulsive interaction. The observed positive deviation in A_{ex} and ΔG_{ex} confirmed the accumulation of OLA in the palisade layer of the mixed lipidic system.

3.2. Dynamic light scattering studies

Size of the NLC formulations govern the stability during storage and inter cellular activity. [1, 2, 8] In the present study the conventional phospholipid (SLC) of the NLC formulations were partially substituted with IPA. In other word, SLC was used in combination with suitable amount of IPA in the preparation of NLC_{IPA}. Considering the extent of miscibility obtained from Langmuir monolayer study, systems with SLC/IPA ratio of 40 : 60, 30 : 70 and 20 : 80 were considered for the preparation of NLC_{IPA}. The size of the NLC_{IPA} formulations containing different ratio of SLC/ IPA and conventional NLC without having IPA were monitored with respect to time and compared. All the formulations were found to stable for 90 days of storage as no significant change in the size of the NLC formulations were noted during this time period. The size vs. time profile of the NLC and NLC_{IPA} having different SLC/IPA ratio have been presented in the panel A of Fig. 4.

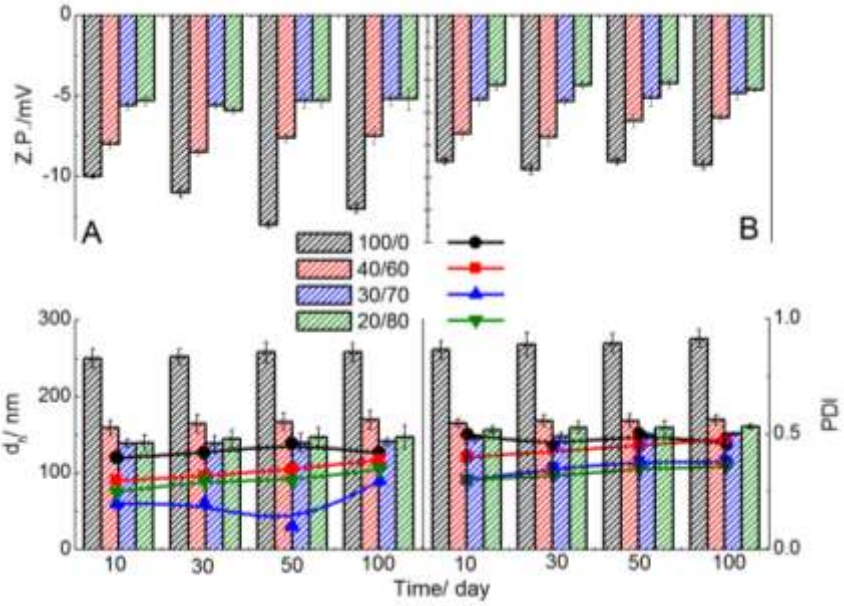

Figure 4. Variation in size (d_h), PDI and zeta potential (Z.P.) with time for 1 mM NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) stabilized by 2 mM aqueous Tween 60 solution in the absence (panel A) and presence (panel B) of OLA at 25 ºC. SLC/IPA ratios for different systems have been mentioned inside the figure. [OLA]: 10 µM.

The size of NLC without having IPA was found in the range 250 to 258 nm. But in the presence of IPA the size of the NLCs were found in the range of 139 to 150 nm. The observed significant reduction in the size with the addition of IPA, indicated the mutual association among the lipidic components in the presence of IPA. [13, 20] The association assisted by IPA was also supported by the langmuir monolayer studies. NLC_{IPA} having SLC/IPA ratio equal to 30 : 70 was found to show the maximum reduction in size. Further enhancement in the IPA proportion has not shown any significant variation in the size of $NLC_{IPA.}$ Hence, SLC/IPA ratio 30 : 70 was considered as the optimum and provide the maximum stability to the NLC_{IPA} system. Being optimum NLC_{IPA} system having SLC/IPA ratio 30 : 70 was subjected for the incorporation of OLA. The size vs. time profile of the OLA loaded conventional NLC and NLC_{IPA} have been presented in the panel B of the Fig. 4. The size of the OLA loaded formulations were found in the range of 260 to 265 nm and 140 to 160 nm for conventional NLC and NLC_{IPA} respectively. In all the case, an overall enhancement in size was observed with the incorporation of OLA. The significant enhancement in the size of the drug loaded NLC_{IPA} indicated the accumulation of

OLA in the palisade layer. [12] The drug loaded formulations were also found to stable for 90 day as no significant change in size was observed during the storage time.

PDI of the studied formulations were also monitored with respect to time and the PDI vs. time profiles have been presented in the Fig. 4. Observed PDI for the studied systems were found in the range of 0.3 to 0.5. The obtained value of PDI indicated the formation of the stable and homogeneous dispersion. [1, 4, 6, 11] PDI for the NLC_{IPA} were found to be lower than the conventional NLC formulations. The lowering in the PDI also indicated the formation of compact molecular association among the lipidic components in the presence of IPA. [20] The NLC_{IPA} having the SLC/IPA ratio 30 : 70 was found to show the lowest PDI among other studied formulations. Observed minimum PDI also confirmed the additional stability of the system due to the maximum association among the lipidic constituents in the presence of IPA at this specific composition. PDI of the OLA loaded formulations were also monitored with respect to time and no significant variation in PDI vs. time profile was noted. But enhancement in the PDI was observed for the OLA loaded NLC as well as the NLC_{IPA} systems. The enhancement in PDI also supports the accumulation of OLA on the palisade layer of the NLC and NLC_{IPA}. [12, 21] The accumulation of OLA in the palisade layer disturbed the surface homogeneity and hence enhancement in the PDI was observed.. The obtained result clearly indicated the formation of the shell enriched type NLC and NLC_{IPA}. [12]

Z.P. is a direct indication of the stability of the nanocolloidal formulations because it regulates the flocculation and the coagulation rate of the lipid based drug delivery formulations. [3-7]Z.P. of the studied formulations were also recorded with respect to time and presented graphically in the Fig. 4. Z.P. value for the studied conventional NLC was found in the range of -10 to -12mV. But the Z.P. for the NLC_{IPA} were found in the range of -5 to -9 mV. Observed reduction in the negative magnitude of Z.P. in the presence of IPA can be explained on the basis of the dissociation of IPA molecule on the surface of the NLC_{IPA}. The dissociation of the IPA resulted into the formation of the DS^- and $HTMA^+$ ion. DS^- ions got disperse into the dispersion medium and the presence of the $HTMA^+$ ion on the surface was mainly responsible for the reduction in Z.P. [13, 20] With increasing IPA proportion a progressive reduction in the negative magnitude of Z.P. was observed. No significant variation in the Z.P. with storage time also indicated the stability of the studied formulations. [21] In case of OA loaded NLC and NLC_{IPA},

Z.P. were found to lie in the range of -9 to -9.5 mV and -4 to -8 mV respectively. Further reduction in the Z.P. in the presence of OLA also indicated the accumulation of it in the palisade layer of the studied formulations. [12] The adsorbed OLA on the palisade layer masked the NLC and NLC_{IPA} surface and reduced the Z.P. value. The observation also confirmed the formation of the shell enriched $NLC_{IPA.}$

3.3. Morphological studies

In the present set of work the morphology of the studied NLC and NLC_{IPA}were evaluated using conventional TEM and FF-TEM technique. Further detail evaluation of the surface roughness and the three dimensional overview of the studied NLCs were done using AFM technique. The conventional and the FF-TEM images of some representative systems have been given in the panel A and B of Fig. 5 respectively.

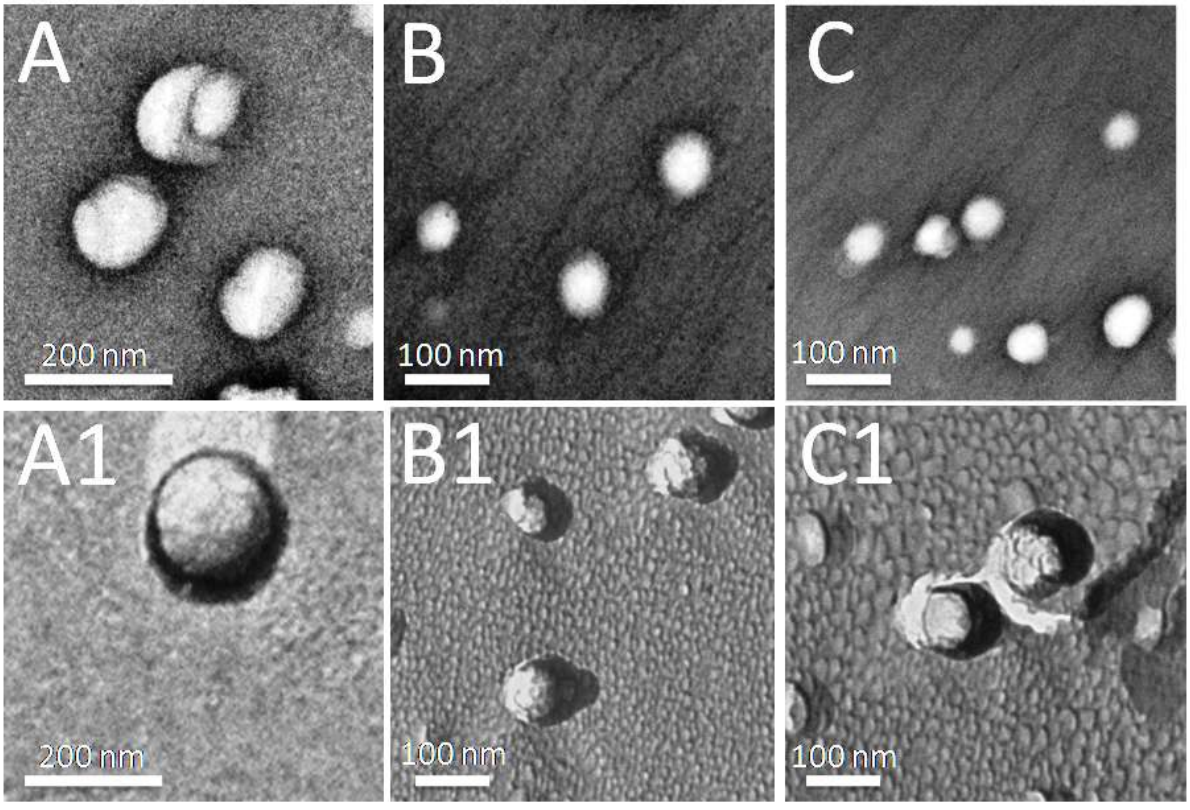

Figure 5. TEM (panel A, B & C) amd FF-TEM (panel A1, B1 & C1) images for (A) conventional NLC (SLC+TS+PA, 2:2:1, M/M/M); (B) NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) and (C) NLC_{IPA} in presence of OLA. SLC/IPA ratio is 30 : 70 (M/M) in NLC_{IPA}. The scale bars are mentioned inside the figure.

