CHAPTER 2

Physicochemical characterization of chrysin derivative loaded nanostructured lipid carriers with special reference to anticancer activity

Abstract

Homologs of long chain chrysin derivatives (LCDs, C_n:8-18) were synthesized and incorporated into nanostructured lipid carriers (NLC) with an aim to treat human neuroblastoma. Mutual miscibility among the NLC components (tripalmitin, cetyl palmitate, oleic acid) and chrysin derivatives at the air-water interface were assessed by Langmuir monolayer approach. There occurred attractive interactions among the components. Having the maximum extent of association the optimum combination for the NLC formulations were found to be 2:2:1 (M/M/M) for tristearin : cetyl palmitate: oleic acid respectively. NLC formulations, both in the absence and presence of chrysin derivatives, were characterized by combined dynamic light scattering, electron microscopy, atomic force microscopy and differential scanning calorimetry. Size and zeta potential of the NLC formulations were found in the range 200 – 350 nm and -12 to -18 mV respectively. Chrysin and LCDs, when loaded in the NLCs, were further assessed by the evaluation of its encapsulation and release kinetics. C_{18} derivative of chrysin showed maximum incorporation, drug loading capacity and sustained release profile. All the drug loaded formulations showed potential anticancer activity again human neuroblastoma cell lines (SHSY5Y). Superior incorporation efficiency and sustained release profile of LCDs were able to enhance their anticancer activity in comparison to pure chrysin, putting them forward as the promising agent to combat cancer.

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1. Introduction

To double check the progress in the therapeutic efficacy, development of new drugs alone is not sufficient.[1-4] On the other hand, insufficient bioavailability of the drug substance are very challenging issue to encounter the paradigm.[4] To overcome those problems nanostructured lipid carriers (NLC) were introduced at the beginning of 1990.[5-8] They are also known as the modified solid lipid nanoparticles (SLN). These are the nanocolloidal suspension having the size range 10-1000 nm.[9-11] The main difference between SLN and NLC: only the symmetric hydrocarbon chain containing lipid components are used for the preparation of SLN, unlike the NLC, where two or more structurally different lipids (solid and/or liquid) are used. NLCs have superiority over the other drug delivery systems like liposomes, nanoemulsion, microemulsion, nanocapsule, nanosponge, polymeric nanoparticles, *etc*.[4, 9-11] Adequate pharmaceutical loading capacity, possibility of specific targeting and sustained release characteristics over the other delivery systems make NLC superior. Additionally, NLCs are capable in providing chemical stability and steadiness of the incorporated pharmaceutical. Beyond the mentioned advantages, NLC formulations suffer from serious limitations.[1, 5-8] The higher aggregation rate due to ongoing lipid modification leads to the stability problem.[9, 10] So formulation of stable NLC by judiciously choosing lipidic components with suitable composition is a challenging task in the area of pharmaceutical research.

Generally, the biocompatible solid lipids, namely wax, phospholipid, monoglycerides, diglyceride, triglyceride in combination with fatty acid were mainly used for the preparation of NLC.[5-12] However, NLC formulation comprising cetylpalmitate (CP), tripalmitin (TP) and oleic acid (OA) as the core lipidic materials have yet not been explored to the best of our knowledge. Selection of the suitable lipid component and the fatty acids are not the only criteria for the preparation of stable NLC formulations.[9] Determination of the suitable lipid composition by exploring the nature of the interaction among the lipid components and fatty acids are also very much warranted. However, the detail investigation among the lipid interaction in formulating stable NLC systems is lagging and fragmented in nature.[9] On the other hand detailed physicochemical characterization of NLCs involving the solution phase and thermal stability are warranted in developing a stable NLC formulation.

Chrysin (5,7-dihydroxy flavone) as a natural flavonoid, generally obtained from several wild and edible plants, honey and propoles.[13, 14] It has various biological activities like anti-inflammation, anti-oxidant and anticancer activity. Some recent studies have proved that it can markedly decrease the malondialdehyde level and elevate antioxidant enzyme activity.[13-20] But lower bioavailability of chrysin is a major issue. Several chrysin loaded NLC formulations were developed to overcome the limitation and enhance the activity. But due to its amphiphilic nature the loading and the encapsulation efficiency of the chrysin were not satisfactory.[17, 20] To enhance the encapsulation efficiency, people have synthesized chrysin derivatives having long hydrocarbon chain to enhance the lipid solubility of the compound and enhanced the encapsulation efficiency.[15] In the present study four long hydrocarbon chain (8, 10, 16 and 18 carbon atoms) chrysin derivatives have been synthesized. Pure chrysin and its derivatives were incorporated in the NLC formulation for a comparative evaluation of effectiveness of chrysin derivatives over the parent compound from the view point of drug incorporation, drug loading and the sustained release behavior.

In the present set of study CP, TP and OA have been subjected for the preparation of NLC formulation. Before the preparation of the NLC formulation the lipid composition was optimized by studying their mutual miscibility and interaction at different composition at the air-water interface. Langmuir monolayer approach was employed for the study. Also the interfacial interaction between the lipid mixture (considered as a pseudo single component) and the chrysin derivatives at different proportions were assessed by way of surface pressure – area measurements. Aqueous solution of the nonoionic surfactant Tween 60 was used as stabilizer. The prepared NLC formulation, in the absence and presence of chrysin (and its derivatives) were then subjected for the determination of size, polydispersity index and zeta potential by dynamic light scattering technique (DLS). Differential scanning calorimetric studies were employed for the evaluation of the thermal properties of the NLC formulations and the determination of the thermodynamic parameters, *viz.*, phase transition temperature (T_m), enthalpy change of the phase transition (ΔH) and the heat capacity changes (ΔC_p) as well as the peak width of the chain melting at half maximum ($\Delta T_{1/2}$). Morphology of the NLCs were studied by transmission electron microscopy (TEM), freeze fractured transmission electron microscopy (FF-TEM) and atomic force electron microscopy (AFM). Chrysin and the LCDs have been incorporated in the optimized NLC formulation and the drug loaded NLC formulations were subjected to the

physicochemical characterizations. Chrysin and the LCDs of chrysin loaded formulations were also subjected for the determination of entrapment efficiency, drug loading capacity and the release kinetic studies. For the evaluation of the biological activity, chrysin as well as the LCD loaded formulations were subjected for *in vitro* cytotoxicity through MTT based assay against human neuroblastoma cell lines (SHSY5Y). It is believed that such a combined set of studies would eventually help in formulating a suitable drug delivery system in the treatment of cancer.

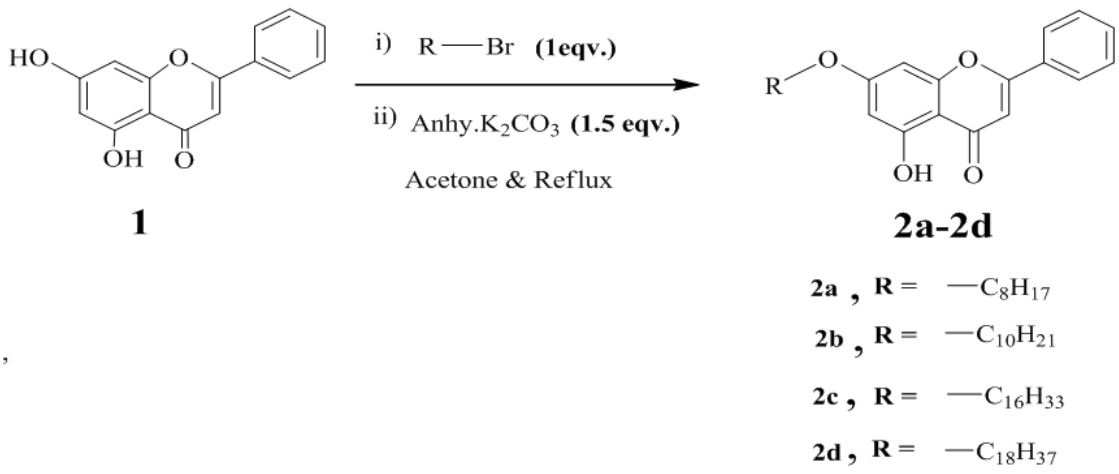
2. Materials and method

2.1. Materials.

CP, TP, OA and chrysin were purchased from Sigma-Aldrich Chemicals (USA). AR grade Tween 60 was procured from Sisco Research Laboratory (SRL), India. All the chemicals were obtained as received and stated to be >99.5% pure. HPLC grade solvents and double distilled water having specific conductance of 2-4 μS (at 25^0C) were used in preparing the solution.

2.2. General method for synthesis of chrysin (1) derivatives (2 a-d).

To a solution of **1** (0.1 g, 93 mM) in 10 ml of anhydrous acetone was added alkyl bromide (0.076 g, 0.394mM), and anhydrous K_2CO_3 (0.0815g, 59.1mM) followed by refluxing for 24 h until the starting material disappeared on TLC. The reaction mixture was filtered and the filtrate was dried by distillation. The solid residue was suspended in water and extracted three times with ethyl acetate. The mixed ethyl acetate extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue obtained was purified with silica gel column chromatography eluting with petroleum ether-ethyl acetate.



Scheme 1. Synthesis of C (7) modified derivatives (LCD's) of chrysin.

