CHAPTER 6

SYNTHESIS AND CHARACTERIZATION OF CURCUMIN LOADED POLYMER/LIPID BASED NANOPARTICLES AND EVALUATION OF THEIR ANTITUMOR EFFECTS ON MCF-7 CELLS

6.1 INTRODUCTION

In this chapter, the focus is on synthesis and characterization of curcumin loaded PHEMA/Stearic Acid (C-PSA-NPs) conjoint-based nanoparticles, through environmentally benign approach. The efficacy of the synthesized nanoparticles is evaluated with MCF-7 cell line. The use of amphiphilic pluronic F-68 (Gao et al 2011) as an emulsifier and a stabilizer is investigated to understand the means to provide an apt drug delivery system for a hydrophobic drug like curcumin.

6.2 MATERIALS AND METHODS

Choline formate ionic liquid (IL) was synthesized as per procedure (Jensen et al 2009). The purity and the presence of moisture in Choline Formate (CF) were verified by comparing the FT-IR and $^1$H-NMR standard spectra. HEMA (2- Hydroxyethylmethacrylate) and curcumin ($\geq 94\%$) were obtained from Sigma Aldrich, Bangalore. Stearic acid and pluronic F-68 were procured from Merck India Ltd, Bangalore. Stearic acid was purified by recrystallization from 95 % ethanol.
MCF-7 cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. Minimum Essential Medium Eagle (MEM), 10 % Fetal Bovine Serum (FBS), Trypsin-EDTA and Penicillin (100 U/mL)/Streptomycin (100 μg/mL) were obtained from Himedia, India, and used without further purification, for cell line studies. MTT (4,5dimethyl thiazol 2, 5 diphenyl tetrazolium bromide), Dimethylsulphoxide (DMSO), glycine buffer were obtained from Sigma-Aldrich, Bangalore. All other chemicals and reagents were of analytical grade. Double distilled water was used for all the experiments.

6.2.1 Preparation of Curcumin Loaded PSA Nanoparticles (C-PSA-NPs)

The polymer matrix was synthesized using HEMA and IL (volume ratio: 1:1), the mixture was poured into polystyrene plate and kept in a dessicator; it was exposed to nitrogen for 24 h (Jensen et al 2009). Curcumin loaded PSA nanoparticles (C-PSA-NPs) were prepared by emulsification-solvent evaporation method (Senna et al 1998). Precisely, 200 mg of polymerized PHEMA gel was dissolved in a binary solvent (acetone and ethanol in the ratio of 4:1) with 50 mg of SA and curcumin. The mixture was stirred for 10 min in a magnetic stirrer (IKA, Germany). The above mixture was added drop by drop to an aqueous solution of 1% pluronic F-68 (w/v), simultaneously homogenizing at 15,000 rpm for 3 min using Homogenizer (IKA, Germany), to form an emulsion. Subsequently, the organic solvent was evaporated by stirring the emulsion for 8 h at 40 °C until the formation of C-PSA-NPs. The nanoparticle suspension was then dispensed into 10mL vials and transferred to a VirTis freeze-dryer (New York, USA). After the drying process lasted for 48 h at -70 °C, the lyophilized powder was stored. The control sample of PSA-NPs (without curcumin) was synthesized by adopting the same procedure.
6.2.2 Physicochemical Characterization

The physicochemical characterization such as particle size, zeta potential, scanning electron microscopy, atomic force microscopy, FT-IR, X-ray diffraction, thermal analysis, in vitro release study, DNA fragmentation and cell cycle analysis of the curcumin, PSA-NPs and C-PSA-NPs were performed according to the procedure reported in chapter 5 - (Section 5.2.2.1, 5.2.2.5-9, 5.2.4 & 5.2.5).

6.2.3 Loading Efficiency and Solubility Study of Curcumin

In order to determine the loading efficiency (%) of curcumin in C-PSA-NPs, UV-Vis spectrophotometer (Shimadzu UV 2101 PC) studies at 426 nm were carried out. A standard curve in the range of 2-10 µg/mL curcumin was plotted. The curcumin content present in C-PSA-NPs was calculated as loading efficiency using the Equation (6.1). Aqueous solubility of curcumin and C-PSA-NPs was determined by dissolving it separately in 0.01 M of PBS in pH 7.4.

