Chapter 4

From Micron to Nano-Curcumin by sophorolipid co-processing: Highly enhanced bioavailability, fluorescence, and anti-cancer efficacy

Co-sonication of curcumin and acidic sophorolipid in aqueous solution is shown to lead to a dramatic enhancement of curcumin bioavailability through size reduction and encapsulation. The cytotoxicity effects of curcumin on breast cancer cell lines, MCF-7 and MDA-MB-231, are shown to be significantly enhanced by the formation of its complex with sophorolipid. The relative cytotoxicity of curcumin with its SL(A) complex is more due to the presence of glucose moiety. The results further suggest that sophorolipid based formulations, which solubilize and nano-encapsulate curcumin after lipid digestion, show great potential for curcumin cell entry.
4.1 Introduction

Curcumin is known to have antioxidant, anti-inflammatory, chemo-preventive and chemo-therapeutic properties.\(^1\) The bioavailability\(^2\) of curcumin is determined by the rate and concentration at which it enters the plasma and reaches the target sites. The oral bioavailability\(^3\) of curcumin is low because a major portion of the compound remains unabsorbed due to a fairly low intestinal absorption capacity. Even a minor amount which is actually absorbed is rapidly metabolized in the liver and thrown out of the body by the gall bladder.\(^4\)-\(^6\) Several studies have verified that even a very high oral dose of curcumin (up to 1 g/kg\(^{-1}\) of the body weight) is almost completely eliminated by the human metabolic system.

Curcumin (diferuloylmethane), a bright orange yellow pigment, is the main active ingredient of turmeric; an ancient spice known for its medicinal uses.\(^7\) Curcumin exhibits tautomerism in its molecular structure and thus exists in the enol form in non-polar solvents, because of intra-molecular hydrogen bond formation. In polar solvents, however, it is observed in the diketo form.\(^8\) The keto form of Curcumin acts as a proton donor in acidic and neutral media, whereas at pH values above 8.0, the enol form dominates and acts as an electron donor.

The existence of the phenolic, b-diketone, as well as the methoxy groups of Curcumin contribute to its free-radical scavenging property. This property imparts the anti-cancer nature to this compound.\(^9\)-\(^10\) However, as mentioned previously, these results have not been reflected well in clinical studies mainly due to the low oral bioavailability of Curcumin. Therefore several soft materials systems including liposomes,\(^11\)-\(^13\) microspheres,\(^14\) dendrimres,\(^15\) micelles,\(^16\) hydrogels and solid lipid nanoscale particles\(^17\)-\(^20\) have been explored to design specific drug-delivery systems for Curcumin. These nano-assembly forming procedures designed to improve the bioavailability of curcumin are all inherently expensive and hence there is a strong urge to obtain cost-effective replacements for this system.

Biosurfactants\(^21\) derived from microorganisms are an interesting category of bio-organic systems with potential applications in biomedical science. They can be produced from renewable feedstock or waste material\(^22\)-\(^23\) by natural fermentation. These amphiphilic compounds are known to easily form self-assemblies at different
pH conditions in aqueous environment. Sophorolipids are an eco-friendly and biocompatible class of amphiphilic biosurfactants which easily form emulsions in aqueous solution to reduce the surface tension and interfacial energies.\textsuperscript{24} They possess unique structures that can be engineered to suit specific application domains.\textsuperscript{25} Sophorolipid exists in two forms: acidic and lactonic. Acidic sophorolipids (SL(A)), are known to form micelles, which interact depending on the pH of the system.\textsuperscript{26-27} SL(A), is composed of a sophorose unit attached to an oleic acid moiety through an ether bond on the C17 carbon atom of the fatty acid chain. This particular characteristic leaves the COOH group available and responsive to changes in pH of the solution giving rise to the possibility of a series of self-assembled structures.