2.3. Characterization of the derivatives.

LCDs, after purification were characterized by checking their melting points, solubility. Subsequently, the compounds were exposed the FTIR, ^{1}H and ^{13}C NMR studies. The observations are mentioned below:

2a: Yield 98 %, yellow solid, soluble in $CHCl_3$, molecular weight 366.18 corresponding to molecular formula $C_{23}H_{26}O_4$, mp. 105^0C. IR (ν cm^{-1}): 3457 (hydroxyl), 1662 (α,β-unsaturated carbonyl), 1620, 1590, 816 (aromatic), 2961, 2927, 2856 (C-H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.70 (1H, s, 5-OH), 7.89 and 7.86 (2H, d, J = 2.8 *Hz*, C-2′, C-6′), 7.51 (3H, m, C-3′, C-4′, C-5′), 6.65(1H, s, H-3), 6.65 (1H, d, J = 2.8 *Hz*, H-6), 6.35 (1H, d, J = 2.8 *Hz*, H-8), 4.02 (2H, t, J = 8.8 *Hz*, -OCH_2), 1.30-1.87 (12H, m, 6 × -CH_2) and 0.89 (3H, t, J = 9.2 *Hz*, -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 182.4 (C-4), 165.2 (C-7), 163.9 (C-3), 162 (C-5), 157.8 (C-9), 131.8 (C-1′), 131.3 (C-4′), 129 (C-3′, C-5′), 126.3 (C-2′, C-6′), 105.8 (C-3), 105.5 (C-10), 98.6 (C-6), 93.0 (C-8), 68.7 (-OCH_2), 22.6 – 31.8 (6 × -CH_2) and 14.05 (-CH_3).

2b: Yield 97 %, yellow solid, soluble in $CHCl_3$, molecular weight 394.21 corresponding to molecular formula $C_{25}H_{30}O_4$, mp. 90^0C. IR (ν cm^{-1}): 3415 (hydroxyl), 1666 (α, β-unsaturated carbonyl), 1624, 1580, 829 (aromatic), 2960, 2919, 2852 (C-H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.80 (5-OH), 7.89 (2H, C-2′, C-6′), 7.55 (3H, m, C-3′, C-4′, C-5′), 6.68 (1H, s, H-3), 6.47 (1H,

brs, H-6), 6.31 (1H, brs, H-8), 3.70 (2H, brs, -OCH_2), 0.86-1.56 (16H, m, 8×-CH_2) and 0.83 (3H,m, -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 182.4 (C-4), 165.2 (C-7), 163.9 (C-3), 162 (C-5), 157.7 (C-9), 131.8 (C-1′), 131.3 (C-4′), 129 (C-3′, C-5′), 126.3 (C-2′, C-6′), 105.7 (C-3), 105.5 (C-10), 98.6 (C-6), 93.0 (C-8), 68.7 (-OCH_2), 22.6 – 31.9 (8 × -CH_2) and 14.06 (-CH_3).

2c: Yield 97 %, yellow solid, soluble in $CHCl_3$, molecular weight 478.30 corresponding to molecular formula $C_{31}H_{42}O_4$, mp. 80^{0}C. IR (ν cm^{-1}): 3409 (hydroxyl), 1666 (α, β - unsaturated carbonyl), 1615, 1586, 825 (aromatic), 2940, 2918, 2850 (C-H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.70 (5 - OH), 7.90 (2H, C-2′, C-6′), 7.55 (3H, m, C-3′, C-4′, C-5′), 6.67 (1H, s, H-3), 6.50 (1H, brs, H-6), 6.36 (1H, brs, H-8), 4.05 (2H, brs, -OCH_2), 0.88 - 1.82 (28H, m, 14 × -CH_2) and 0.86 (3H, brs, -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 182.5 (C-4), 165.2 (C-7), 163.9 (C-3), 162 (C-5), 157.7 (C-9), 131.8 (C-1′), 131.3 (C-4′), 129 (C-3′, C-5′), 126.3 (C-2′, C-6′), 105.7 (C-3, C-10), 98.6 (C-6), 93.0 (C-8), 68.7 (-OCH_2), 22.6 – 31.9 (14 × -CH_2) and 14.06 (-CH_3).

2d: Yield 96 %, yellow solid, soluble in $CHCl_3$, molecular weight 506.33 corresponding to molecular formula $C_{33}H_{46}O_4$, mp. 97^{0}C. IR (ν cm^{-1}): 3453 (hydroxyl), 1662 (α, β - unsaturated carbonyl), 1615, 1586, 820 (aromatic), 2919, 2856 (C-H); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.70 (5-OH), 7.90 (2H, C-2′, C-6′), 7.54 (3H, m, C-3′, C-4′, C-5′), 6.67 (1H, s, H-3), 6.50 (1H, d, J= 2.8 *Hz*, H-6), 6.36 (1H, d, J = 2.8 *Hz*, H-8), 4.05 (2H, t, J = 8.8 *Hz*, -OCH_2), 1.26-1.82 (32H, m, 16 × -CH_2) and 0.85 (3H, t, J = 8.8 *Hz* -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 182.4 (C-4), 165.2 (C-7), 163.9 (C-3), 162 (C-5), 157.7 (C-9), 131.8 (C-1′), 131.3 (C-4′), 129 (C-3′, C-5′), 126.3 (C-2′, C-6′), 105.7 (C-3), 105.5 (C-10), 98.6 (C-6), 93.0 (C-8), 68.7 (-OCH_2), 22.6 – 31.9 (16 × -CH_2) and 14.06 (-CH_3).

2.4. Preparation of NLC.

NLCs were prepared using hot homogenization followed by ultrasonication method. The detailed of the preparative procedure is available in our recent publications.[9-11] In the NLC formulation, CP and TP was used in equimolar ratio and the mole% of OA was varied from 10 to 30 mole% with an increment of 10 mole%. The overall concentration of NLC formulations were fixed at 1mM. 2 mM aqueous Tween 60 solution was used as the dispersion medium for the studied NLC formulation. 20 mole% OA comprising formulations were subjected for the

incorporation of chrysin and the synthesized LCDs. The concentration of chrysin and LCD were fixed at 10 µM in the present set of study.

2.5. Analytical Instruments:

Surface pressure-area isotherms were recorded on a Langmuir surface balance (Micro Trough X, Kibron, Finland), with an initial open trough having area of 137 cm^2 (232 x 59 mm). Monolayer were generated by spreading quantitative volume of 1 mM solution of lipid mixture in chloroform-methanol mixed solvent system (3:1) over the surface with a Hamilton (USA) micro syringe. The solvent was allowed to evaporate for 20 min after which the monomolecular film was compressed at a barrier speed of 5mm min^{-1}. Surface pressure area isotherms were constructed with a 2 mM aqueous Tween 60 solution as subphase. A dynamic light scattering spectrometer, DLS (Nano ZS 90, Malvern, UK) was used for the determination of the hydrodynamic diameter, polydispersity index and the zeta potential of the NLC formulations. Morphological studies were made using transmission electron microscopy (Hitachi H-600, Japan) and the freeze fractured transmission electron microscopy (H-7650, Hitachi Science Systems Ltd., Japan). Detailed investigations of the morphology and to get a three dimensional overview, samples were also analyzed by atomic force microscopy (Bruker Nanoscope V Multimode SPM). All the AFM images were recorded in noncontact mode and the images were analyzed further using nanoscope software package. Thermal properties of the NLC formulations were also evaluated using differential scanning calorimeter, DSC (Mettler Toledo, Switzerland). The scan rate was fixed at 2ºC min^{-1}. Aluminum pan having maximum capacity of 40 µL was used for the study. Identical pan containing the dispersion medium (2 mM aqueous Tween 60) was used as the reference. All obtained thermograms were analyzed using DSC1 $Star^e$ software.

2.6. Entrapment Efficiency and Drug Loading Capacity Studies.

Method of centrifugation was adopted for the evaluation of the entrapment efficiency and corresponding loading capacity. Chrysin and the LCDs loaded NLC formulations were subjected for centrifugation for 25 min at a speed of 20,000 rpm at 4ºC to separate the lipid phase

containing drug from the dispersion medium. The supernatant was collected and analyzed colorimetrically for the estimation of free drug. The entrapment efficiency and the loading capacity was calculated using the following equations:[9-11]

$$EE\% = \frac{W_{total\ \ CHR} - W_{free\ \ CHR}}{W_{total\ \ CHR}} \times 100\ \% \quad (1)$$

$$DL\% = \frac{W_{total\ \ CHR} - W_{free\ \ CHR}}{W_{total\ \ CHR} - W_{free\ \ CHR} + W_{total\ \ lipid}} \times 100\ \% \quad (2)$$

here, $W_{total\ CHR}$, $W_{freeCHR}$ and $W_{total\ lipid}$ represent the total amount of drug, free drug present in the dispersion medium and total amount of lipid in the NLC formulation respectively.

2.7. *In vitro* Release Kinetics Studies.

Standard dialysis bag (12kD, Sigma-Aldrich Chemicals, USA) method was adopted for the evaluation of release kinetics of incorporated drug.[9-11, 21] 2 mM aqueous Tween 60 solution was used as the release medium. 10 mL of the NLC formulation was suspended in 20 mL release medium. Sink condition was maintained throughout the experiment. The experiment was performed under constant starring at room temperature. The released chrysin and the LCDs were quantified colorimetrically.

2.8. *In vitro* Cell Viability Test.