Observed size of the studied NLC and NLC_{IPA} were found to be in accordance with the previously observed DLS data. Slide deviation in the observed size was due to the difference in the technique of measurement. In all the cases, NLC and NLC_{IPA} were found to be spherical. No significant difference in morphology was noted in the presence of IPA. The incorporated OLA did not influence the morphology of the studied formulations.

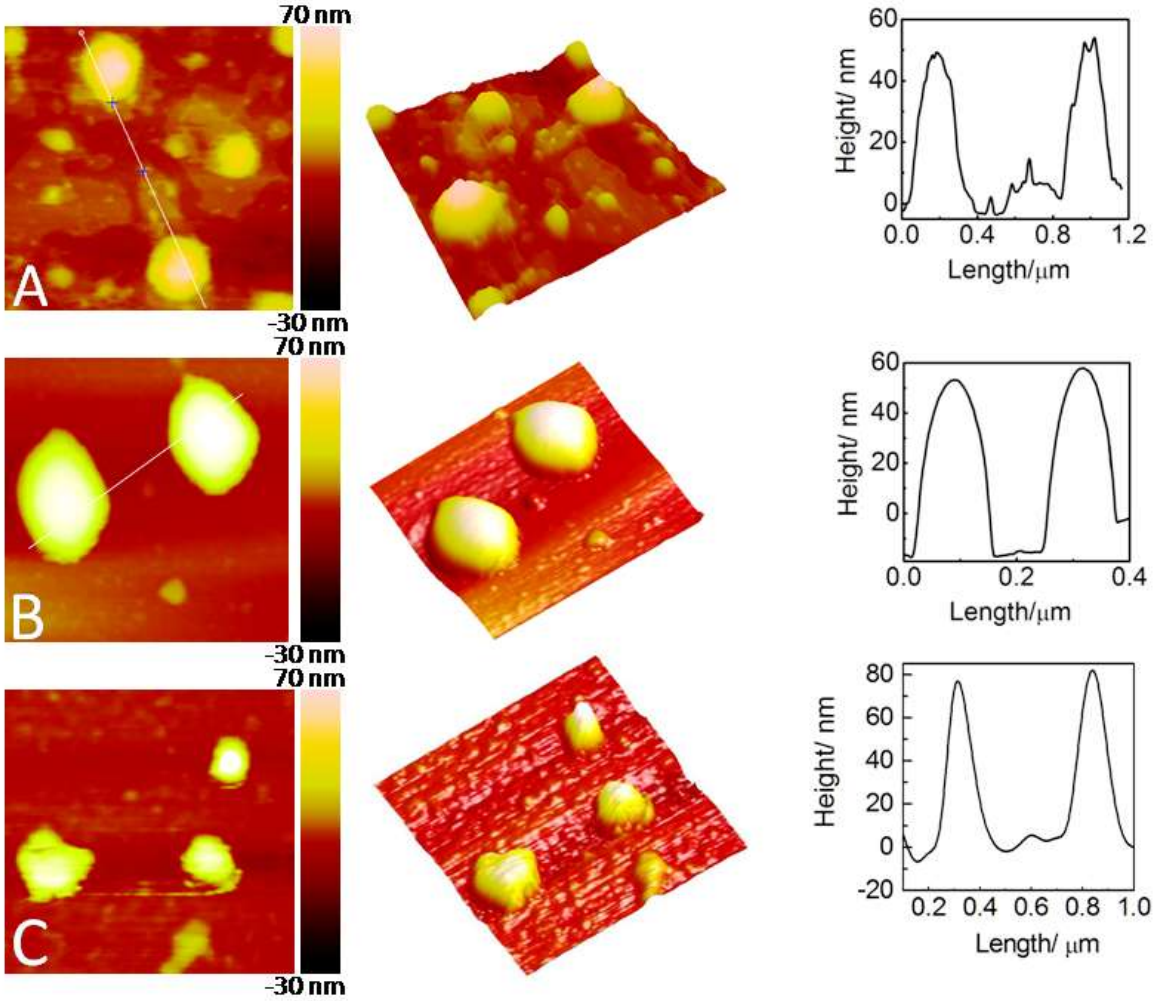

Figure 6. AFM images of (A) conventional NLC (SLC+TS+PA, 2:2:1, M/M/M); (B) NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) and (C) NLC_{IPA} in presence of OLA. SLC/IPA ratio was 30 : 70 (M/M) in $NLC_{IPA.}$ Corresponding three dimensional view and roughness profiles were also presented in the Figure.

Fig. 6 showed some representative AFM images along with the three dimensional representation of them. The obtained bright spots in the AFM images indicated NLC. No significant difference in size was noted from previously performed TEM and FF-TEM studies.

The height of NLCs were found in the range of 30 to 40 nm. The collapse of NLC and NLC_{IPA} during the drying process under vacuum in the AFM analysis was mainly responsible for the reduction in the observed height. [22, 23] NLC and NLC_{IPA} showed smooth surface morphology. No difference in the surface morphology observed in the presence of IPA in NLC_{IPA}. Corresponding roughness analysis of the NLC and NLC_{IPA}were also represented along with the AFM image in Fig. 6, The smooth hump observed in the roughness analysis indicated the even surface for the studied formulations, But NLC and NLC_{IPA} loaded with OLA showed a reduction in the surface smoothness. Observed folds in the roughness analysis indicated the presence of OLA on the palisade layer of NLC_{IPA}. The accumulated OLA on the palisade layer of NLC_{IPA} made the surface rough. The observation also indicated the formation of the shell enriched type NLC_{IPA}.

3.4. Differential scanning calorimetric studies

DSC is a very sensitive technique in determining the internal morphology and related thermodynamics for the lipid based drug delivery systems like NLC. In the present set of studies, DSC thermograms of NLC and NLC_{IPA} having different SLC/IPA ratio were constructed and compared to evaluate the effect of added IPA in the internal morphology and thermal properties. Some representative DSC cooling thermograms of conventional NLC and NLC_{IPA} with three different SLC/IPA ratio have been presented in the panel A Fig. 7.

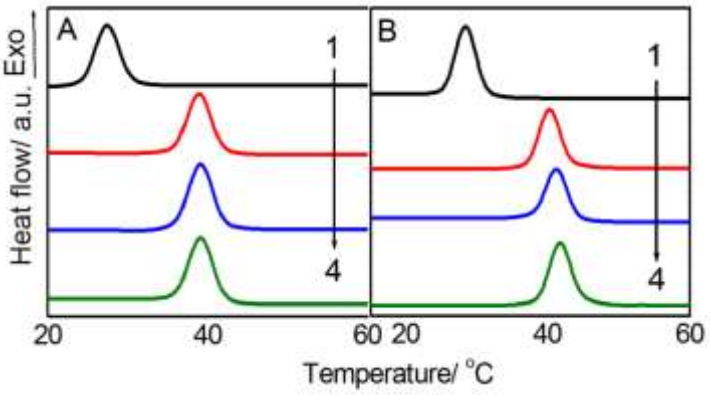

Figure 7. DSC cooling thermograms for NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) formulations having different SLC/IPA ratio in absence (panel A) and presence (panel B) of OLA. SLC/IPA (M/M) ratios (panel A & B): 1, 100 : 0; 2, 40 : 60; 3, 30 : 70 and 4, 20 : 80. [NLC_{IPA}] and [OLA] : 1 mM and 10 µM respectively. Scan rate, 2.5 $^{o}C\ min^{-1}$.

In addition to this, a heating cooling DSC thermogram of NLC_{IPA} has been shown in Fig. 8.

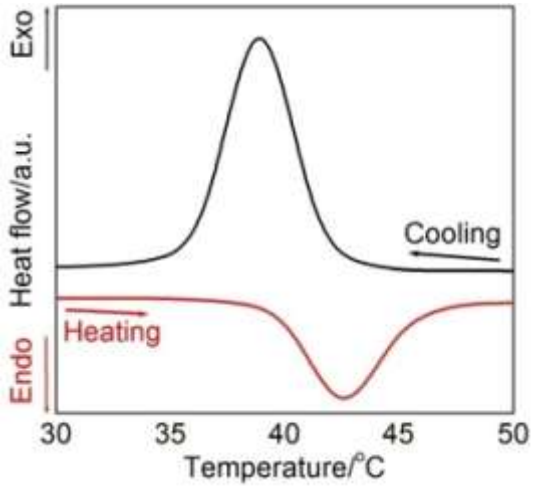

Figure 8. Representative heating cooling DSC thermogram for NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) formulations having SLC/IPA (M/M) ratio 30 : 70. [NLC_{IPA}] : 1 mM. Scan rate, 2.5 $^{o}C\ min^{-1}$.

A significant reduction in the temperature of maximum heat flow (T_m) was observed for the NLC and NLC_{IPA} from the bulk lipid mixture. Downshift in T_m was due to the reduction in size and subsequent enhancement in surface area in the form of NLC. [12, 21] Similar to the liquid crystalline systems, observed cooling thermograms were found to be more prominent than the heating thermograms for conventional NLC as well as NLC_{IPA}. [12, 21, 24-27] Due to the prominent nature, cooling exothermic thermograms were used for the determination of different thermodynamic parameters. Evaluated thermodynamic parameters were presented graphically in the Fig. 9.