### 4.2 Experimental

#### 4.2.1 Synthesis of acidic sophorolipid (SL(A))

Crude sophorolipid mixture was synthesized by \textit{Candida bombicola} processed by alkaline hydrolysis to convert it as acidic sophorolipid under heat treatment. In brief 20 gm. crude sophorolipid mixture was added in 50 mL 5M NaOH solution and reflux under stirred condition. Temperature of the solution was rises to 90 °C at constant rate 2°C/min, and then leaves the solution for 10 minutes and finally it was cool to room temperature. Color of the solution was yellow/pale yellow from the start point to the end. Temperature control is a crucial parameter because once it reaches beyond 90 °C, would reduce the final product due to the formation of oxidized species. Acidic sophorolipid was collected at pH 4 by adding 40 mL 18.5 wt% HCL solutions.

#### 4.2.2 Synthesis procedure for the SL(A)+Cur assembly

SL(A) dissolved in 25 ml distilled water (1 mg/ml) was taken in a 50 ml beaker, and was kept for bath sonication for 30 minutes. 5 ml Curcumin solution (1mg/ml) in distilled water was mixed drop wise during sonication at the rate of 0.5 ml/min. The final volume was dried by rota-vapor and then 5 ml water was added for complete dispersion. The solution was filtered through a 0.22 µm filter paper to make sure that only the well-dispersed compound will go across the membrane.
4.3 Characterization

In this study, we have developed a novel formulation, namely a complex of acidic sophorolipid and curcumin ((SL(A)+Cur), to improve the water solubility, stability and bioavailability of curcumin in order to enhance its effectiveness in the context of anti-cancer activity. SL(A)+Cur complexes were prepared by sonication driven supramolecular self-assembly (Figure 4.1), and were characterized by using UV-Vis and photoluminescence (PL) spectroscopy, dynamic Light Scattering (DLS), Zeta potential, Fourier-transform infrared (FTIR), x-ray diffraction and scanning, Transmission electron microscopy (SEM and TEM, respectively). The as-synthesized
curcumin formulation showed significantly improved bioavailability in cancer cells compared to the curcumin in ethanol. The optimized curcumin formulation also exhibited more cytotoxicity in cancer cells. This study thus suggests a new cost-effective nanoscale self-assembly approach for improved curcumin delivery and therapeutic efficacy in cancer.

4.3.1 UV-Vis and Photoluminescence studies

UV-Vis absorption spectra were recorded on Varian CARY 100 Bio UV-Vis spectrophotometer, with 10 mm quartz cell at 25±0.1 °C. For recording the spectra, 3 ml solutions of SL(A), Curcumin and SL(A)+Cur solution were prepared with concentration of 100 µg/ml. The solutions were mixed gently and subsequently the spectra were recorded.

4.3.2 DLS measurements

DLS measurements were carried out on Brookhaven Instrument model 90 Plus Particle Size Analyzer.

4.3.3 Zeta Potential

The surface charges of the SL(A), Curcumin and SL(A)+Cur were determined using a Zeta potential analyzer (Brookhaven Instruments Corporation, NY). The average Zeta potentials of the nano self-assembly dispersions were determined without any dilution.

4.3.4 FTIR analysis

FTIR spectra were recorded with KBr pellets in transmission mode using a Nicolet Magna IR-750 spectrophotometer at 4 cm⁻¹ resolution with 64 scans between 4000 and 400 cm⁻¹. Two milligram of dried powder was mixed with 198 milligram KBr and analyzed.

4.3.5 Scanning Electron Microscopy (SEM)

Field emission scanning electron microscopy images were acquired on FEI QUANTA 200 microscope, equipped with a tungsten filament gun, operating at WD 10.6 mm and 20 kV. 10 µL aliquots of all three sample solution were placed on silicon wafer
and these were fixed on copper stubs with the help of carbon tape. The samples were dried at room temperature overnight and images were recorded without gold coating.

4.3.6 Sample preparation for NMR

Chloroform was added in SL(A)+Cur aqueous solution to extract curcumin from nano-complex. The sample was rotator evaporator and vacuum dried for 60 min to remove chloroform. Curcumin crystals, as a control, were dissolved in chloroform and vacuum-dried as above the encapsulated curcumin. The obtained curcumin powders were dissolved in deuterated chloroform for 1H NMR study.

4.3.7 Cell lines and reagents

The human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231 used in the study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO₂ incubator at 37°C. Tissue culture plastic ware was purchased from BD Biosciences, CA, USA. Curcumin, Dulbecco's Modified Eagles Medium (DMEM), Fetal Bovine Serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin and L-glutamine were obtained from Gibco BRL, CA, USA.