Cytotoxicity of chrysin and its derivatives loaded in NLC formulation on human Neuroblastoma (SHSY5Y) cancer cell line, obtained from National Center for Cell Science, Pune, India, were determined by MTT assay.[21] The cells (5 x 10^3/ well) were plated in 200µL Ham's F 12 medium per well in 96 well plate, incubated at 5% carbon dioxide incubator for 24hr. After incubation, 10 µL of the studied NLC formulations were added. The experiments were performed with three different drug concentrations. All the measurements were performed in triplicate. Blank NLC formulation was used as a control. After treatment, media was replaced with MTT solution (10 µL of 5 mg /mL/well) prepared in PBS and incubated further for 3 hr at 37^{o}C in a humidified incubator with 5% CO_2. Then 50 µL of isopropanol was added to the each well and plates were gently shaken for 1 min and absorbance was taken at 595 nm by micro titerplate. The effect of chrysin and the LCD of chrysin loaded NLC formulations on the studied cell lines were expressed by calculating the % cytotoxicity using the following equation:[21]

$$\%\ cytotoxicity = \frac{Abs\ for\ the\ control\ \ cell - Abs\ for\ the\ treated\ \ cell}{Abs\ for\ the\ control\ \ cell} \times 100\% \qquad (3)$$

The minimum concentration of the drug required for the 50% inhibition of the treated cell is called inhibitory concentration (IC_{50}) value of the drug. IC_{50} of chrysin and the LCDs of chrysin in the NLC formulation were also determined separately.

3. Results and discussion

3.1. Langmuir Monolayer Studies.

Mutual miscibility and interaction of mixed lipidic systems with chrysin/LCDs were assessed by Langmuir surface pressure-area measurements. Representative surface pressure-area isotherms of LCDs of chrysin in water and 2 mM Tween 60 solution have been presented in the panel A of Fig. 2 .The lift off area for chrysin, CHR8, CHR10, CHR16 and CHR18 were recorded at 0.64, 0.66, 0.61, 0.66 and 0.63 $nm^2 molecule^{-1}$ respectively in water. In case of Tween 60 as subphase the observed lift off area for chrysin, CHR8, CHR10, CHR16 and CHR18 appeared at 0.68, 0..73, 0.71, 0.75 and 0.74 $nm^2 molecule^{-1}$ respectively. The surface pressure area isotherm for CP, TP and OA using water and Tween 60 as subphase have been presented in the Fig. 1.

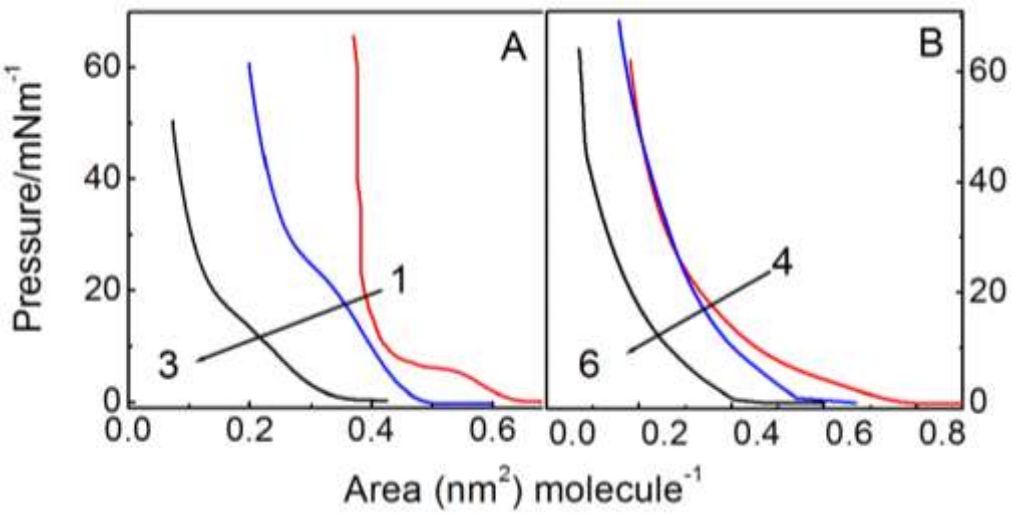


Figure 1. Surface pressure (π)- area (A) isotherms of pure lipids using water (panel A) and 2mM aqueous Tween 60 solution (panel B) as the subphase at 25ºC. Systems: 1 & 4, TS; 2 & 5, OA; 3 & 6, CP.

The lift off area for CP, TP and OA were observed at 0.4, 0.63 and 0.5 nm^2 respectively in water. The observation was found in good agreement with the literature.[22, 23] In case of Tween 60 solution as subphase, the observed lift off area for CP, TP and OA were 0.42, 0.76 and 0.6 $nm^2 molecule^{-1}$ respectively. The surface active nature of Tween 60 was mainly responsible for the observed increment in the recorded limiting area in all the cases.[9]

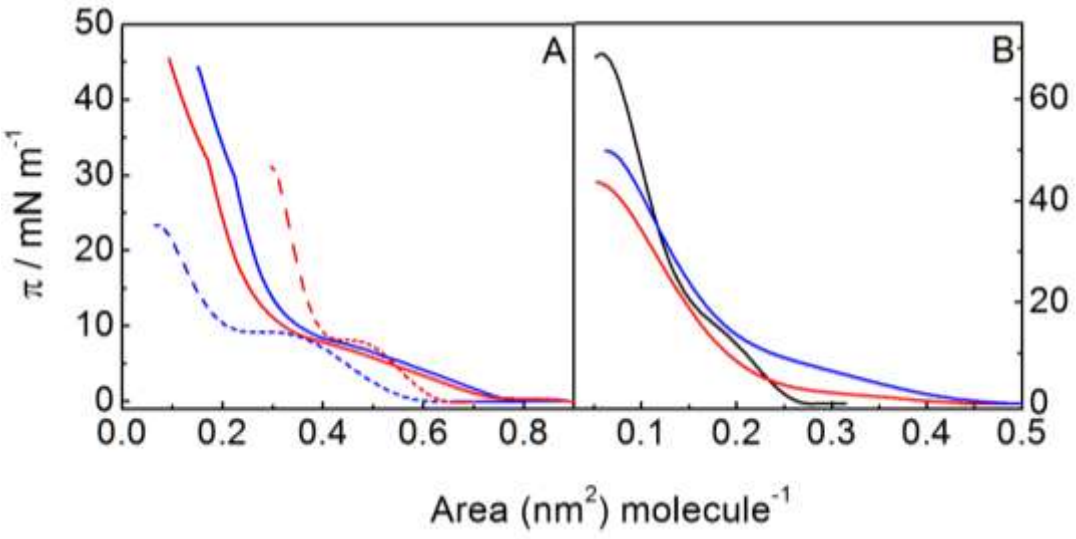


Figure 2. Surface pressure (π) – area (A) isotherm of CHR 10 (blue) and CHR 16 (red) using water (dotted line) and 2 mM aqueous Tween 60 solution (solid line) as subphase (panel A). Panel B represented the π – A isotherms of mixed lipidic system (CP+TP+OA, 2:2:1, M/M/M) in absence (black) and presence of 50 mole% CHR 10 (blue) & CHR 16 (red) using 2mM aqueous Tween 60 as subphase. Temperature 25 ºC.

The surface pressure-area isotherms of the mixed lipidic system (CP+TP+OA) were studied by varying the mole% of OA. Equimolar mixture of CP and TP were taken as the component 1 and OA was taken as the component 2. 2 mM aqueous Tween 60 solution was taken as subphase. For the equimolar mixture of CP+TP, the lift off area was found at 0.6

$nm^2 molecule^{-1}$. With the progressive addition of oleic acid there occurred a condensation, although in a nonsystematic way,[9] suggesting associative interaction between the hydrophobic part of the lipids.

Elasticity moduli (C_s^{-1}) of the studied monolayers were calculated using the following equation:[9, 24-26]

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)_T \quad (4)$$

Some representative C_s^{-1} vs. % compressed area (% A) profile has been presented in the Fig. 3.

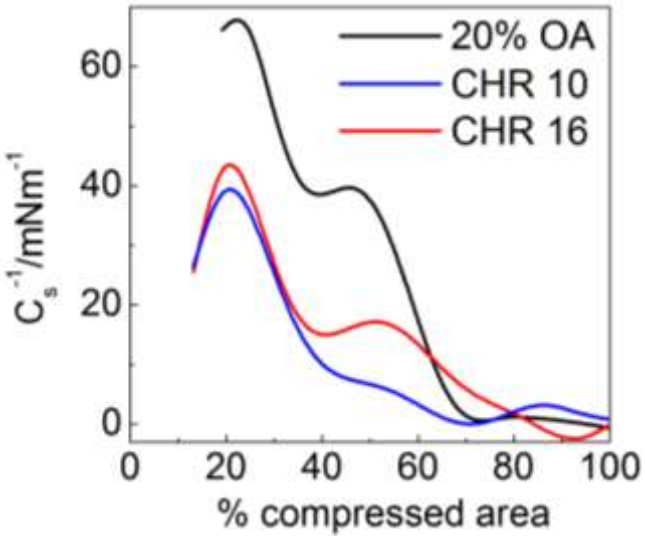


Figure 3. Variation of elasticity moduli (C_s^{-1}) with % compressed area upon addition of 50 mole% different LCDs of chrysin on the mixed lipidic system (CP+TP+OA, 2:2:1 M/M/M). 2mM aqueous Tween 60 was used as subphase. Different systems have been mentioned inside the figure.