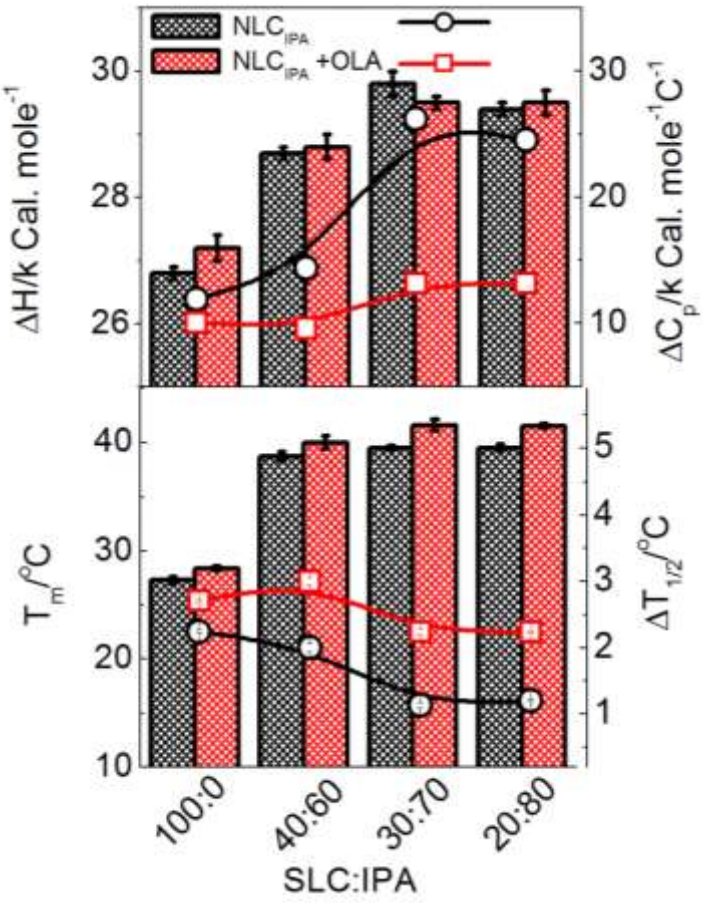

Figure 9. Variation in different thermodynamic parameters with the change in SLC/IPA tatio of NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) in the absence (black) and presence (red) of OLA. The individual systems were mentioned inside the figure.

In case of the conventional NLC, T_m was found at 27.3 ºC. But in case of NLC_{IPA}, significant enhancement in the T_m was observed. NLC_{IPA} having SLC/IPA ratio 40:60, 30:70 and 20:80 were showed T_m at 38.7, 39.5 and 39.5 ºC respectively. Increment in T_m in the presence of IPA indicated the structural compactness and lesser lipidic modification. Maximum value of T_m was observed for NLC_{IPA} having SLC/IPA ratio 30:70. Further enhancement in the IPA proportion has no effect on the observed T_m value. The observation indicated that SLC/IPA ratio 30:70 was the optimum for the stable structural arrangement of NLC_{IPA}. ΔH and ΔC_p for the conventional NLC and NLC_{IPA} were also evaluated to gather further information regarding the structural properties and the multicrystalline nature. The ΔH and ΔC_p for NLC_{IPA} were found to be higher than the conventional NLC. The lesser multicrystallinity and structural compactness of

NLC_{IPA} were responsible for the observed result. NLC_{IPA} having SLC/IPA ratio 30:70 was found to show the maximum ΔH and ΔC_p. Further enhancement in IPA had no effect in the ΔH and ΔC_p for NLC_{IPA}. The observed result also confirmed that, SLC/IPA ratio 30:70 was optimum and indicated the saturation limit of IPA for NLC_{IPA}. The reduction in $\Delta T_{1/2}$ value for NLC_{IPA} than conventional NLC also indicated the structural compactness and reduced multicrystallinity. The observed minima in $\Delta T_{1/2}$ for NLC_{IPA} having SLC/IPA ratio 30:70 also confirmed the saturation limit of IPA for the stable NLC_{IPA} formulation.

Thermal properties of the OLA loaded conventional NLC as well as the NLC_{IPA} were also evaluated and the obtained thermograms were presented in the panel B of Fig. 7. The calculated thermodynamic parameters were also presented in Figure 9 along with the base formulations. In case of the DSC thermogram of the OLA loaded conventional NLC and NLC_{IPA}, no significant change was observed. But the incorporation of OLA caused a right shift in the T_m value for NLC as well as NLC_{IPA}. The enhancement in T_m indicated the formation of the shell enriched NLC formulations. [11, 12] The shell formed by the incorporated OLA, delayed the phase transition of NLC. The observed repulsive interaction of OLA with the lipid system in the Langmuir monolayer studies also support the accumulation of OLA in the palisadelayer of NLC and NLC_{IPA}. No observable variation in ΔH, ΔC_p and $\Delta T_{1/2}$ for OLA loaded NLC and NLC_{IPA} clearly indicated the presence of any interaction of OLA with the lipid matrix and confirmed the formation of shell enriched type NLC and NLC_{IPA}.

3.5. Entrapment efficacy and drug loading capacity

Entrapment efficiency (EE%) and the drug loading capacity (DL%) are the two key parameters from the stand point of a lipid base drug delivery system. In the present set of work, EE% and DL% of OLA in conventional NLC and NLC_{IPA} were determined and compared. NLC_{IPA} having SLC/IPA ratio 40 :60, 30 : 70 and 20 : 80 along with conventional NLC without having IPA were subjected for the determination of EE% and DL%. The obtained results were presented graphically in the panel A of Fig. 10.

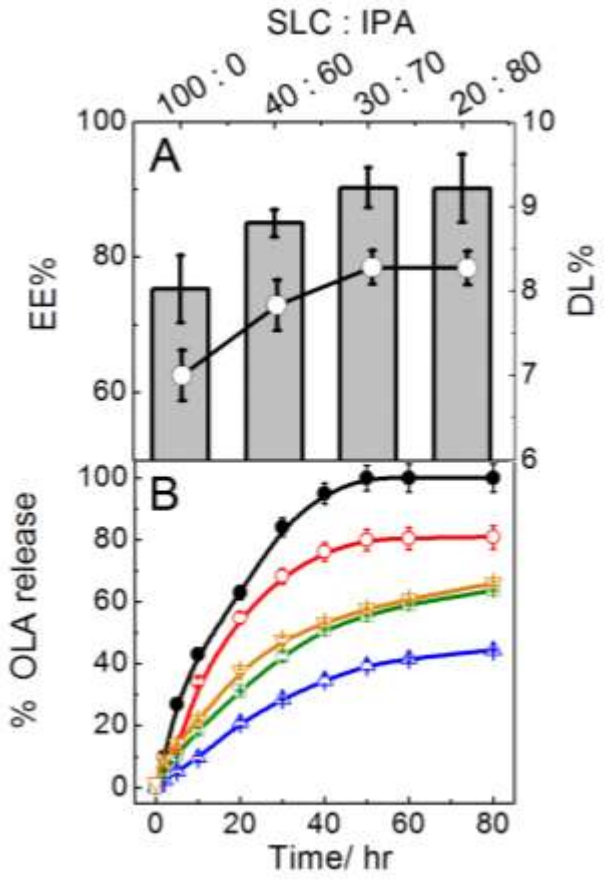

Figure 10. Variation in entrapment efficiency (EE%), drug loading (DL%) capacity (panel A) and release profile (panel B) of OLA with the variation in the SLC/IPA ration for NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) formulation at 25 ºC . SLC/IPA (M/M) ratio (panel B): ●, controlee ○,100 : 0; □, 20 : 80; Δ, 30 : 70 and ▽,40 : 60.

EE% and DL% of the NLC_{IPA} were found to improve in the presence of IPA. Amphiphilic OLA molecules preferentially got accumulated on the surface of NLC and forms shell enriched type NLC. The polar lipid head groups on the surface provided polar environment to host the drug in NLC. In case of NLC_{IPA}, enhancement in the surface accumulation of OLA was observed. Enhancement in the polar nature of NLC_{IPA} surface in the presence of IPA was mainly responsible for the observed result. Enhanced polarity of the surface provided better hold to the surface accumulated OLA and reduced the drug expulsion. EE% and DL% of the NLC formulations were also found to be dependent on the SLC/IPA ratio. With increasing IPA proportion, enhancement in EE% and DL% were observed up to SLC/IPA ratio of 30 : 70. In other word the maximum EE% and DL% were observed for the NLC_{IPA} having SLC/IPA ratio 30:70. Further enhancement in IPA proportion did not have any significant influence in the EE%

and DL%. Hence, NLC_{IPA} formulation having SLC/IPA ratio 30 : 70 was the optimum one and signifies the saturation limit of IPA for NLC_{IPA}.

3.6. Release of OLA:

Release profiles of OLA from the studied conventional NLC and NLC_{IPA} having different SLC/IPA ratio have been presented in the panel B of Fig. 10. The simple diffusion of OLA through dialysis membrane was also presented along with the release profile of NLC and considered as the controlee. Studied conventional NLC and NLC_{IPA} were found to sustain the release of OLA to a considerable extent. The release profile of the studied formulations was monitored up to 80 hr. In case of conventional NLC a biphasic release pattern was noted. [12] Initial burst release of the loosely surface bind OLA and then sustain release of rather strongly adsorbed OLA in the surface of NLC. In case of NLC_{IPA} having IPA in combination with SLC, release was found to get sustained in comparison to the conventional NLC. In addition to this, NLC_{IPA} were found to show different release pattern. A systematic homogeneous release pattern was noted for NLC_{IPA}. The observation clearly supported the homogeneous surface accumulation of OLA in $NLC_{IPA.}$ The presence of IPA at the surface of NLC_{IPA} enhanced the dipolar interaction between polar sides of the surface accumulated OLA and provided the desirable sustained release rate of OLA. In addition to these the release of OLA from the NLC_{IPA} was also dependent on the SLC/IPA ratio. Maximum sustained release was observed for the NLC_{IPA} having SLC/IPA ratio 30 :70. Further enhancement in IPA proportion did not encourage more sustained release of OLA from NLC_{IPA}.

In order to get proper understanding regarding the release mechanism of OLA from conventional NLC and NLC_{IPA}, obtained release profiles were fit in five different release models *viz.*, first order, zero order, higuchi, weibull and korsmeyer-peppas model using DD Solver 1.0. [28, 29] All the regression data of four different dissolution models have been presented in table 1.

Table 1 Release kinetics of OLA from conventional NLC and NLC_{IPA} having different SLC/IPA ratio.

Different	First order		Zero order		Weibull		Korsmeyer-Peppas			Higuchi	
SLC/IPA (M/M) ratios of OLA loaded NLC_{IPA}	k_1/ h^{-1}	R^2	k_0/ mol $lit^{-1}.h^{-1}$	R^2	k_w/ h^{-1}	R^2	k_k/ h^{-n}	R^2	n	k_h/ $h^{-0.5}$	R^2
100 : 0	0.021	0.989	1.190	0.853	1.247	0.997	6.212	0.998	0.590	8.766	0.968
40 : 60	0.016	0.957	1.061	0.998	1.159	0.996	3.641	0.965	0.627	5.931	0.946
30 : 70	0.011	0.946	0.979	0.997	0.949	0.994	2.098	0.966	0.546	4.270	0.963
20 : 80	0.018	0.905	0.809	0.993	1.104	0.990	3.131	0.965	0.465	5.987	0.962

[NLC_{IPA}] (SLC/IPA+TS+PA, 2:2:1, M/M/M): 1 mM, and [OLA]: 10 µM.