4.3.8 MTT assay

The cell viability was determined by MTT dye uptake as described previously. Briefly, the cells were seeded at a density of 1 × 10⁵ cells/ml density in 96-well plates. An untreated group was kept as a negative control. The cells were treated with different concentrations (0-160 µg/ml) of SL(A), Curcumin (dissolved in ethanol) and SL(A)+Cur. MTT solution (5 mg/ml) was added to each well and the cells were cultured for another 4 h at 37°C in 5% CO₂ incubator. The formazan crystals formed were dissolved by addition of 90 µl of SDS-DMF (20% SDS in 50% DMF). After 15 min, the amount of colored Formazan derivative was determined by measuring optical density (OD) using the ELISA micro plate reader (Biorad, Hercules, CA) at 570 nm (OD570-630 nm). The percentage viability was calculated as:
% Viability = \[\frac{\text{OD of treated cells}}{\text{OD of control cells}}\] \times 100

**Statistical Analysis**

All the experiments were performed in triplicates and repeated twice and the data are presented as mean ± SD. Statistical analysis was conducted with the Graph Pad 4 prism program using one-way ANOVA. The $p$-values used for comparisons were <0.05. IC$_{50}$ values were calculated using Kyplot software.

### 4.4 Result and discussions

#### 4.4.1 Optical properties

The optical properties of sophorolipid acidic SL(A), curcumin (Cur) and sophorolipid-curcumin (SL(A)+Cur) complex show distinct significant differences. SL(A) appears transparent, Curcumin solution looks turbid, whereas the SL(A)+Cur solution appears transparent yellow indicating curcumin solubilization. The physophysical properties of curcumin are very sensitive to the medium. Curcumin has extensive absorption around 420 nm in organic solvent. However, its absorbance decreases in aqueous solution due to degradation of Curcumin in water by a reaction at the keto-enol group. Interestingly though, the sophorolipid shell imparts a hydrophobic surface to the curcumin core in the SL(A)+Cur complex and the outer hydrophilic portion of the sophorolipid assists in the solubility of the complex in water (Figure 4.2 A). This assembly in aqueous solution greatly assists in stabilization of the complex giving rise to the enhanced absorption at 420 nm in the aqueous medium.$^{[8, 11]}$

Photoluminescence (PL) of the SL(A), Curcumin and SL(A)+Cur samples was recorded for comparison by excitation at the same wavelength of 420 nm in an aqueous solution. SL(A) sample exhibits no PL, while Curcumin exhibits weak excitonic emission at 550 nm due to low solubility.$^{[28]}$ However, on addition of SL(A) in Curcumin aqueous solution a strong emission is seen at 500 nm reflecting tremendous enhancement of the fluorescence intensity (Figure 4.2 B). The fluorescence maximum shifts from a broad unremarkable 550 nm band to a remarkable blue shifted band at 500 nm.$^{[11, 29]}$ To confirm the improvement in PL, we performed some additional experiments.
Figure 4.2 (A) UV-Visible spectra of SL (A), Curcumin and SL(A)+ Cur solutions. SL(A) solution shows absorbance at \( \lambda = 234 \) nm and Curcumin solution at \( \lambda = 344 \) and 420 nm while SL(A) + Cur showing increase absorption at one peak at 420 nm; (B) Photoluminescence study of SL(A), Curcumin and SL(A)+ Cur solutions. SL(A) solution shows no PL, Curcumin solution shows PL at 550 nm while SL(A) + Cur showing very strong Photoluminescence at 500 nm; (C) Photoluminescence quenching and right shift of the PL of SL(A)+Cur self-assembly on gradually addition of ethanol solvent;

When we add ethanol gradually in this mixture, photoluminescence quenching is observed. This is probably due to disturbance of nonpolar region around Curcumin nanoparticles created by SL(A) self-assembly. Furthermore, a red shift is observed (from 500 nm to 550 nm) with the addition of ethanol to the SL(A)+Cur aqueous solution, clearly indicating a gradual degradation of the self-assembly yielding the original structure itself (Figure 4.2 C).