In all the cases, C_s^{-1} was found to pass through maxima (in the range of 41 to 67 mNm^{-1}) upon compression. The value of C_s^{-1} for the mixtures indicated the presence of liquid expanded state of the monolayer.[9, 24] In case of the 20 mole% OA comprising system was found to show the highest value of C_s^{-1}.[9, 24, 25]

In order to evaluate extent of association and miscibility among the components the excess area (A_{ex}) and changes in the excess free energy of mixing (ΔG_{ex}^{0}) values were evaluated. The mean molecular area (A_{id}) can be calculated as:[9, 24-26]

$$A_{id} = x_1 A_1 + x_2 A_2 \quad (5)$$

where, x and A represent the mole fraction and area of pure component. The A_{ex} value was calculated as:[9, 24-26]

$$A_{ex} = A_{12} - A_{id} \quad (6)$$

where, A_{12} signifies experimentally obtained mean molecular area. Excess free energy of mixing were calculated using the following expression:[9, 24-26]

$$\Delta G_{ex}^{0} = \int_0^{\pi} [A - (x_1 A_1 + x_2 A_2)] d\pi \quad (7)$$

The obtained A_{ex} and ΔG_{ex}^{0} values, as shown in Fig. 4 were found to be dependent on the mole% of OA.

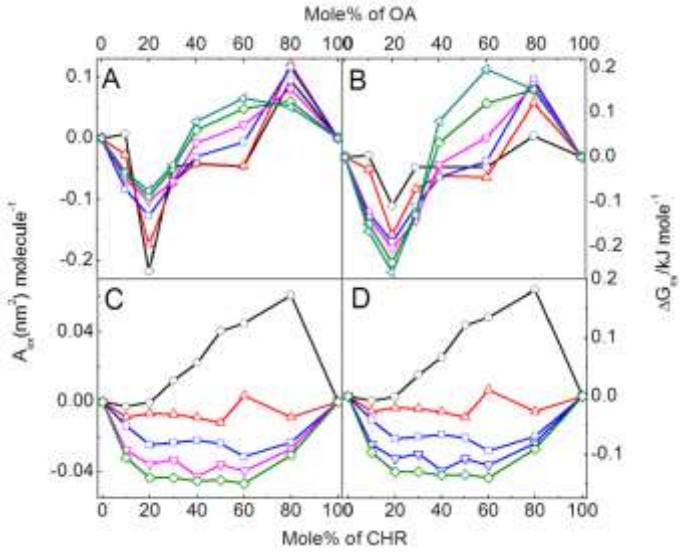


Figure 4. Variation of excess molecular area (A_{ex}) and changes in excess free energy (ΔG_{ex}^{0}) of the mixed lipidic system with the mole% OA (panel A, B) and chrysin, LCDs of chrysin (panel C, D). For panel A, B: Component 1: CP+TP (1:1, M/M), Component 2: OA. For panel C, D: Component 1: CP+TP+OA (2:2:1, M/M/M), Component 2: chrysin and LCDs of chrysin. Surface pressure for panel A & B (mNm^{-1}): O, 5; Δ, 10; □, 15; ▽, 20;◊, 25 and ◁, 30. Systems for panel C & D: O, CHR; Δ, CHR 8; □, CHR 10; ▽, CHR 16 and◊, CHR 18. Temperature: 25 °C.

Addition of OA caused association among the lipidic components of the mixed lipidic system. At 20 mole% OA comprising system, observed minima in the A_{ex} and ΔG_{ex}^{0} vs. mole% of OA profile, indicated the maximum association and the stability of that lipid composition respectively.[9] Further increase in the mole% of OA reduced the extent of association and ultimately showed repulsive type interaction at 80 mole%. The result indicated the maximum miscibility of the lipidic components and stability with 20 mole% OA. Hence this composition was regarded as the optimum lipid composition for the mixed lipidic system in order to formulate NLCs.

To investigate the nature of interaction between chrysin and synthesized four different LCDs of chrysin viz., CHR8, CHR10, CHR16 and CHR18 with the lipid system (CP+TP+OA, 2:2:1, M/M/M), surface pressure area isotherms were constructed by considering the lipid system as the component 1 and chrysin and the LCDs of chrysin as the component 2 separately using aqueous Tween 60 solution (2mM) as subphase. Surface pressure area isotherms of the mixed lipidic systems in presence LCDs of chrysin were graphically presented in the panel B of Fig. 2 as representative. In the surface pressure area isotherm no significant change in the limiting area was observed in the presence of chrysin and different LCDs of chrysin. Hence, compression modulii of the monolayer in the presence of chrysin and LCDs of chrysin were calculated and plotted against the % compressed area (Fig. 3) to get information regarding compressible nature of the monolayer studied. The magnitude of C_s^{-1} was found to rise with the surface compression and obtained a maximum value. The maximum vale for C_s^{-1} was found to lie in the range of 23 to 65 mNm^{-1}. Observed value was an indicative of liquid expanded state.[9, 24] The obtained maximum value of compression modulii was found comparatively higher in case of the monolayer in the presence of the LCDs than pure chrysin. The higher value of C_s^{-1} indicated the association and the formation of the condensed monolayer.[24] The hydrocarbon chain present in the LCDs provided higher hydrophobic attraction and assisted the formation of condensed monolayer.

To have a quantitative idea regarding the nature interaction and the extent of miscibility of chrysin and LCDs of chrysin with the lipid system, A_{ex} and ΔG_{ex}^{0} values were calculated and plotted against the mole% of chrysin and the LCDs of chrysin and presented in the panel C and D of Fig. 4.The A_{ex} and ΔG_{ex}^{0} data at 30 mNm^{-1} pressure has been presented as representative. In

case of pure chrysin repulsive type interaction was observed with the studied lipid system.Obtained positive value for A_{ex} and ΔG_{ex} indicated the repulsive type interaction. Presence of the hydroxyl group at the 5 and 7 position of chrysin make the molecule amphiphilic in nature and restrict the interaction with the hydrophobic part of the lipidic system. Hence, it showed repulsive type interactions. In contrast associative type of interaction has been observed for the monolayer in the presence of LCDs of chrysin.The obtained negative value of A_{ex} and ΔG_{ex}^{0} indicated the association. The extent of association was also found to increase progressively with increasing the hydrocarbon chain length in the LCDs of chrysin. The maximum association was observed for the system containing CHR 18 having the longest hydrocarbon chain (C18) among the synthesized LCDs. The enhanced lipophilicity in the presence of long hydrocarbon chain enhanced the hydrophobic interaction in the system and assisted molecular association.[9] The observed result give a clear indication of the enhanced miscibility of the LCDs of chrysin than pure chrysin itself with the lipid system. The repulsive interaction of chrysin lead chrysin molecule to reside in the palisade layer of the lipid system. In contrast, due to the higher missibility and lipophilic nature, LCDs of chrysin got inserted into the lipid system.

3.2. Dynamic light scattering studies

The hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) are the important characteristic of NLC formulations, govern the physicochemical stability and biological performance.[5, 6, 9-11] In the present study mean size of the NLC formulation was found to lie in the range of 200 to 350 nm which is in good agreements with the pharmaceutical literature.[9-11] To get information regarding the stability of the NLC formulation, size of the studied formulations were recorded with respect to time. The size *vs.* time profile of some representative NLC formulations has been presented in the Fig. 5. The formulations were found to stable up to 90 days and during storage no significant change in the d_h value for the NLC formulations were observed. The stability of the NLC formulations were also indicated from the monomodal size distribution of the studied formulations (data not shown).

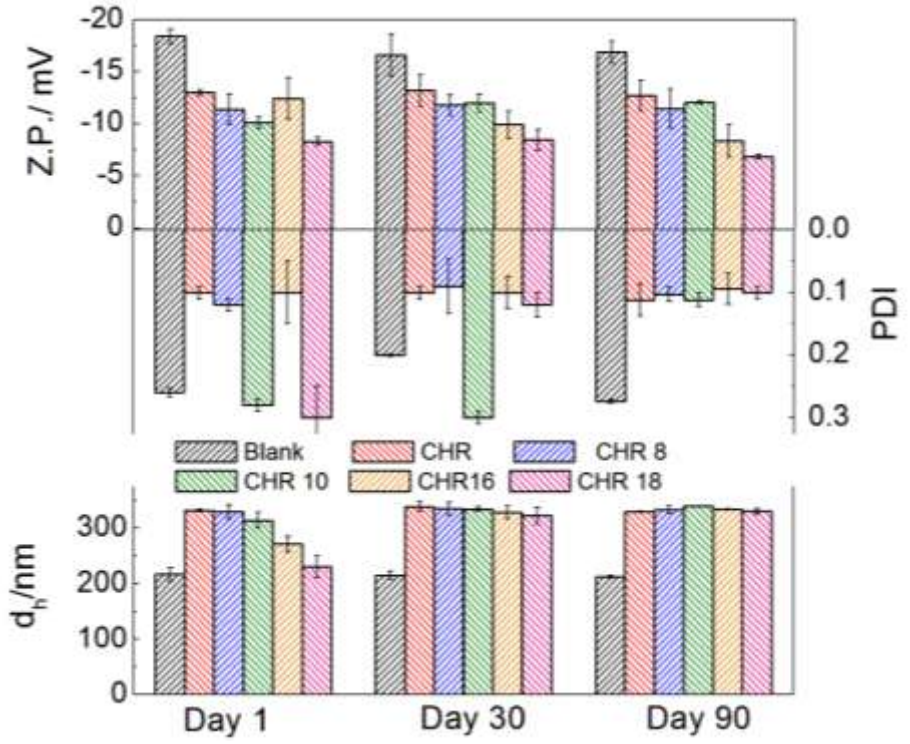


Figure 5. Variation of hydrodynamic diameter, PDI and zeta potential with time for 1 mM NLC formulation (CP+TP+OA, 2:2:1, M/M/M) stabilized by 2 mM aqueous Tween 60 solution in the absence and presence of chrysin and LCDs of chrysin at 25 ºC. Individual systems are mentioned inside the figure. [Chrysin] & [LCDs of chrysin]: 10 µM.