The obtain data indicated that, the simple diffusion of OLA was found to follow higuchi release formalism. This type of release governs by the fick diffusion. In other word it controlled by the difference in concentration of OLA in either side of the dialysis membrane. By comparing the non liner regression coefficient values for the different release models, it was observed that the conventional NLC formulation followed korsmeyer–peppas release mechanism. [11, 12, 21] On the other hand, release of NLC_{IPA} was found to follow the zero order release mechanism. The observed result indicated the uniform distribution of OLA over the NLC_{IPA} surface. Due to the homogeneous surface distribution, OLA was found to get released at constant rate. The observed release mechanism indicated the drug enriched shell model of NLC_{IPA}. [12]

3.7. Anticancer activity of OLA loaded conventional NLC and NLC_{IPA} against GIT cell lines

To investigate the potentiality and applicability of the OLA loaded NLC and NLC_{IPA} as drug delivery system, *in vitro* cytotoxicity of the studied formulations were evaluated on three different GIT cell lines such as hepatocellular carcinoma cell (HepG2), hepatocyte-derived carcinoma (Huh-7) and human colorectal carcinoma (HCT-116) cell line using MTT assay technique. [20] OLA loaded conventional NLC, NLC_{IPA} and OLA in the native form were subjected for the cytotoxicity studies. Obtained results were summarized graphically in the Fig. 11 and Fig. 12.

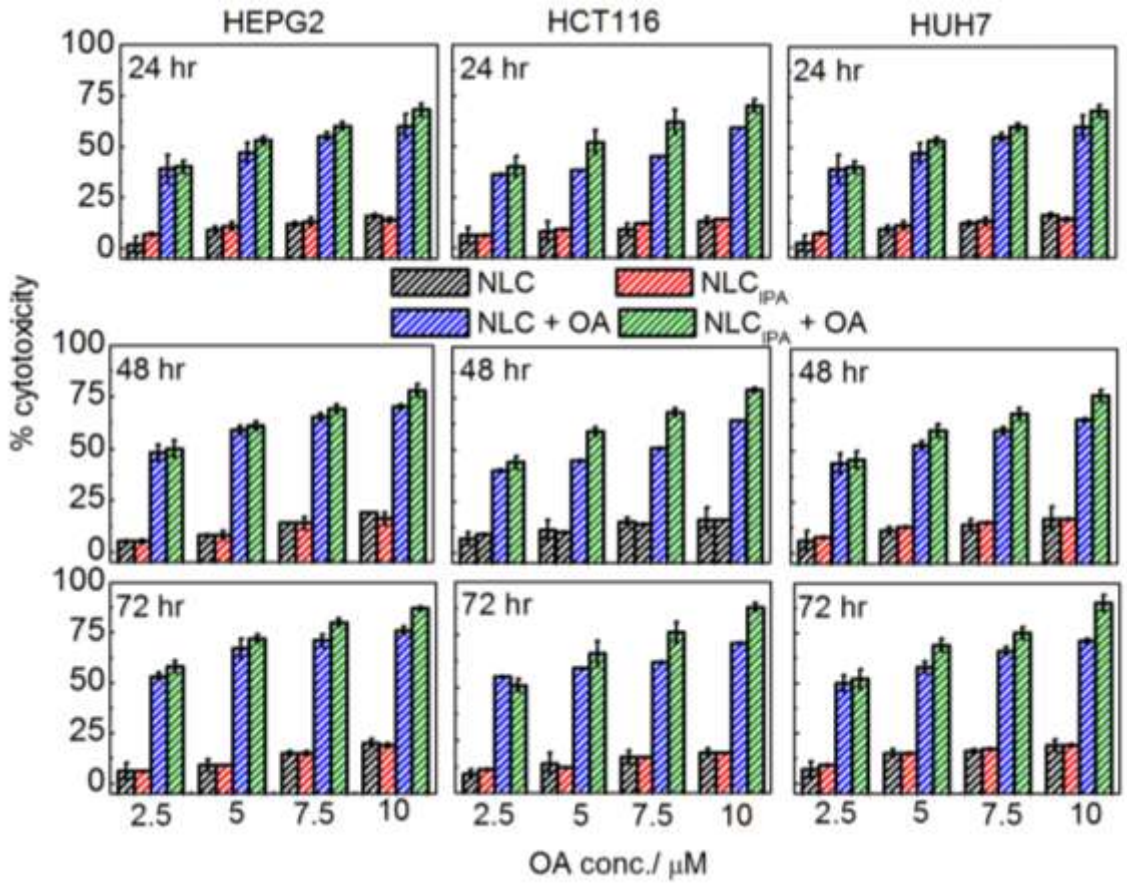

Figure 11. *In vitro* cytotoxicity of the conventional NLC (SLC/IPA+TS+PA, 2:2:1, M/M/M) and NLC_{IPA} (SLC/IPA+TS+PA, 2:2:1, M/M/M) in absence and presence of OLA on three different cell lines at three different incubation times. The different systems, cell lines and incubation times were mentioned in the figure. NLC_{IPA} having SLC/IPA ratio 30 : 70 was used for the cytotoxicity studies.

In all the cases the dispersion medium of the studied formulation (2 mM aqueous Tween 60 solution) was used as controlee. Conventional NLC and NLC_{IPA} without having OLA were also subjected for the cytotoxicity studies and they were found to show no significant toxic effect on the studied cell lines after 72 hr of incubation time. The observation indicated the biocompatibility of base NLC and $NLC_{IPA.}$ In the present set of work, OLA loaded conventional NLC and NLC_{IPA} showed enhanced cytotoxicity in comparison to the OLA in native form.

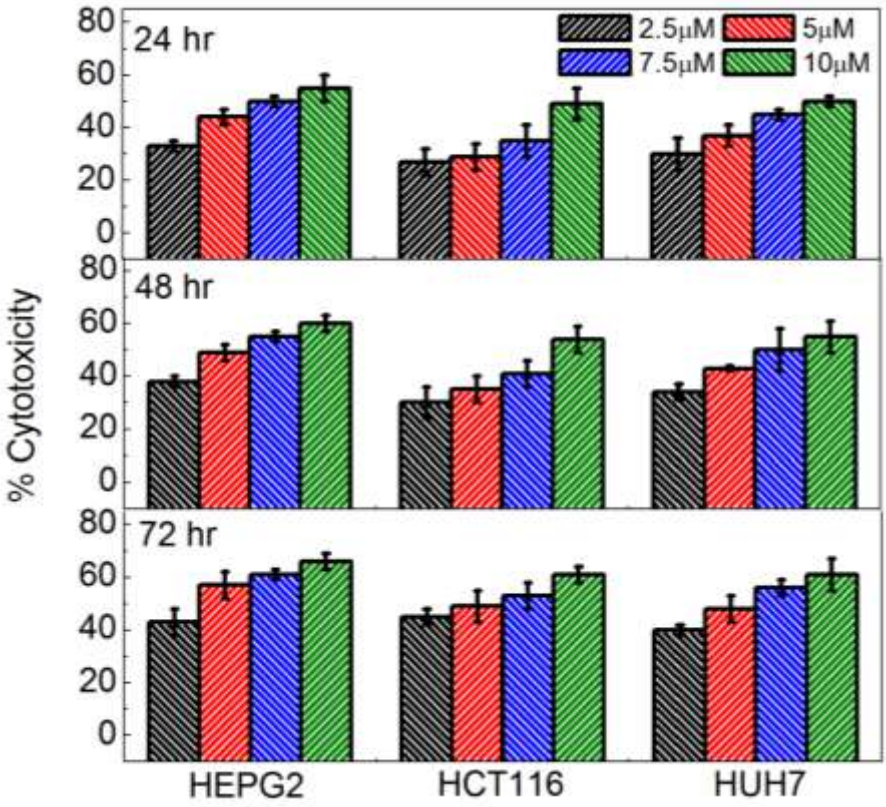

Figure 12. *In vitro* cytotoxicity of OLA alone on three different cell lines at three different concentrations of OLA. Different cell lines and incubation times were mentioned in the figure.

Moreover, the NLC_{IPA} loaded with OLA showed even higher activity than OLA loaded conventional NLC. The superior activity of OLA loaded NLC_{IPA} formulations can be explained on the basis of higher penetration power and maximum amount of OLA internalization into the cancer cell. The smaller size, higher drug loading and drug incorporation efficacy was mainly responsible for the observed superior activity than the conventional NLC formulations. In addition to this the sustained release of the incorporated OLA was also helped in enhancing the cytotoxicity.

For the better understanding on the cytotoxic activity of the studied formulations on GIT cell lines, IC_{50} values for free OLA, OLA loaded conventional NLC and NLC_{IPA} were calculated at three different incubation times (24 hr, 48 hr and 72 hr) and compared. Obtained results were summarized in table 2.

Table 2. IC_{50} values of pure OLA, conventional NLC and NLC_{IPA} loaded with OLA on different cell lines.

Systems	IC_{50} on different cell lines (μM)								
	HEPG2			HCT116			HUH7		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Pure OLA	6.51	5.88	5.25	8.54	7.42	5.78	7.31	6.52	5.80
Conventional NLC	5.36	4.93	4.48	6.71	5.96	4.90	5.91	5.37	4.88
NLC_{IPA}	5.36	4.61	4.01	5.28	4.73	4.21	5.36	4.72	4.14

The lowest IC_{50} values were observed for OLA loaded NLC_{IPA} in all the cases. The reduced IC_{50} value of the OLA loaded NLC_{IPA} confirmed the superior activity of it over the free OLA and OLA loaded conventional NLC. No significant variation in the activity was observed with different GIT cell lines studied.

Moreover, a progressive increment in cytotoxicity was observed for all the formulations with increasing OLA concentration and incubation time. The enhancement was observed for all the GIT cell lines studied. The observation indicated that the anticancer activity of the studied formulations was a concentration and time dependent phenomenon. The enhancement in activity may be explained on the basis of better internalization of OLA inside the cell with increasing drug concentration and incubation time. Further investigation on the detailed mechanism of anticancer activity against GIT cancer cell lines , more sophisticated biological studies were warranted and those are taken as the future prospective of our research group.