As described earlier, a large blue shift was observed when Curcumin was bound to the SL(A) micelles signifying that Curcumin in SL(A) micelles creates nonpolar
environment, possibly by binding to the hydrophobic regions of SL(A) micelles. Besides the shift in the fluorescence maximum, there was a remarkable improvement in the fluorescence intensity of Curcumin upon formation of the self-assembly with SL(A) (Figure 4.3). As seen from Figure 4.3 B a very feeble fluorescence is seen in the case of Curcumin particulates, while the well-dispersed and therefore well distributed SL(A)+Cur nanoparticulates exhibit enhanced fluorescence ( Figure 4.3D).

**Figure 4.3** (A and C) Optical Microscopy images of Curcumin and SL(A)+Cur self-assembly; (B) and (D) Fluorescence images of Curcumin and SL(A)+Cur self-assembly. (scale bar 100 µm).

### 4.4.2 Particles Size Distribution and Zeta Potential

In this experiment, all solutions were analyzed at a constant shutter opening diameter in the DLS apparatus. DLS measurements for Curcumin, SL(A) and SL(A)+Cur (10 mg of each dissolved in 10 ml H2O) exhibit hydrodynamic radii of about 818 nm, 6.8 nm and 15.5 nm, respectively (Figure 4.4 A, C, E). This was also confirmed with the results obtained by SEM and TEM analysis. These data clearly show that the size of the SL(A)+Cur particles is ~6-7 nm indicating that there is a decreased agglomeration of Curcumin in aqueous solution due to its capping by SL(A) and there
is a definite increase in the size of SL(A)+Cur complex as compared to only SL(A) because of the additional encapsulation of Curcumin nanoparticles.

To understand the stability of SL(A)+Cur, Zeta potential measurements were done on all the three samples. The zeta potential values for the three sample were SL(A) = -17.30 mV, Curcumin = -15.14 mV, SL(A)+Cur= -24.38 mV (Fig. 4 B, D, F respectively). The increase in the zeta potential of SL(A)+Cur compared to individual SL(A) and Curcumin can be taken as an indication of an increased stability of the self-assembled complex.

Figure 4.4 DLS and Zeta Potential results for (A) Curcumin showing hydrodynamic radius of 818.6 nm and (B) corresponding Curcumin zeta potential; (C) SL(A) showing hydrodynamic radius of 6.8 nm and (D) corresponding SL(A) zeta potential; (E) SL(A)+Cur showing hydrodynamic radius 15.5 nm and (F) corresponding SL(A)+Cur zeta potential.

4.4.3 Microscopy studies
SL(A), Curcumin and SL(A)+Curcumin samples were further examined by scanning electron microscopy (Figure 4.5 A, B and C). SL(A) exhibits a ribbon type
morphology (Figure 4.5 A), Curcumin appears as large chunks forming undefined shape (Figure 4.5B), whereas SL(A)+Cur shows a fibrous morphology (Figure 4.5 C) which differs distinctly from that of SL(A). Remembering that these morphologies evolve in the solution drying process and do not represent the situation in the solution, it is clear that morphological organization of SL around Curcumin with hydrophilic groups protruding outside would change their organization and therefore the morphology during the drying process as compared to SL itself which does not have such preferential molecular organization in the solution. The TEM pictures at different levels of magnification (Figure 4.5 D-E-F) apparent in SEM (Figure 4.5 C) due to the corresponding limited resolution show tiny fairly uniformly dispersed Curcumin nanoparticles (< about 20 nm, some agglomerated on TEM grid) which were not apparent in SEM (Figure 4.5 C) due to the corresponding limited resolution.