In the present study, equimolar mixture of CP and TP were considered as the core lipidic material for the NLC formulations and OA was used as FA. The mole% of OA was varied from 10 mole% to 30 mole% to obtained optimum NLC composition having balanced rigidity and fluidity of lipid matrix. The d_h value of the NLC formulations were also found dependent upon the mole% of OA. With increasing mole% of OA from 10 to 20 mole % a reduction in the d_h value was observed. But further increase in mole% of OA (30 mole%), increase in d_h value was observed. The observed reduction in d_h value indicated the saturation limit of OA in the NLC matrix. The compact molecular arrangement at this composition was the main reason for the observed size constriction.[9] Detected maximum association at this lipid composition for the mixed lipidic system in the monolayer experiment was also support the observation.

Being the optimum NLC formulation, 20 mole% OA comprising NLC formulations were subjected for the incorporation of chrysin and synthesized LCDs of chrysin *viz.*, CHR8. CHR10, CHR16 and CHR18. The size of the loaded NLC formulation was found in the range of 300 to 350 nm. The size of the drug loaded formulations was found higher than the base NLC formulation. This observation was the preliminary indication for the incorporation of drug. The stability of the chrysin and LCDs of chrysin loaded NLC formulations were also studied by recording the size with respect to time and the size *vs*, time profile of the formulations were presented in the Fig. 5. The formulations were also found to stable for 90 days of storage. The size of the chrysin and the LCDs of chrysin loaded formulations were compared and it was observed that the size of NLC formulations loaded with pure chrysin was higher than the LCD loaded NLC formulation. In addition the size of the LCD loaded NLC formulation were found to decrease progressively with increasing the hydrocarbon chain length present in the derivative. NLC formulation loaded with CHR 18 was found to show least d_h. The observation indicated the inside penetration of the LCDs of chrysin into the NLC matrix due to the enhanced lipophilicity. [10, 11] In addition, the assisted association among the lipidic component in the presence of LCDs of chrysin was also responsible for the reduction in the d_h value. The enhanced van der Wall attraction in the presence of the long hydrocarbon chain of chrysin derivatives was mainly responsible for the observed phenomenon.[9] The size constriction has also been reflected in the associative type interaction of the LCDs of chryin with the mixed lipidic systems in the monolayer studies. The observation becomes insignificant with the storage time indicated the lipidic modification in NLC with time.[9]

Presence of the two hydroxyl group in the 5 and 7 position of chrysin make the molecule amphilic in nature and restrict the inside penetration and formed a shell enriched NLC system. The higher size of the pure chrysin loaded formulation was an indicative of the shell enriched model. The formation of the shell enriched structure was also justified from the repulsive type interaction of pure chrysin and the mixed lipid system in the monolayer studies.

PDI is a quantitative approach in estimating the homogeneity in the NLC dispersion. The PDI value of the studied NLC formulations was found to lie in the range of 0.2 to 0.3. The observed value indicated the formation of homogeneous NLC formulations.[1, 5-7] The incorporation of chrysin and the LCDs of chrysin did not influence the PDI value of the formulations. The result

signifies that, incorporation of drug did not alter the surface homogeneity of the NLC formulations. The stability of the base as well as the drug loaded NLC formulations were also monitored by recording the PDI value with respect to the storage time (Fig. 5). In all the cases no significant increase of PDI was observed. The observed fluctuation of the PDI value during storage indicated the internal lipidic modification.[11]

Zeta potential is an important parameter in evaluating the stability of the NLC formulation. The zeta potential is mainly depend on the potential on the surface of NLC.[11] In all the cases negative value of zeta potential was observed. Z.P. of the studied formulations were found to lie in the range of -12 to -18 mV. The observed value of zeta potential indicated the steric stabilization assisted by the stabilizer (2 mM aqueous Tween 60) used in the formulation.[9-11] The Z.P. vs. time profile of the NLC formulations have been presented in the Fig. 5 as representative. The Z.P. of the formulations was found to decrease with time. The reduction in the magnitude indicated the lipidic modification inside the NLC matrix. In case of 20 mole% OA comprising NLC formulation no significant change in the magnitude of Z.P. indicated the lesser lipidic modification for this composition. The higher association among the lipidic component and the formation of compact molecular arrangement was mainly responsible for the observation. With the incorporation of chrysin and LCDs of chrysin a reduction in negative magnitude of Z.P. was observed. In case of the pure chrysin loaded formulation, surface accumulation of the incorporated chrysin was found to mask the surface charge of NLC formulation and reduced the magnitude of Z.P. In case of the LCDs of chrysin, further reduction in negative magnitude of Z.P. was observed. In case of the LCDs of chrysin loaded formulations, the increased lipophilicity of the LCDs in the presence of long hydrocarbon chain suppress the dissociation of OA, which was the major source of the surface potential. CHR 18 having the longest hydrocarbon chain among the synthesized derivatives was found to reduce the Z.P. value of the NLC formulation to a considerable extent. The zeta potential of the chrysin and the LCDs of chrysin loaded formulations were also recorded with respect to time and have been presented in Fig. 5. The profile was found almost similar to the base NLC formulations. Here also reduction of Z.P. with time indicated the internal structural modification with storage time.[11]

3.3. Morphology of the NLC formulations:

The morphology of the studied NLC formulations was investigated using transmission electron microscopy. Further investigations were carried out using the freeze fractured transmission electron microscopic technique. Irrespective of the used methods of investigation spherical morphology of the NLC systems with smooth surface was observed. The representative TEM and FF-TEM images for base and CHR 16 loaded formulations have been presented in the panel A, B and C, D of Fig. 6 respectively.

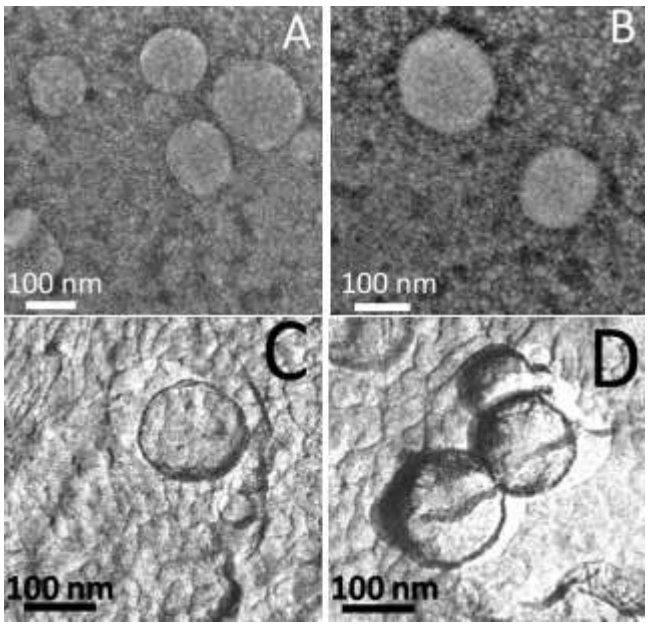


Figure 6. TEM (panel A, B) amd FF-TEM (panel C, D) images for NLC (CP+TP+OA, 2:2:1, M/M/M) formulations in the absence (panel A, C) and presence (panel B, D) of chrysin. The scale bars are mentioned inside the figure.

Observed size of the NLC formulations were in good agreement with the previously performed DLS data. No significant change in the surface morphology with the incorporation of chrysin and the LCDs were observed for the studied formulations.

Further investigation on the surface morphology and to get the topological information regarding the studied formulations AFM studies were also performed. AFM image of the base NLC formulation have been presented in the Fig. 7 as representative.