4. Conclusion

In the present set of studies, conventional NLC and NLC_{IPA} were prepared using hot homogenization followed by ultrasonication method. Conventional NLC was prepared using SLC, TS and PA with molar ratio 2: 2: 1. The conventional NLC was modified and NLC_{IPA} formulations were prepared using IPA in combination with SLC. Before the preparation, the ratio of SLC and IPA were quantitatively estimated by considering the mutual interaction among the lipid constituents and IPA at the air water interface by Langmuir monolayer approach. Three different systems with SLC/IPA ratio 40 : 60, 30 : 70 and 20 : 80 were selected for the

preparation of NLC_{IPA}. The prepared conventional NLC and NLC_{IPA} were characterized using DLS, DSC, TEM, FF-TEM and AFM. The results indicated that, in the presence of IPA, the stability of NLC_{IPA} enhanced in comparison to conventional NLC. Reduction in the lipid modification in the presence of IPA stabilized the NLC_{IPA} formulation. Among the different NLC_{IPA} formulations, system with SLC/IPA ratio 30 : 70 was found to be the optimum having the least structural disorder and the maximum compactness among the lipid components suggested by the solution as well as the thermal behavior of the systems. OLA as drug was incorporated into the studied formulations. The detailed physicochemical characterization of the OLA loaded system indicated the preferential surface accumulation of OLA on conventional NLC and NLC_{IPA}. The amphiphilic nature of OLA due to the presence of the phenolic hydroxyl and carboxylic acid group was responsible for the accumulation at palisade layer. Due to the surface adsorption of OLA, shell enriched type NLC system was proposed for the studied systems. The EE% and DL% of the NLC_{IPA} were found to be superior over the conventional systems. The release of OLA was also get sustained in the presence of IPA in NLC_{IPA}. The anticancer activity of the OLA loaded conventional NLC and NLC_{IPA} were carried out on Hepatocellular carcinoma (HepG2), hepatocyte-derived carcinoma (Huh-7) and colorectal carcinoma (HCT-116) cell lines. The activity of NLC_{IPA} was found to be higher than the conventional NLC. In summary, it can be said that the IPA is a promising alternate of conventional phospholipid in enhancing the stability and performance of OLA loaded NLC_{IPA}. But further studies were warranted for the exhaustive evaluation of these systems with various synthetic ion pair amphiphiles. In addition, their activity and other related biological parameters with different category of drugs is also warranted for their comprehensive evaluation as successful drug delivery system.

References

References are given in BIBLIOGRAPHY under references for CHAPTER 3 (pp. 154-155).

CHAPTER 4

Role of PEG 2000 in the surface modification and physicochemical characteristics of pyrazinamide loaded nanostructured lipid carriers.

Abstract

Hydrogenated soy phosphatidylcholine (HSPC), tristearin (TS) and oleic acid (OA) (2:2:1, M/M/M) were employed for the preparation of the nanostructured lipid carriers (NLC). Surface modified NLC formulations (NLC_{PEG}) were formulated by adding polyethylene glycol 2000 (PEG 2000) in the dispersion medium (2 mM aqueous Tween 60) of NLC. 0.0001, 0.001, 0.01 and 0.1 (W/V)% of PEG 2000 were used to obtained the effect of PEG 2000 on the physicochemical characteristics of NLC_{PEG}. In all the cases hot homogenization followed by ultrasonication technique was used as preparative procedure. 0.01 (W/V)% PEG 2000 was found to be the saturation limit for the studied formulations. Pyrazinamide (PYZ) was used as drug in the present work and successfully incorporated in NLC and NLC_{PEG} systems. The base and the drug loaded formulations were subjected for detailed characterizations using dynamic light scattering (DLS), differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The stability of the NLC_{PEG} formulations were found higher than the conventional NLC formulations. Added PEG 2000 provided extra steric stability to the NLC_{PEG} systems by introducing an additional layer over NLC_{PEG}. The presence of the additional layer of PEG 2000 offered a preventative barrier towards easy expulsion of the surface accumulated PYZ. Hence, considerable improvement in entrapment efficiency (EE%), drug loading (DL%) and desirable sustained release profile were observed for NLC_{PEG} formulations. .

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1. Introduction

Nanocolloidal lipid based drug delivery systems have enormous possibility in the improvement of phermacodynamics and pharmacokinetics of a large number of active pharmaceutics. [1-5] Some commonly used drug delivery systems are microemulsion, liposomes, nano suspensions, polymeric nanoparticles *etc*. But more or less all the mentioned lipid based drug delivery systems suffer from serious limitations like low drug incorporation, fast release of the incorporated drug, low affinity towards hydrophilic drug and biocompatibility in some cases. [6-10] The mentioned common disadvantages of the lipid based drug deliver are successfully overcome in nanostructured lipid carriers (NLC). NLCs are the modified form of solid lipid nanoparticles, also known as the second generation solid lipid nanparticle. [1-5] Blend of two or more structurally dissimilar biocompatible lipids (solid or liquid) is used for the preparation of NLC. The created structural imperfections by the stericaly different lipid molecules effectively host the incorporated pharmaceutics to a considerable extent and effectively reduce the drug lose and unwanted drug leakage from NLC surface. NLC systems thus effectively overcome all major limitations of the conventional drug delivery systems and prove them as the most promising lipid based drug delivery of recent age. Although NLCs are advantageous, but problems like lipid modification, high rate of coagulation, separation of the lipid phase, poor incorporation of the hydrophilic drug etc., have restricted the wide participation of NLC and restrains the market availability of it. [1-3, 11-13] These shortcomings are the serious challenge to a pharmacist in the development of NLC systems as nobel drug delivery agent.

Several attempts have been made by various research groups in enhancing the stability and performance of NLC formulation by employing different surfactants, polymers and surfactant in combination with polymer in the dispersion medium. [9, 14, 15] In case of the surfactant – polymer stabilized NLC, non ionic surfactants and polymers were found to give the best performance due to their biocompatibility and non toxicity. [9] In addition to this, the nonionic polymers provide a great deal of steric stability and effectively reduce the lipid modification which is directly related to the coagulation rate of NLC. It has been established that, the added non ionic polymer produced a layer over the NLC surface and effectively reduce the coagulation. The polymer layer is also effective in reducing the easy escape of the surface accumulated hydrophilic drug components. But detailed study regarding the effect of nonionic polymers on

the stability of the NLC formulations and the exact role of the polymer layer on the drug loading and release mechanism of the hydrophilic drugs are still lagging in the literature. To uncover those facts, different concentration of a nonionic biocompatible polymer PEG 2000 has been used in combination with the non ionic surfactant Tween 60 in developing NLC_{PEG} systems loaded with a hydrophilic drug PYZ.

PYZ is a well known first line drug in the treatment of the active tuberculosis. Generally PYZ is used in combination with isoniazid, rifampicin and ethambutol in the treatment of active tuberculosis. [16-19] It is also used as a potent drug for ueicosuric disuses. In addition to this hypouricemia and hyperuricosuria have been effectively diagnosis using PYZ. [17, 18] But high water solubility, high rate of execration and high dose frequency have restricted its pharmaceutical use. [17, 18] Hence, a suitable delivery system is a need for PYZ. Researchers have taken a number of attempts to develop a suitable solid lipid based drug delivery systems to overcome the limitations. [16-19] Most of the proposed formulations suffer from several serious limitations like low EE%, DL% and fast release of incorporated PYZ. So, the focus of the present work is given in the development of suitable NLC system for PYZ and attempt has been made to improve the performance by employing surface modification using nonionic polymer PEG 2000. No such prior attempt has been made in developing surface modified NLC_{PEG} for the delivery of PYZ to the best of our knowledge.

In the present study, surface modified NLC_{PEG} formulations were prepared and their stability and efficiency as a delivery agent of hydrophilic pharmaceutics PYZ were compared with the conventional NLC without having any surface modifications provided by nonionic polymer PEG 2000. In this work, HSPC, TS and OA (2 : 2 : 1, M/M/M) were used as lipidic components for the preparation of NLC and NLC_{PEG}. 2 mM aqueous Tween 60 solution was used as the dispersion medium for the studied formulations. In case of the surface modified NLC_{PEG}, four different concentrations (0.0001, 0.001, 0.01 and 0.1 W/V %) of nonionic polymer PEG 2000 was used along with 2 mM aqueous Tween 60 solution in the dispersion medium. Conventional NLC and surface modified NLC_{PEG} formulations were prepared using the hot homogenization followed by ultrasonication method. The obtained formulations were subjected for detailed physicochemical characterizations using DLS, DSC, conventional TEM and AFM. Water soluble PYZ was used as drug for the studied formulations. The PYZ loaded formulations

were also characterished using the mentioned analytical techniques to gather information regarding the location of the incorporated PYZ. Conventional NLC and NLC_{PEG} formulations were further characterized for the evaluation of DL% and EE%. The release of the incorporated PYZ was also studied to evaluate the potentiality of the studied formulations as a delivery system for PYZ. The obtained release profiles were further analyzed using different release models for the evaluation of the exact release mechanism of the incorporated PYZ. Such comprehensive set of work can be helpful in providing a concept in the development of different surface modified NLC formulations for water soluble drugs PYZ and other drugs of this family.

2. Material and methods

2.1. Materials

HSPC, tristearin (TS) and oleic acid (OA) have been purchased from Sigma-Aldrich Chemicals (USA). Tween 60 and polyethylene glycol 2000 (PEG 2000) were procured from Sisco Research Laboratory (SRL), Pyrazinamide (PYZ) was obtained from Merck Specialties Pvt. Ltd, India. All the solvents of analytical grade have been used throughout the study. Double distilled water having molar conductivity of 2 mS cm^{-1} at 25 ºC was used for the experiments.

2.2. Preparation of NLC and NLC_{PEG}

Conventional hot homogenization followed by ultrasonication technique was used for the preparation of the studied formulations. Details regarding the preparative procedure can be obtained in our recent publications. [6-9] HSPC, TS and OA were used in the molar ratio 2 : 2 : 1, M/M/M and the concentration of the studied formulations were maintained at 1 mM. In case of the NLC_{PEG} formulation, PEG 2000 was introduced by dissolving it in the dispersion medium of NLC. 0.0001, 0.001, 0.01 and 0.1 (W / V) % of PEG 2000 was used separately in the dispersion medium for the evaluation of the effect on the stability for NLC_{PEG} formulations. 2 mM aqueous Tween 60 solution was used as dispersion medium for the studied formulations. During the preparation of PYZ loaded NLC and NLC_{PEG} formulations, PYZ were introduced in the lipid physical mixture. In this study, PYZ concentration was fixed at 10 µM for the conventional NLC and NLC_{PEG} formulations.