4.4.4 FTIR Analysis

Figure 4.6 shows the FTIR spectra for SL(A), Curcumin and SL(A)+Cur after sonication treatment for 30 minutes. The SL(A) reveals a broad band at 3350 cm⁻¹...
corresponding to the O-H stretch frequency in the glucose moiety of the molecule. The asymmetrical and symmetrical stretch modes of methylene (CH$_2$) groups occur at 2928 and 2854 cm$^{-1}$, respectively. Sophorolipid has two strong absorption bands arising from C-O- and C-O stretching; the C-O absorption band at 1744 cm$^{-1}$ may include contributions from acid group.

\[
\text{Figure 4.6 FTIR analysis of SL(A), Curcumin and SL(A)+Cur.}
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Moreover, sugar C-O- stretch of C-O-H groups is found at 1048 cm$^{-1}$ and the band at 1452 cm$^{-1}$ corresponds to the C-O-H in-plane bending of carboxylic acid (-COOH) in the structure of the product. All these details are in conformity with the literature reports.\textsuperscript{29} The FTIR spectrum of Curcumin shows a sharp one peak at 3508 cm$^{-1}$ indicating the presence of OH. The strong peak at 1626 cm$^{-1}$ has a predominantly mixed (C=C) and (C=O) character. Another strong band at 1601 cm$^{-1}$ is attributed to the symmetric aromatic ring stretching vibrations (C=C ring). The 1508 cm$^{-1}$ peak is assigned to the (C=O), while enol C-O peak was obtained at 1272 cm$^{-1}$, C-O-C peak at 1023 cm$^{-1}$, benzoate trans-CH vibration at 959 cm$^{-1}$ and cis CH vibration of aromatic ring at 713 cm$^{-1}$.\textsuperscript{30} The FTIR spectrum of SL(A)+Cur shows all the peaks
related to SL(A) and Curcumin peaks are seen to have been suppressed due to nano-encapsulation.

### 4.4.5 X-ray diffraction (XRD) analysis

To examine the crystallinity of micelle-encapsulated SL(A)+Cur, XRD analysis was performed. XRD analysis of samples was done over broad angle range (2θ = 10-80 degrees). The powder X-ray diffractograms of SL(A), Curcumin and SL(A)+Cur dried powders are shown in Figure 4.7

![XRD spectra of SL(A)+Cur self-assembly](image)

**Figure 4.7** XRD spectra of SL(A)+Cur self-assembly. Black line explain the Curcumin XRD pattern, red SL(A), green SL(A)+Cur while blue SL(A)+Cur after dissolve in chloroform solvent. Blue line showed unaffected nature of curcumin encapsulated in SL(A) self-assembly

The characteristic peaks of Curcumin appeared at diffraction angles of 2θ at 7.96, 8.90, 12.26, 14.54, 17.24°, etc. indicating that Curcumin is present as a crystalline form. It is found that the characteristic diffraction peaks of the Curcumin are absent in the spectrum of the SL(A)+Cur nano-encapsulation, which suggests that Curcumin being nano-encapsulated its peaks are too broadened and the XRD pattern in dominated by the structure of sophorolipid component. Interestingly, the diffraction patterns of the material obtained after dissolution in chloroform showed a pattern
similar to that of pure Curcumin, indicating that the complex is broken down and the encapsulated Curcumin is released.

4.4.6 Nuclear magnetic resonance analysis

We have confirmed the stability of curcumin after four month storage in refrigerator condition through NMR study of as synthesized and after four storage sample study.

**Figure 4.8** (A) NMR spectra of curcumin in SL(A)+ Cur complex and (B) curcumin in SL(A)+ Cur complex after four month stability.

Sample preparation for the NMR study perform by addition of chloroform in
SL(A)+Cur self-assembled compound. Chloroform break the assembly and extract curcumin which was further dried in high vacuum condition. Figure 4.8 A illustrate the as synthesized curcumin (from SL(A)+Cur assembly) which match with reference NMR spectrum. $^1$H NMR spectrum of curcumin (200 MHz, CDCl$_3$) was recognized by chemical shift ($\delta$) of 3.96 (6H, s, OCH$_3$), 6.53 (1H, s, C(OH)=CH), 6.92 (2H, d, 2,6-H), 6.96 (2H, d), 7.11 (2H, d), 7.56 (2H, s), 7.64 (2H, d, 1,7-H). Rather than a β-diketone, $^1$H NMR spectra of the curcumin denoted hydroxyl group, at $\delta$ 6.45 corresponding to enol-keto tautomer. Self-assembled structure of SL(L)+cur showed both SL(L) and curcumin identical peak without any contamination. Stability of SL(A)+Cur complex performed by four month old sample NMR spectra analysis (Figure 4.8 B), which shows identical peaks with as synthesized self-assembled complex.