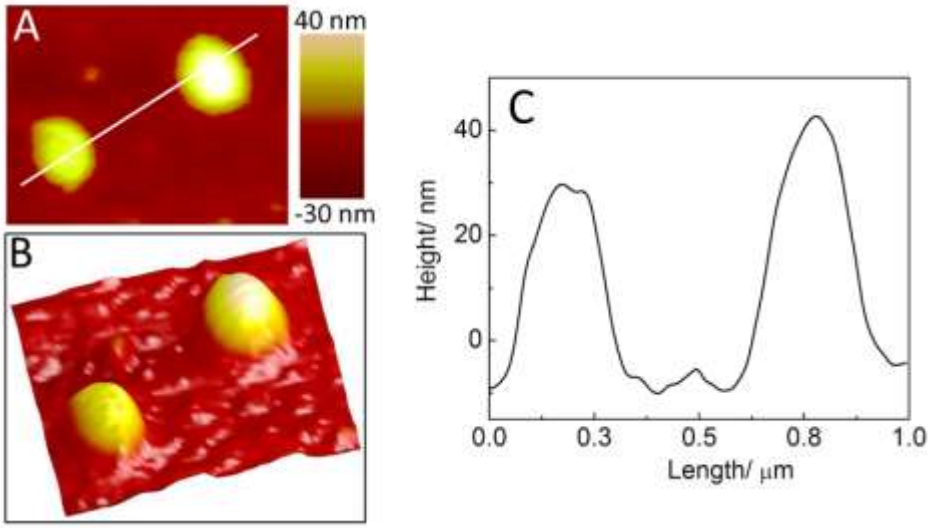


Figure 7. AFM image of NLC (CP+TP+OA, 2:2:1, M/M/M) formulation where panel Aabd B represented the two and three dimensionalview respectively. Panel C represented the roughness profile for the NLC formulation. Height scale is given inside the figure. Scane area: (1X1) μm.

Panel A and B of the figure represented the two dimensional and the three dimensional over view of the NLC. The observed bright spots in the figure represented the NLC. The size of the NLCs was also found in good agreement with the DLS, TEM and FF-TEM data. The height of the NLCs was found to be in the range of 40 to 45 nm. The observed value was found to be in good agreement with the reported data for this type of systems in the literature.[27-29] The roughness of the surface in the studied formulations was also evaluated and presented graphically in the panel C of Fig. 7. The observed smooth humps in the graph indicated the existence of the smooth surface for the investigated system. No significant change in the surface morphology and topology was observed with the incorporation of chrysin and LCDs of chrysin in AFM studies.

3.4. Differential scanning calorimetric studies:

To evaluate the extent of the polymorphic nature and to know the extent of crystallinity of the NLC formulation, DSC studies were performed. The obtained results have been further correlated with the encapsulation parameter, release profile and the physicochemical stability of the studied formulations.[9-11, 21, 23, 24] A representative heating cooling DSC thermogram of the NLC formulation have been presented in the Fig. 8. DSC of individual lipids (CP, TP and OA), chrysin and the synthesized LCDs of chrysin e.g., CHR8, CHR10, CHR16 and CHR18 have been recorded separately. The phase transition temperature of the pure lipidic components was in good agreement with the previous reports on the literature.[30-32] The phase transition temperature for chrysin was obtained at 286.07 and 282.8 ºC in the heating and cooling thermograms respectively. The phase transition temperature for LCDs of chrysin *e.g.,* CHR8, CHR10, CHR16 and CHR18 were found to be 100.8, 91.80, 88.50 and 93.83 ºC respectively in the heating thermogram. The phase transition temperature in the cooling thermograms was observed 2-3 ºC lower than the heating thermogram. The lowering in the phase transition temperature for the LCDs of chrysin indicated the development of the liquid crystalline behavior in them.[33-35] In the present study the DSC thermograms of the NLC formulation along with the physical mixture were compared (Fig. 8).

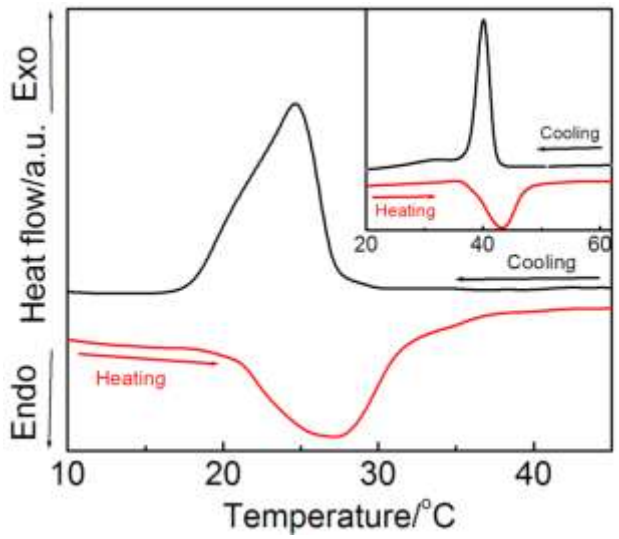


Figure 8. DSC thermogram of a 5 mM NLC formulation (CP+TP+OA, 2:2:1, M/M/M, stabilized with 2 mM aqueous Tween 60 solution). Inset represented the DSC thermogram of the corresponding physical mixture.

A reduction in the T_m value for the NLC formulation was observed in comparison to the physical mixture. Reduction in T_m value was mainly due to the enhancement in multicrystallinity and reduction in size with simultaneous increase in the surface area, according to the Thomson proposition.[9-11] In case of all the NLC formulation the cooling thermograms were obtained 2-3 ºC lower temperature than the heating thermogram. The observation indicated the liquid crystalline nature of the studied formulations. Similar to the liquid crystalline system, obtained cooling thermograms were found to be more prominent in comparison to the heating thermograms.[33-35] For this reason the cooling thermograms were used further for the calculation of the thermodynamic parameters.[9]

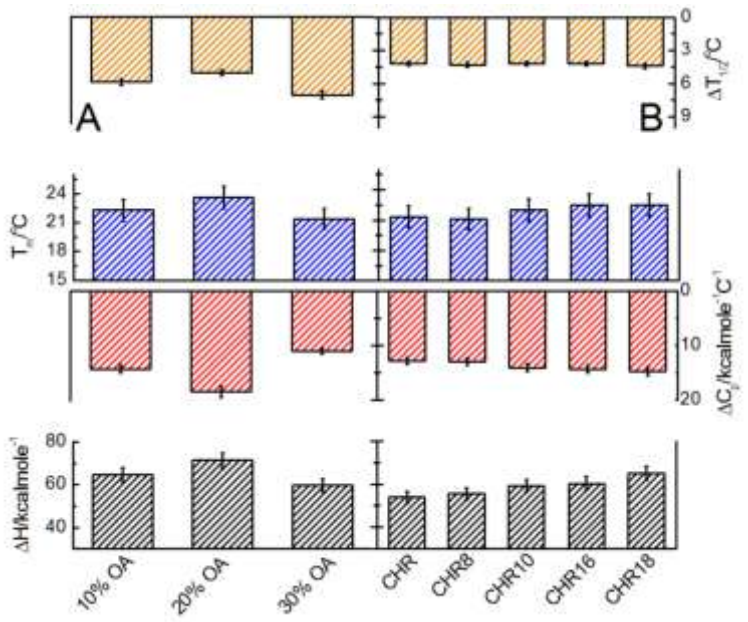


Figure 9. Change in the thermodynamic parameters for NLC formulations in the presence of different mole% of OA (panelA). Panel B represents the change in the thermodynamic parameters for the NLC (CP+TP+OA, 2:2:1, M/M/M) formulation with the incorporation of chrysin and LCDs of chrysin. The systems are mentioned in the figure.

Panel A of Fig. 9 represented the thermodynamic parameters of the NLC formulation having three different mole % of OA (10, 20 and 30). The T_m value associated with the cooling thermogram of the NLC formulations were observed at 22.3, 23.62 and 21.4 °C respectively for 10, 20 and 30 mole% OA comprising NLC formulations respectively. The T_m value for the system having 20 mole% OA was found higher than the other studied compositions. The formation of the compact molecular arrangement enhanced the T_m. With further increment in mole% of OA a reduction in T_m value was observed. The enhanced mole% of OA contain (beyond 20 mole%) mainly enhanced the disturbance in the crystalline arrangement of the NLC matrix by making increasing number of liquid lipid compartments. The result was found further correlated with the calculated thermodynamic parameters *e.g.*,ΔH, ΔC_p and $\Delta T_{1/2.}$. The maximum value of ΔH and ΔC_p was found in case of 20 mole% OA comprising NLC system. At 30 mole% OA comprising system, reduction in the ΔH and $\Delta C_{p,}$ indicated the increased multicrystalline nature of NLC. $\Delta T_{1/2}$ of the DSC thermograms with increasing mole% of OA was also determined. On going from 10 mole% to 20 mole% OA, observed reduction in the $\Delta T_{1/2}$ value indicated the compact molecular arrangement of the NLC formulation having 20 mole% OA. This indicated the enhanced crystallinity of the said NLC formulation. Beyond 20 mole%,

flattening of the thermograms were observed. The result indicated the reduction in the crystalinity of the NLC formulation with increasing OA content beyond 20 mole%. The result indicated the 20 mole% OA comprising NLC formulation was the optimum lipid composition for NLC. The results were found further correlated with the Langmuir monolayer and dynamic light scattering studies.

To get quantitative idea regarding the crystallinity of the NLC formulations, crystallinity index (CI%) of the NLC formulations were also calculated using the following equation[10, 11]

$$CI\% = \frac{\Delta H_{NLC}}{\Delta H_{lipid\ \ mixture}} \times 100\% \quad (8)$$

Where ΔH_{NLC} and $\Delta H_{lipidmixture}$ represented the phase transition enthalpy of NLC formulation and the corresponding lipid physical mixture respectively. The CI% *vs.* OA mole % graph has been presented in the panel A of Fig. 10.