2.3. Analytical instrumentations

Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) of the studied formulations were evaluated using dynamic light scattering technique (Zetasizer Nano ZS90 ZEN3690, Malvern Instruments Ltd., U.K.). Morphology of the prepared NLC and NLC_{PEG} systems were studied using conventional TEM (Hitachi H-600, Japan) and AFM (Bruker Nanoscope V Multimode SPM). Thermal behavior of the studied formulations were analyzed using a differential scanning calorimeter (DSC, Mettler Toledo, Switzerland). Obtained thermal data were further analyzed using DSC1 $STAR^e$ software for the evaluation of different thermodynamic parameters.

2.4. Entrapment efficiency (EE%) and drug loading capacity (DL%)

Method of centrifugation was used for the evaluation of EE% and DL% for NLC and NLC_{PEG} formulations. The drug loaded NLC and NLC_{PEG} formulations were subjected for high speed centrifuge at 15000 rpm. During centrifugation, the temperature of the studied formulations were maintained at 4 ºC. Collected supernatant of the centrifuged samples were analyzed using a specrophotometer to determine the amount of free drug. After that, EE% and DL% of the studied formulations were determined using the following equations[6-9]

$$EE\% = \frac{W_{loaded\ \ PYZ}}{W_{totaal\ \ PYZ}} \times 100\ \% \quad (1)$$

$$DL\% = \frac{W_{loaded\ \ PYZ}}{W_{lipid}} \times 100\ \% \quad (2)$$

Where $W_{total\ PYZ}$, W_{lipid} and $W_{loaded\ PYZ}$ were represented the weight of total PYZ added in the studied formulations, amount of lipids used in NLC, NLC_{PEG} and amount of entrapped PYZ in NLC, NLC_{PEG} respectively.

2.5. *In vitro* release study

The release of the incorporated PYZ from NLC and NLC_{PEG} were studied using conventional dialysis bag approach (12 kD). [6-9] 5 mL of the PYZ loaded NLC and NLC_{PEG} formulations were taken inside the dialysis bag and immersed into 20 mL of release medium. 2 mM aqueous Tween 60 solution was used as release medium for the present study. The release

experiment was carried out by maintaining sink condition under constant stirring. The released drug was quantified colorimetrically.

3. Results and discussions

3.1. Solution phase behavior of NLC and NLC_{PEG}

Hydrodynamic diameter (d_h) of the NLC formulation is a very important quality control parameter because it mainly determines their solution phase stability, loading capacity, entrapment efficiency and the release rate of the incorporated drug. In the present study HSPC, TS and OA (2 : 2: 1, M/M/M) were used for the preparation of the NLC and NLC_{PEG} formulations. 2 mM aqueous Tween 60 solution was used as the dispersion medium for the studied formulations. In case of the NLC_{PEG} formulations, different concentration of PEG 2000 was added in the dispersion medium. To evaluate the solution phase stability of the studied formulations, d_h was monitored with respect to time and presented graphically in the panel A of Fig 1.

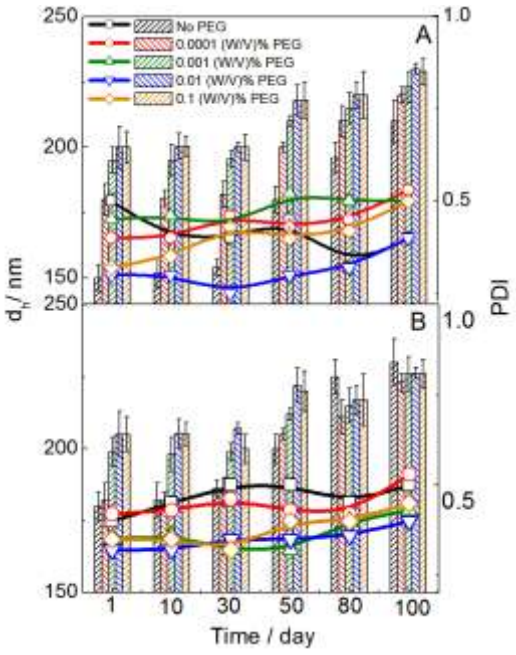

Figure 1. Variation in d_h and PDI with respect to time for the NLC (HSPC : TS : OA, 2 : 2 : 1, M/M/M) and NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) systems in the absence (panel A) and presence (panel B) of PYZ at 25 ^{o}C. The different concentration of PEG 2000 was mentioned inside the figure.

The d_h of the studied conventional NLC formulations was found in the range of 150 - 210 nm. The d_h of the PEG 2000 coated formulations were found in the range of 200 - 230 nm. The d_h of NLC_{PEG} formulations were found to be higher than the conventional NLC. Increase in d_h was due to the presence of a additional layer of the added PEG 2000 in NLC_{PEG}. The d_h of NLC_{PEG} formulations were also get governed by the concentration of PEG 2000. Progressive enhancement in d_h was noted with increasing concentration of PEG 2000 in the dispersion medium. Maximum d_h was observed for the NLC_{PEG} formulation having 0.01 (W/V)% PEG 2000 in the dispersion medium. Further increase in the amount of PEG 2000 was found to show no effect in d_h of the studied formulations. The result clearly indicated the saturation point of PEG 2000 for the studied NLC_{PEG} formulations. At this concentration, palisade layer of NLC_{PEG} get saturated with PEG 2000 and no additional accumulation of PEG 2000 was noted with further addition of PEG 2000.

A clear idea regarding the solution phase stability during the storage time can be obtained from the d_h vs. time profile (panel A of Fig. 1) of the studied formulations. The d_h of the conventional NLC and NLC_{PEG} formulations were monitored for 100 days and no significant fluctuation in d_h value was observed. The observation indicated the formation of stable nanocolloidal suspension. In addition to this, a smooth progressive increment in the d_h value was noted with the storage time for all the studied formulations. The coagulation of the nanocolloidal suspension was mainly responsible for the observed phenomenon. Observed growth rate of the NLC_{PEG} formulations were found to be lower than the conventional NLC system. Reduction in growth rate indicated the reduced coagulation in the presence of additional PEG 2000 layer along with the non ionic surfactant Tween 60. The additional layer of PEG 2000 provided extra steric stability to the NLC_{PEG} and restricted the coagulation phenomenon among the suspended lipid particles to a considerable extent.

d_h vs. time profiles for PYZ loaded NLC and NLC_{PEG} formulations were also presented in the panel B of Fig 1. d_h of PYZ loaded conventional NLC and NLC_{PEG} formulations were found in the range of 180 - 230 and 185 - 233 nm respectively. The observed enhancement in the d_h value in case of the conventional NLC, indicated the accumulation of PYZ on surface and suggested a shell enriched NLC formulation. On the other hand, no significant influence was noted in the d_h value of PYZ loaded NLC_{PEG} formulation. Obtained result indicated the inside

penetration of the incorporated PYZ in between the palisade layer of NLC_{PEG} and PEG 2000 layer.

PDI of the nanocolloidal drug delivery systems is another crucial parameter because it regulates the homogeneity of nanocolloidal suspensions. In the present study PDI for the studied formulations were also monitored with respect to time and presented in the panel A of Fig. 1. The observed PDI for the studied formulations were found in the range of 0.3 – 0.5. Observed PDI for the prepared formulation indicated the formation of the well homogenized suspensions. Observed fluctuation in the PDI vs. time profile for the studied formulation signified the ongoing crystalline modification inside NLC and NLC_{PEG} systems. The crystalline modifications in NLC, make the lipid core mobile and disturbed the homogeneity of the formulation during storage. In case of NLC_{PEG}, fluctuation in PDI was considerably less. This observation indicated the enhancement in stability for NLC_{PEG} formulation in comparison to the conventional NLC. PDI of PYZ loaded formulations were also determined and presented in panel B of Fig. 1. In case of PYZ loaded NLC formulation, a small increase in the PDI was noted. The increased PDI indicated reduction in the surface homogeneity due to the accumulation of PYZ on the palisade layer of NLC. On the other hand, no significant variation in PDI was noted for the PYZ loaded NLC_{PEG} from the corresponding base systems. The observation indicated the accumulation of PYZ in between the lipid palisade layer and the additional polymer layer. Due to the inside penetration of incorporated PYZ into the PEG 2000 layer, no surface irregularity was noted in the surface of PYZ loaded NLC_{PEG} formulations.

Z.P. is directly related to the surface charge and electrostatic stability of lipid based nanocolloidal drug delivery systems. In the present study, Z.P. of the studied formulations were monitored with respect to time and presented graphically in the panel A of Fig. 2.

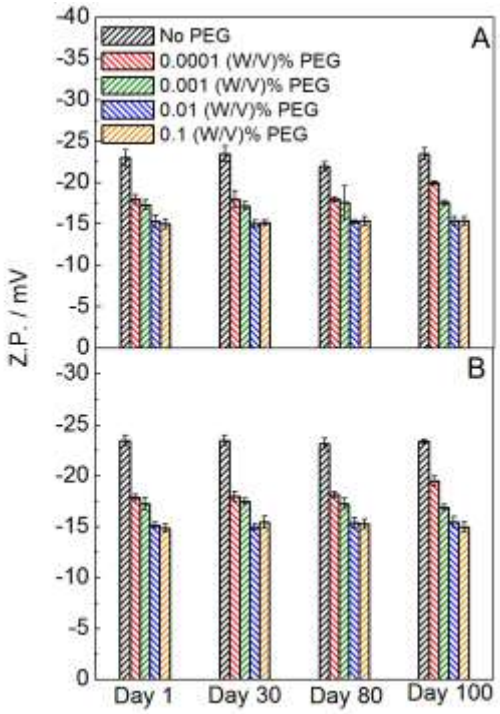

Figure 2. Z.P. vs. time profile for NLC (HSPC : TS : OA, 2 : 2 : 1, M/M/M) and NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) systems in the absence (panel A) and presence (panel B) of PYZ at 25 ºC. The different concentration of PEG 2000 was mentioned inside the figure.