4.4.8 Cytotoxicity Assay

To evaluate whether SL(A) enhanced the bioavailability of curcumin in the cancer cells, cytotoxic potential of aqueous SL(A)+Cur was tested in breast cancer cell lines, MCF-7 and MDA-MB-231 and compared with that of aqueous SL(A), curcumin in water, and curcumin dissolved in ethanol, the latter being used as a positive control. Curcumin was administered to the cells at nontoxic doses of ethanol. After treatment with SL(A), both the breast cancer cell lines exhibited 80-100% viability upto the concentration of 160 µg/ml showing that it was almost non-toxic to the cells. It can be observed that in SL(A)+Cur nano-complex, curcumin exhibited anticancer activity at extremely low doses starting from 6.66 µg/ml compared to 40 µg/ml concentration of curcumin dissolved in ethanol in MCF-7 cells (Figure 4.9 A).

As seen in the case of MCF-7 cells, curcumin dissolved in ethanol is more toxic than nano-complexes at moderate and low concentrations. In the low to moderate dose range (5-20 µg/ml), curcumin dissolved in ethanol has more concentration compared to the concentration of curcumin present in the SL(A)+Cur nano-complexes (0.83-3.33 µg/ml). Thus the nano-complexes do not show effective killing within this concentration. However, at higher dose range (40-160 µg/ml), the concentration of curcumin in SL(A)+Cur is also increasing (6.66 to 26.66 µg/ml) and thus we observe
the increase in cytotoxicity. Similarly, in MDAMB-231 cells (Figure 4.9 B), SL(A)+Cur exhibits cytotoxicity at 3.33 µg/ml compared to 20 µg/ml concentration of curcumin dissolved in ethanol. We have also added the data of curcumin (in water) that shows no cytotoxicity since curcumin does not go in water. These results clearly show that the bioavailability of curcumin is increased in the SL(A)+Curcumin complex as compared to the of curcumin dissolved in ethanol.

Interestingly, MCF-7 shows increased susceptibility to anticancer drugs at lower doses compared to MDAMB-231, the reason being the difference in estrogen and progesterone (ER/PR) receptor status in the cell lines. MCF-7 is ER/PR positive while MDAMB-231 is ER/PR negative, thereby being slightly insensitive to lower concentrations of anticancer drugs.

*Figure 4.9* Cytotoxicity analysis of SL(A), Curcumin and SL(A)+Curcumin by MTT assay in (A) HEK 293 cells (B) MCF-7 and (C) MDA-MB-231. All the data are presented as mean±SD of five independent experiments at p<0.0001, indicating statistically significant differences compared to the control untreated group.

Moreover, SL(A) makes the curcumin present in the NPs more bio-available at lower
doses as compared to higher doses of curcumin dissolved in ethanol which explains the increased cytotoxicity of nano-complex.\textsuperscript{32-33} This improved cytotoxicity of SL(A)+Cur complexes compared to curcumin in ethanol in cancer cell lines may be due to tautomeric molecular form of curcumin after SL(A) encapsulation.

We have compared the cytotoxicity of curcumin in water, curcumin in ethanol, SL(A) and SL(A)+Curcumin in non-cancerous cell line HEK 293 (Figure 4.9 C). Interestingly, except for Curcumin in ethanol (at higher doses), all others are non-toxic to the cells.\textsuperscript{34-40}

4.5 Conclusions

Sophorolipid, an environmentally friendly, biocompatible and important class of biosurfactants is complexed with Curcumin to increase its solubility, stability, fluorescence and bioavailability. Particle size distribution, TEM and Zeta potential analyses suggest that the increase in cellular uptake could be attributed to the nano-encapsulation of Curcumin, its solubilization and stability in aqueous solution. It has been clearly established that the complex formation of Curcumin with SL(A) significantly reduced its therapeutic index, reflecting its increased bioavailability. This study emphasizes that solubilization by nano-encapsulation is an effective aspect of designing drug delivery systems.

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