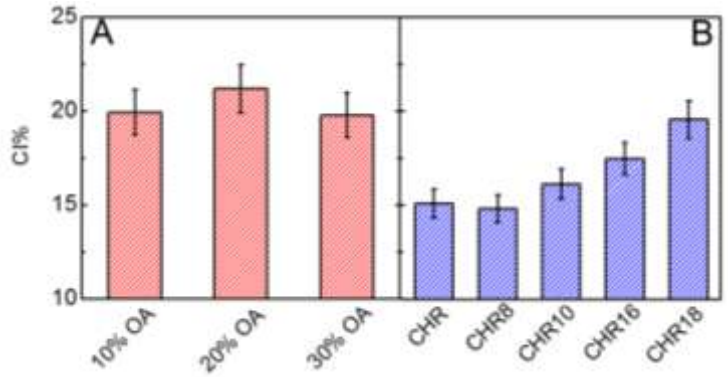


Figure 10. Crystallinity index (CI%) of the NLC formulations (CP+TP+OA, 2:2:1, M/M/M) in the absence (panel A) and the presence (panel B) of chrysin and LCDs of chrysin. The individual systems are mentioned in the figure.

The highest CI% was observed for the system comprised with 20 mole% OA. The result was found to be in good agreement with the calculated thermodynamic parameter for the NLC formulations comprising different mole% of OA.

No significant change in the nature of thermograms from the base NLC formulation were observed with the incorporation of chrysin and LCDs. DSC thermograms of the lipid physical mixture in the presence of chrysin and the LCDs of chrysin were also recorded and no separate peak for chrysin and the LCDs were observed. The result indicated the solubility of chrysin and

the LCDs of chrysin in the lipid system. Like the base NLC formulations, the cooling thermograms were more prominent than the heating thermogram. Hence cooling thermograms were used for the evaluations for the thermodynamic parameters.

It has been observed that with the incorporation of chrysin and the chrysin derivative, an overall reduction in the T_m was observed. The reduction in T_m indicated the enhancement in the multricryatallinity in the presence of drug. In case of pure chrysin loaded NLC the T_m was found at 21.32 °C. T_m value for CHR8, CHR10, CHR16 and CHR18 loaded NLC formulations were observed at 21.11, 22.07, 22.46 and 22.5 °C respectively. Progressive increase in the T_m value indicated the enhanced association among the lipidic components in the presence of LCDs of chrysin.The obtained maximum value for CHR18 loaded NLC formulation attributed to the maximum association. The similarity in the hydrocarbon chain between CHR18 and OA also influence the compact molecular arrangement.[9] Repulsive type interaction of pure chrysin with the lipid system made the system unorganized. The reduced phase transition enthalpy of chrysin loaded NLC formulations indicated the less associated nature of the system. The amphiphilic nature of chrysin was mainly responsible for this. The result indicated the surface accumulation of chrysin in NLC formulation. Progressive increment of the phase transition enthalpy and the heat capacity value with the increasing hydrocarbon chain length of the chrysin derivatives indicated the formation of compact molecular arrangement. The enhanced hydrophobic interaction and the van der Wall attraction with the increasing hydrocarbon chain length of the derivative assisted the formation of well-organized liquid crystalline system.[9-11] The results were also found with the good agreement with the observed T_m value. In case of chrysin and LCDs of chrysin loaded NLC formulation the CI% were also calculated and presented in the panel B of Fig. 10. An increase in the CI% was noted in case of the LCDs of chrysin loaded NLC formulations in comparison to the pure chrysin loaded NLC formulation. Progressive enhancement in CI% was also noted with increasing the length of hydrocarbon chain incorporated in LCD. The observation was not at all unexpected. The enhanced molecular association led to the systematic molecular organization in the lipid aggregated systems.

3.5. The drug incorporation efficiency (EE%) and the drug loading capacity (DL%) studies:

The drug incorporation efficiency and the drug loading capacity are the important parameter from the view point in the development of a successful drug delivery system.[5, 6, 9-12] In the present study, the incorporation efficiency and the loading capacity of the chrysin and the LCDs of chrysin viz., CHR8, CHR10, CHR16 and CHR18 loaded NLC formulations were determined and presented graphically in the panel A of Fig. 11.

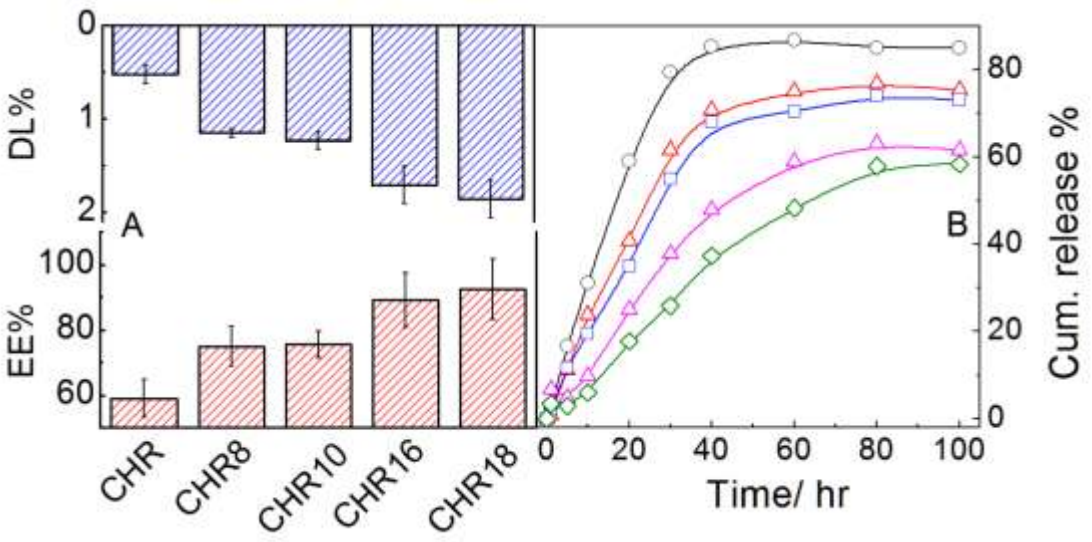


Figure 11. Entrapment efficiency (EE%), drug loading (DL%) capacity (panel A) and release profile (panel B) of chrysin and different LCDs of chrysin form NLC formulation at 25 ºC . systems (panel B): O, CHR; Δ, CHR 8; □, CHR 10; ▽, CHR 16 and◊, CHR 18.

The drug incorporation efficiency and the drug loading capacity for the pure chrysin in the NLC formulation were found to be 59 and 0.52% respectively. But incorporation efficiency of LCDs of chrysin was found higher than pure chrysin. The entrapment efficiency for CHR8, CHR10, CHR16 and CHR18 loaded NLC formulations were found to be 75, 75.6, 89.2 and 92.5%respectively. The corresponding drug loading values were 1.15, 1.23, 1.70 and 1.85% respectively. The enhanced lipophilicity of the chrysin derivatives leads to the higher

incorporation and the drug loading capacity. Progressive enhancement in drug incorporation and loading capacity was observed with increasing the length of the hydrocarbon chain on the chrysin derivative. The enhanced miscibility and the lipid solubility of the LCDs with increasing length of incorporated hydrocarbon chain were mainly responsible for the observed result. The higher lipid solubility of the drug, restrict the drug expulsion during the NLC hardening and recrystallization during NLC preparation.[11] Hence, provide better encapsulation parameters. The obtained result was found to be in good agreement with the monolayer, DLS and DSC studies performed previously on the studied formulations.

3.6. *In vitro* release studies

The release profile of chrysin and individual LCDs of chrysin (CHR8, CHR10, CHR16 and CHR18) from NLC formulations were studied using 2mM aqueous Tween 60 solution as the release medium. The release profile of chrysin and the LCDs of chrysin have been presented graphically in the panel B of Fig. 11. The simple diffusion of the chrysin and the LCDs of chryin through the dialysis membrane in Tween 60 solution were also presented in the Fig. 12.

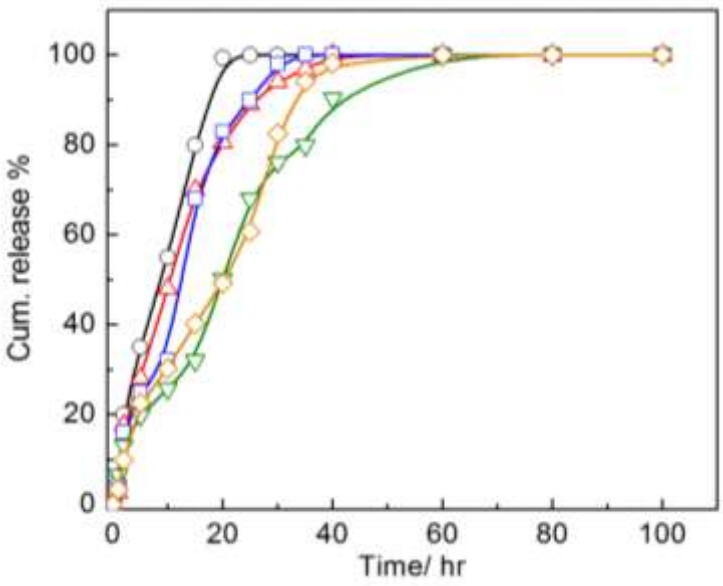


Figure 12. Simple difusin of chrysin and LCDs of chrysin in 2 mM aqueous Tween 60 solution through dialysis membrane (12KD) at 25 ºC. Systems, ○, CHR, Δ, CHR8; □, CHR10;▽, CHR16 and ◊, CHR 18.. 10 µM chrysin and the LCDs were used for the experiment.