The Z.P. of the conventional NLC formulation were found in the range of -22 to -24 mV. In case of the NLC_{PEG} formulations, Z.P. was formed in the range of -15 to -20 mV.. Reduction in the negative magnitude of Z.P for NLC_{PEG} indicated the pressure of polymer layer (non ionic PEG-2000) which effectively masked the surface change of NLC_{PEG}. Z.P. value of the studied NLC_{PEG} formulation was also found to get influenced by the amount of added PEG 2000. With increasing concentration of PEG 2000, lowering of surface charge was noted and the this effect get continued till the concentration of PEG 2000 reached at 0.01 (W/V)%. Further enhancement in PEG 2000 has no significant influence in the reduction of surface charge of the studied formulations. The increasing concentration of the nonionic polymer was found to increase the thickness of polymer layer over the NLC surface and progressively reduced the surface charge. After the saturation point, no further accumulation of PEG 2000 occurred over NLC_{PEG} and no further reduction in the ZP was observed. In case of PYZ loaded conventional NLC and NLC_{PEG} formulations no significant influence on the Z.P. value was noted in comparison to the base systems (panel B of Fig. 2). Absence of any significant fluctuation in the Z.P. vs. time profile of

the base and the drug loaded formulations also signifies the considerable solution phase stability for the formulations under experiment.

3.2. Morphological studies

The surface morphology of the NLC and NLC_{PEG} were analyzed using conventional TEM and AFM. In all the cases spherical morphology and a smooth surface was noted. Some representative TEM images have been given in the panel A and B of Fig. 3.

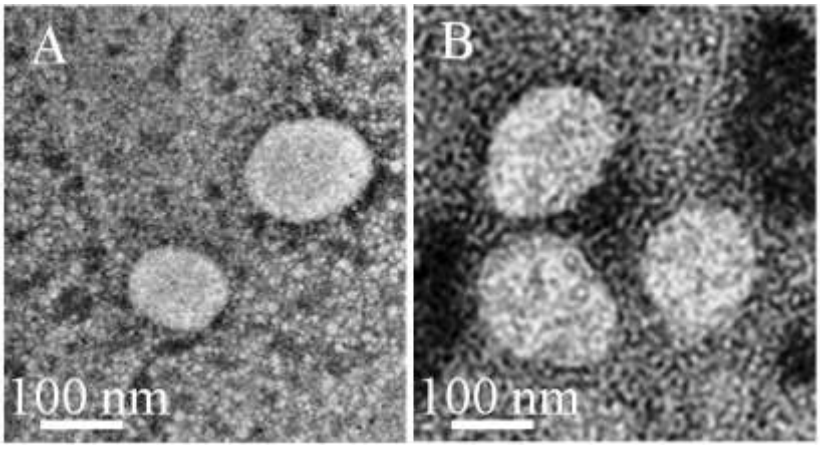

Figure 3. TEM images of the NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having 0.01 (W/V)% of PEG 2000 in the absence (A) and presence (**B**) of PYZ. Scale bars are given inside the figures.

No significant difference in the size of the studied NLC and NLC_{PEG} systems were observed from the previously performed DLS studies. A small reduction of size in case of TEM indicated the loss of the hydration sphere associated with the NLC and NLC_{PEG} during the drying process of sample preparation. [6-8] The loss of hydration sphere result a little contraction in size of NLC and NLC_{PEG} systems. The morphology of the studied formulations were further investigated using AFM. AFM image of a representative NLC_{PEG} formulation have been presented in the Fig. 4 along with the three dimensional over view and roughness analysis profile.

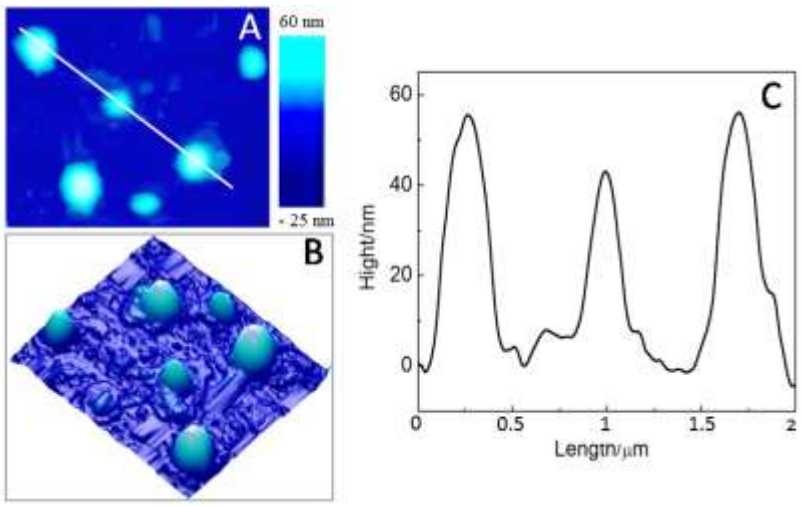

Figure 4. AFM image (A) NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having 0.01 (W/V)% of PEG 2000. Panel B and C represented the three dimensional surface morphology and corresponding roughness analysis profile respectively.

The observed results were found to be in accordance with the previously performed DLS and TEM studies. The observed height of the studied formulations was found in the range of 40 - 60 nm. The significant reduction in height can be explained by the collapse of NLC during the vacuum drying process. [20, 21] The presence of additional polymer layer was not detected clearly in TEM and AFM analysis. During the loss of hydration sphere, the layer of PEG 2000 also gets ruptured. More sophisticated morphological analysis like FF-TEM and cryo TEM are expected to be helpful in getting the image of the polymer layer. Our research group is looking forward for those sophisticated studies in near future.

3.3. Thermal behavior of NLC and NLC_{PEG}

DSC is a very useful technique in collecting information regarding internal morphology and crystallinity of the lipid based nanocolloidal drug delivery systems. Representative DSC thermograms of the conventional NLC and PEG 2000 coated NLC_{PEG} formulations have been presented in the Fig. 5.

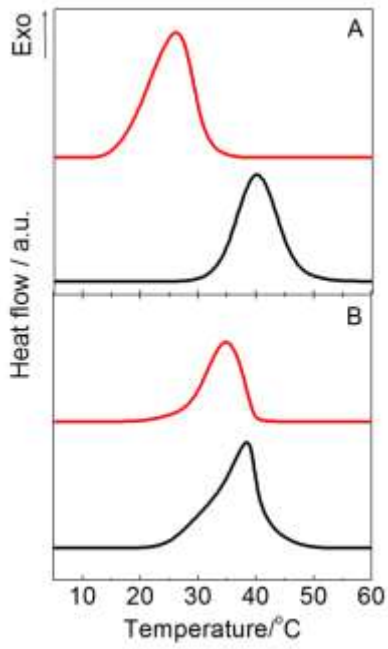

Figure 5. DSC cooling thermograms of NLC (red) and NLC_{PEG} (black) (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having 0.01 (W/V)% of PEG 2000 in absence (panel A) and presence (panel B) of PYZ. The scan rate was fixed at 2 ºC min^{-1}.

In all the thermograms, only one prominent peak was noted. The appearance of a single prominent peak indicated negligible lipidic modification inside NLC and NLC_{PEG} . The phase transition temperature (T_m) for the NLC and NLC_{PEG} were found in the range of 25 - 35 and 32 – 40.1 ºC respectively. A marked reduction in T_m value was noted for NLC and NLC_{PEG} from the bulk lipid mixture. The observed reduction can be explained on the basis of structural reorganization and subsequent generation of structural imperfections during the formation of NLC and NLC_{PEG}. In addition to this, significant reduction in size is also an important cause for the reduction in T_m value for NLC and NLC_{PEG} systems. [6-8] Observed cooling thermograms were found to be more prominent in comparison to the heating termograms. Down shift in T_m value for the cooling thermogram indicated the similarity of the studied NLC and NLC_{PEG} with liquid crystalline systems. [22-25] Due to the prominent nature, cooling thermogram were further analyzed to determine different thermodynamic parameters. The calculated thermodynamic parameters have been presented in the table 1.

Table 1. Phase transition temperature (T_m), half peak width ($\Delta T_{1/2}$), change in enthalpy (ΔH) and heat capacity (ΔC_p) of the base and PYZ loaded NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having different concentration of PEG 2000.

	PEG2000/(W/V)%	T_m/°C	$\Delta T_{1/2}$/ °C	ΔH/ kcal.mol^{-1}	ΔC_p / kcal.mol^{-1}C^{-1}
NLC_{PEG}	0	27	8.5	32	3.76
	0.0001	32	8.3	32.9	3.96
	0.001	37	9.2	33	3.58
	0.01	40	9	32.5	3.61
	0.1	40.1	9.2	33.4	3.63
PYZ loaded NLC_{PEG}	0	35	9.5	33.1	3.48
	0.0001	31.3	9.5	32.5	3.42
	0.001	36.2	9	32.3	3.58
	0.01	39.2	8	33.2	4.15
	0.1	39.3	9.3	33.3	3.58

Concentration of the NLC_{PEG} was fixed at 1 mM. All the DSC studies were performed on day1. Scan rate: 2 °C min^{-1}.

The T_m value for the NLC was found at 25 ºC. T_m value for NLC_{PEG} formulations were found in the range of 32 – 40.1 ºC. The observed enhancement in T_m indicated the presence of additional layer of PEG 2000 over the NLC_{PEG} surface. The presence of additional layer of polymer delayed the phase transition phenomenon and enhanced the T_m for NLC_{PEG}. T_m values of NLC_{PEG} were also found to be dependent on the amount of the added PEG 2000. With increasing the concentration of PEG 2000, enhancement in T_m indicated the formation of thicker layer of PEG 2000 with increasing concentration. But no enhancement was noted after the saturation point (0.01 W/V %). Insignificant change in the ΔH and ΔC_p were noted for the NLC_{PEG} from the convectional NLC system. Obtain result clearly indicated that, the added PEG

2000 forms an outer layer over the NLC surface and do not have any significant interaction with the internal morphology of NLC_{PEG}. Hence, no observable change in $\Delta T_{1/2}$ was also observed among the conventional NLC and NLC_{PEG} formulations

Panel B of Fig. 5 represent the DSC thermogram of PYZ loaded NLC and NLC_{PEG} systems. The calculated thermodynamic parameters of the PYZ loaded formulation have been presented in the table 1 along with the base systems. T_m value of the PYZ loaded NLC was found to be higher than the base systems. The enhancement in T_m is a clear indication for the formation of shell enriched NLC system. The presence of the hydrophilic lipid part (head groups) at the surface provided a dipolar attraction to the hydrophilic drug PYZ and increased the surface availability of PYZ. On the other hand, a slide reduction in T_m was observed for PYZ loaded NLC_{PEG} systems form the base formulations. The observed reduction of T_m indicated the penetration of incorporated PYZ in between the PEG 2000 layer and surface of NLC_{PEG}. The presence of PYZ created irregularity in the molecular arrangement in the PEG 2000 layer. The irregularity created by the incorporated PYZ reduced the shielding to some extent and reduction in the T_m was observed. No observable change in ΔH, ΔC_P and $\Delta T_{1/2}$ were noted for the PYZ loaded NLC and NLC_{PEG} systems. Hydrophilic nature of the PYZ restricted its partitioning in the lipid phase of NLC and NLC_{PEG}. Hence, insignificant change in the ΔH, ΔC_P and $\Delta T_{1/2}$ of the PYZ loaded formulations was observed. The observation confirmed the surface accumulation of PYZ and formation of shell enriched NLC system.