The formulated NLCs were found to sustain the release in comparison to the simple diffusion. Chrysin and all synthesized LCDs of chrysin were found to follow almost similar drug release profile. In all the cases, an initial burst release (8 to 10 hr) followed by a sustained release was obtained. The release of chrysin and the LCDs of chrysin from the NLC formulations were monitored for 100 hr. On comparing the release of the chrysin and the LCDs of chrysin, faster release was obtained for chrysin and relatively sustained release was observed for synthesized LCDs. With increasing the chain length of chrysin derivative, progressive reduction in the release rate was noted. The obtained result can be explained on the basis of the mutual miscibility and the lipid solubility of chrysin and the LCDs of chrysin. The lesser miscibility and relative lesser lipid solubility of chrysin leads to the faster elution of it from NLC formulation. The amphiphilic nature of chrysin lead to surface accumulation and promoted faster release. But in case of LCDs, enhanced lipid solubility and grater mutual miscibility with the lipidic aggregates lead to controlled and sustained release profile. CHR 18 having the longest hydrocarbon chain, found to show the slowest release rate. Progressive increase in the hydrocarbon chain length enhanced the lipid miscibility and lead to the compact association among the components. This leads to sustain the release. The enhanced molecular association and the formation of the compact molecular arrangement due to the enhanced van der wall attraction and hydrophobic attraction leads to the sustain release profile for the LCDs of chrysin. The observed release rate of chrysin and the LCDs of chrysin was also found to correlated with the DLS, DSC and the Langmuir monolayer studies.

The obtained release profile of chrysin and LCDs of chrysin were further subjected for the kinetic studies to put light on the release mechanism. The obtained release profiles were fitted into four different release model viz., first order, zero order, Korsemeyer-Peppas, Higuchi and Weibull with the help of an add in software (DD Solver 1.0).[9-11, 36, 37] The obtained release kinetic data have been presented in the table 1. All the systems were found to follow the Korsemeyer-Peppas release formalism (on the basis of observed maximum R^2 value). The calculated release rate according to the Korsemeyer-Peppasformalism for chrysin was found to be 15.88 hr^{-1}. For LCDs of chrysin the release rates were found to be 10.28, 8.73, 4.52 and 2.07 hr^{-1} for CHR8, CHR10, CHR16 and CHR18 respectively. The value of release exponent (n) was also recorded and the value was found to lie in the range of 0.4 to 0.5. The value indicated classical fix release formalism for chrysin and the LCDs of chrysin loaded NLC systems.

Table 1. Release kinetics data of chrysin and LCDs of chrysin from NLC (CP+TP+OA, 2:2:1, M/M/M).

NLC formulations	First order		Zero order		Weibull		Korsemeyer-Peppas			Higuchi	
	R^2	k_H/ h^{-1}	R^2	k_H/ h^{-1}	R^2	k_H/ h^{-1}	R^2	n	k_H/ h^{-n}	R^2	$k_H/ h^{-0.5}$
NLC_{CHR}	0.9449	0.042	0.8206	1.210	0.9854	0.012	0.9859	0.4	15.888	0.9327	10.573
NLC_{CHR8}	0.9371	0.025	0.8736	1.040	0.9641	0.0007	0.9771	0.4	10.265	0.9585	8.925
NLC_{CHR10}	0.9656	0.021	0.8927	0.989	0.9621	0.006	0.9775	0.5	8.734	0.9661	8.424
NLC_{CHR16}	0.9730	0.013	0.9369	0.799	0.9607	0.004	0.9795	0.5	4.521	0.9757	6.662
NLC_{CHR18}	0.9817	0.010	0.9781	0.698	0.9886	0.003	0.9917	0.4	2.076	0.9761	5.676

Concentration of NLC (CP+TP+OA, 2:2:1): 1 mM; chrysin and LCDs of chrysin: 10 µM.

3.7. Anticancer activity of the chrysin and the LCDs of chrysin loaded NLC formulations:

To gather information regarding the anticancer efficacy of chrysin retained after getting incorporated into the NLC formulation and also to know about the effectiveness of the LCDs of chrysin loaded NLC formulations in showing anticancer activity, cytotoxicity of the studied formulations were determined by measuring the percentage of cell inhibition using MTT assay . Human neuroblastoma cell lines (SHSY5Y) were used for the evaluation of the anticancer efficacy of the studied formulations.[21] Percentage of cell inhibition was determined for the chrysin and the LCDs of chrysin loaded NLC formulations at three different concentrations (5, 7.5 and 10 M). This is just the preliminary study regarding the anticancer activity of the studied formulations. The percentage of cell inhibition (% cytotoxicity) obtained for different formulations at three studied concentrations were presented graphically in Fig. 13.

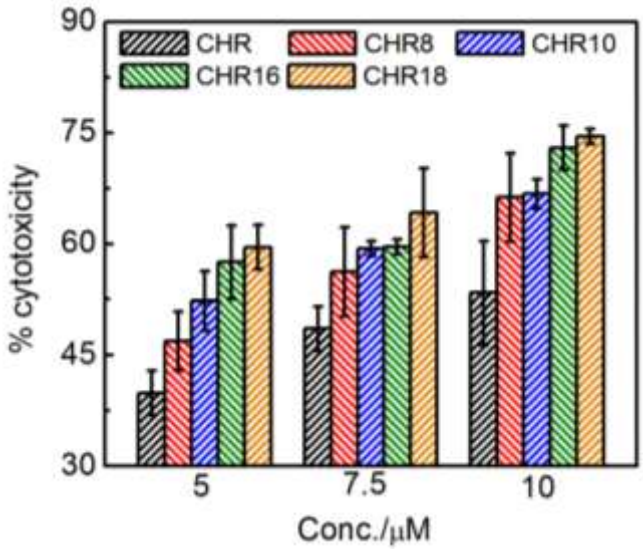


Figure 13. Percentage cytotoxicity at three different concentration of chrysin and LCDs of chrysin loaded in the NLC formulation (CP+TP+OA, 2:2:1, M/M/M) against human neuro blastoma cell lines (SHSY5Y). Blank NLC formulation is taken as control. The individual systems are mentioned inside the figure.

All the studied formulations were found to show superior citotoxic activity than chrysin and LCDs of chrysin alone against the studied cell line (data not shown). Among the studied formulations, cytotoxicity was found higher for LCDs of chrysin loaded NLC formulations.

Cytotoxicity of the studied formulations were found to be concentration dependent. With enhancing the concentration of chrysin and LCDs of chrysin in the NLC formulation, increment in cell inhibition percentage was detected. The calculated IC_{50} value for chrysin and the LCDs of chrysin *viz.*, CHR8, CHR10, CHR16 and CHR18 loaded in the NLC formulations were found to be 7.78, 6.51, 6.19, 5.83 and 5.58 µM respectively. Hence, presence of long hydrocarbon chain in chrysin enhanced the cytotoxic effect when loaded in the NLC formulation. Further studies with different cancer cell lines and *in vivo* evaluation of the anticancer efficacy of chrysin and LCDs of chrysin loaded NLC formulations are warranted for the detailed evaluation of them as anticancer drug delivery systems.

4. Conclusion

To sum up, Chrysin and LCDs of chrysin were successfully incorporated in NLC formulation comprising CP, TP and OA having the molar ratio of 2:2:1 using a hot homogenization followed by ultrasonication method. The monolayer studies were successfully employed to evaluate the interaction among the lipid components and to obtain the accurate lipid composition for the NLC. The nature of interaction between the lipid system, chrysin and LCDs of chrysin were also investigated by monolayer approach. The amphiphilic nature of pure chrysin lead to the repulsive type interaction with the lipid system where as the LCDs showed the associative type interaction and the extent of association was found to increase with increasing hydrocarbon chain length of chrysin derivatives. Base as well as chrysin and LCDs of chrysin loaded NLC formulations were characterized using DLS, DSC, TEM, FF-TEM and AFM studies, All the studies indicated the 20 mole% OA comprising NLC formulations was the optimum one and also confirmed the inside penetration of LCDs of chrysin and surface accumulation of pure chrysin in the studied NLC formulation. The incorporation efficacy of the LCDs of chrysin was found to be higher than the pure chrysin. CHR 18 showed the maximum drug incorporation and the drug loading capacity. *In vitro* release showed that LCDs of chrysin exhibited more sustained release than the pure chrysin from NLC. The higher lipophilicity and the grater association of the LCDs with NLC lead to sustain release profile. Progressive enhancement in hydrocarbon chain in chrysin derivative lead to progressive reduction in observed release rate. Chrysin and the LCDs of chrysin loaded formulations were also subjected for the anticancer activity against human neuroblastoma (SHSY5Y) cell lines. It was observed that LCDs loaded formulations were

equally capable of showing cytotoxicity like the chrysin loaded NLC formulations. Promising cytotoxicity against cancer cell line, higher drug loading capacity and the sustain release made the LCDs more promising than the pure chrysin in case of NLC based delivery systems. With respect to these observations, it can be concluded that LCDs of chrysin loaded NLC formulations are advantageous alternate of pure chrysin loaded NLC formulation for the development of promising anticancer drug delivery agent.

References

References are given in BIBLIOGRAPHY under references for CHAPTER 2 (pp. 152-154).