3.4. Entrapment efficiency and drug loading capacity studies

EE% and DL% of conventional NLC and NLC_{PEG} systems were evaluated and graphically represented in the Fig. 6.

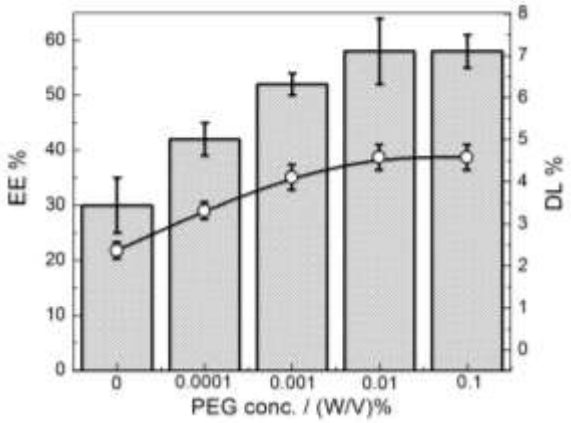

Figure 6. Variation in the EE^ and DL% of the NLC_{PEG} (HSPC : TS : OA, 2 : 2: 1, M/M/M) formulations with changinh the concentration ofPEG 2000.

The effect of concentration of the added non ionic polymer on EE% and DL% of NLC_{PEG} systems were also investigated. In case of conventional NLC formulation the EE% and DL% were found to be 30 % and 2.4 % respectively. The obtained EE% and DL% were found to be less in comparison to the frequently reported NLC formulations loaded with amphipilic drug molecules. PYZ is a hydrophilic drug. Due to its hydrophilic character, physical exclusion rate of the surface adsorbed PYZ is very high. Only a weak dipolar attraction between the amine group of PYZ and the hydrophilic lipid head group mainly the carboxylic acid groups of the OA molecules present at the NLC surface were holding the incorporated PYZ on the surface. EE% and DL% of the NLC_{PEG} formulations were found in the range of 42 – 58 % and 3.3 – 4.6 % respectively. Considerable improvement in the EE% and DL% in case of NLC_{PEG} can be explained on the basis of additional layer of PEG 2000 over NLC_{PEG} surface. In case of the NLC_{PEG}, the incorporated drug get accumulated on the surface as well as in between the lipid palisade layer and the polymer layer. In addition to this, the PEG 2000 layer prevented the easy physical exclusion of the incorporated drug. EE% and DL% of the NLC_{PEG} formulations were also found to be dependent on the concentration of added polymer in the dispersion medium. EE% and DL% of the NLC_{PEG} formulations were found to get increased with increasing concentration of PEG 2000 up to a certain concentration (0.01 W/V%) and after that no further enhancement was noted. Observation clearly indicated the attainment of saturation of the added PEG 2000 in NLC_{PEG} system. With increasing concentration of PEG 2000, the shielding towards

the physical exclusion of the PYZ increases due to the progressive increment in the thickness of the polymer layer. The thickness was found to increase till the attainment of saturation limit.

3.5. Drug release studies

The release of the incorporated PYZ from the conventional NLC as well as NLC_{PEG} formulations were studied and compared. NLC_{PEG} system having 0.01 (W/V)% of PEG 2000 was considered for the release study. The obtained release profile and the simple diffusion of PYZ from dialysis membrane have been presented graphically in the Fig. 7.

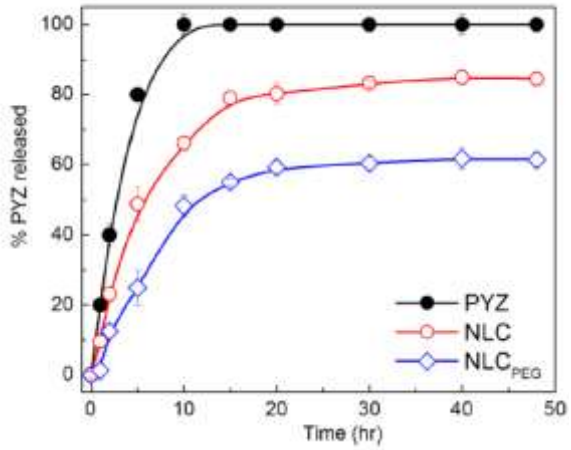

Figure 7. Release profiles of the free PYZ and PYZ from the conventional NLC (HSPC : TS : OA, 2 : 2 : 1, M/M/M) and NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having 0.01 (W/V)% of PEG 2000 at 25 ºC.

The simple diffusion of PYZ was taken as control for the release study to eliminate the effect of dialysis membrane over the release of PYZ. The release of PYZ from NLC and NLC_{PEG} were found to be sustained in comparison to the simple diffusion of PYZ. The release of PYZ from the NLC and NLC_{PEG} were monitored for 48 hrs. In 48 hrs, maximum 84 % of PYZ was found to get released from the conventional NLC system. 61.3 % of PYZ was found to get released from NLC_{PEG} during the same time period. In all the cases a biphasic release pattern was noted. [6, 8, 9] An initial burst release and then a sustained release of PYZ. In case of the conventional NLC 58 % of the incorporated PYZ get released during the initial burst with in 8 hrs. Whereas only 36.3 % of the incorporated PYZ get expulsed as the initial burst in case of NLC_{PEG} formulation. The burst release of the NLC_{PEG} was continued up to 6 hrs from the

beginning of release time. The results indicated that, NLC_{PEG} provided a more controlled and sustained release of the incorporated PYZ in comparison to the conventional NLC formulation. The presence of the additional polymer layer over the NLC_{PEG} surface increased the micro viscosity around it and effectively slowdown the release of surface adsorbed PYZ.

The obtained release profiles were further fitted into different well established release models to get a proper understanding regarding the drug release mechanism. DD solver an add-in program was used to obtained the dissolution data for the studied release profiles. [26, 27] In the present study, first order, zero order, Higuchi, Korsmeyer-peppas and Weibull models were selected to fit the release profiles. The obtained dissolution data have been presented in the table 2.

Table 2. Release kinetic data of PYZ from NLC (HSPC : TS : OA, 2 : 2 : 1, M/M/M) and NLC_{PEG} (HSPC : TS : OA, 2 : 2 : 1, M/M/M) having 0.01 (W/V)% of PEG 2000.

Different formulations	First order		Zero order		Weibull			Korsmeyer-Peppas			Higuchi	
	k_1/ h^{-1}	R^2	k_0/ mol $lit^{-1}.h^{-1}$	R^2	k_w/ h^{-1}	R^2	β	k_k/ h^{-n}	R^2	N	k_h/ $h^{-0.5}$	R^2
NLC	0.03	0.969	2.19	0.873	2.24	0.997	0.465	7.21	0.997	0.40	8.30	0.965
NLC_{PEG}	0.02	0.987	1.05	0.898	1.15	0.996	0.423	4.64	0.996	0.39	4.90	0.976

[NLC] and [NLC_{PEG}]: 1 mM, and [PYZ]: 10 µM.

Obtained regression values suggested that, the release of PYZ from NLC and NLC_{PEG} followed korsmeyer-peppas release formalism. According to this release model, the release rate of PYZ from the conventional NLC and NLC_{PEG} were found to be 7.21 and 4.64 $hr^{=1}$ respectively. Release exponent (n) for NLC and NLC_{PEG} were also evaluated for Korsmeyer-Peppas release model and the values were found to be ≤ 0.5. the obtained result indicate that the release of PYZ from NLC and NLC_{PEG} was controlled by classical Fick diffusion mechanism.

4. Conclusions

The focus of the present work was centered on the development and the physicochemical characterization of the NLC_{PEG} systems designed for the delivery of the hydrophilic drug, PYZ. PEG 2000 a non ionic polymer was used for the surface modification in case of NLC_{PEG} systems. HSPC, TS and OA with a molar ratio 2 : 2: 1, M/M/M was used for the preparation of NLC and NLC_{PEG} formulations. In case of NLC_{PEG} system, the nonionic polymer was introduced in the dispersion medium (aqueous 2mM Tween 60 solution). 0.01 (W/V)% PEG 2000 was found to be the optimum concentration for the preparation of stable NLC_{PEG}. The stability of the NLC_{PEG} formulations was found to be higher than the conventional NLC formulations. The significant reduction in the lipid modification and growth rate indicated the stability of the NLC_{PEG} systems. In addition to this, presence of additional layer of PEG 2000 was found to enhance the steric stability of NLC_{PEG} systems. PYZ was also successfully incorporated in the NLC and NLC_{PEG} formulations. The detailed physicochemical characterization of PYZ loaded formulations indicated the formation of shell enriched NLC and NLC_{PEG} formulations. The EE% and DL% of NLC_{PEG} formulations were found to get improved in comparison to the conventional NLC systems. In addition to this, the release of the incorporated PYZ from the NLC_{PEG} was also get sustained effectively in comparison to the conventional NLC. The layer of PEG 2000 provided a better control over the release of the hydrophilic drug PYZ. Hence, NLC_{PEG} systems were found to be superior than the conventional NLC for the delivery of hydrophilic drug like PYZ. But detailed understanding of the role of nonionic polymer on the stability and performance of NLC formulations, more sophisticated studies like XRD (small and wide angle), FF-TEM, cryo TEM *etc.*, are warranted. The relevant *in vitro* and *in vivo* biological studies are also required for the evaluation of their application potential. Those works are taken as the future prospective of our research group.

R**eferences**

References are given in BIBLIOGRAPHY under references for CHAPTER 4 (pp. 155-156).