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\text{Loading efficiency (\%)} = \frac{\text{amount of curcumin loaded in PSA nanoparticles}}{\text{amount of curcumin initially added}} \times 100 \quad (6.1)
\]

6.2.4 In vitro Cytotoxicity Study

The standard MTT assay was used to determine the cytotoxicity of C-PSA-NPs using MCF-7 cells. MCF-7 cells were grown in DMEM Medium, pH 7.4, with 10 % FBS and 1 % antibiotic (penicillin/streptomycin mixture). MCF-7 cells were seeded on 96 well plates and incubated at 37 °C in a humidified atmosphere of 5 % CO₂. For all experiments, cells were seeded to provide 80 % confluency in 96 well plates and grown for 48 h. On reaching 80 % adherent confluency, cells were treated with a range of concentrations as 2, 5, 10, 15, 20, 25, 30, & 50 µg/mL of curcumin, PSA-NPs and C-PSA-
NPs and incubated for 48 h. The untreated cells and PSA-NPs were kept as control and blank respectively. After this, 200 µL of MTT was added and the plates were incubated at 37 °C, 5 % CO₂ for 4 h. The absorbance was read at 570 nm. Cytotoxicity (IC₅₀) is calculated based on the color yield of treated cells which is proportional to the number of metabolically active cells and indirectly approximates to cell viability. IC₅₀ is defined as the drug concentration required to inhibit growth of cells by 50 % relative to control. A graph was drawn using % of cell viability with respect to the concentration of the sample.

6.2.5 Apoptosis Analysis by Flow Cytometry

Flow cytometry analysis of apoptosis was performed in Annexin V-PE/ 7-Amino-Actinomycin (7-AAD) dual stained MCF-7 cells. The staining was carried out using the PE Annexin V Apoptosis Detection Kit (BD Pharmingen™, US) as per the manufacturer’s instructions. Annexin V-PE binds to phosphatidylserine in the membrane of cells when the apoptotic process begins, and the binding of 7-AAD to the cellular DNA in cells indicates late apoptotic or necrotic cell death. Here, the cells were treated with IC₅₀ concentration of curcumin, PSA-NPs, C-PSA-NPs for 48 h, and then the cells were taken to trypsinization and washed twice with ice-cold PBS. They were finally suspended in 1× binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM CaCl₂) at a concentration of 1 x 10⁶ cells/mL. Five microliters of Annexin V-PE and 7- AAD were added in 100 µL of each cell suspension. The samples were mixed gently and incubated at room temperature for 15 min in the dark. After incubation, 400 µL of ice-cold 1x binding buffer was added and mixed gently and the percentage of apoptotic cells in the population was determined.
6.2.6 High Content Imaging

Localization of C-PSA-NPs and the cytotoxic effect were investigated in MCF-7 cell line. Briefly, 2×10^4 cells/well of was seeded on to 96 well Cell Carrier microplates (PerkinElmer, US). When the cells reached 80% confluence the media were changed and then the cells were treated with PSA-NPs, curcumin and C-PSA-NPs and the plate was incubated for 24 h in humidified incubator at 37 ºC with 5 % CO_2. The cells were washed twice with ice cooled PBS and the drug localized in live cells was analyzed using Operetta High Content Imaging System (PerkinElmer, US).

6.3 RESULTS AND DISCUSSION

6.3.1 Preparation and Characterization of PSA and C-PSA Nanoparticles

As reported earlier (Jensen et al 2009), PHEMA gel was synthesized using choline formate ionic liquid, and PSA-NPs were prepared using optimized concentrations of polymer (PHEMA), lipid (stearic acid) and emulsifier (pluronic F-68) by emulsification-solvent evaporation method. Polymer PHEMA concentration was fixed throughout the study and concentrations of both lipid and emulsifier were different. Of the three lipid concentrations studied (50, 75 and 100 mg), the nanoparticles were found to be stable and homogenous with no aggregation, at 50 mg of lipid concentration. (At 75 and 100 mg lipid concentrations, the nanoparticles obtained were found to be more aggregated leading to unstable dispersions with increased mean diameters of PSA-NPs). A similar study was performed by varying pluronic F-68 concentration (0.5, 1, and 2 %) in aqueous solution. Pluronic F-68 of 1 % concentration provided PSA-NPs in the desired size range of around 184 nm. Curcumin loaded PSA nanoparticles (C-PSA-NPs) were prepared using optimum concentration of lipid (50 mg), and emulsifier (1 %).
Drug loading efficiency was found to be 53.2 %. The high drug loading was believed to be due to the incorporation of stearic acid in the formulation which enabled better entrapment of hydrophobic drug curcumin. Solubility of curcumin and C-PSA-NPs was also determined by dissolving both curcumin and C-PSA-NPs in the aqueous solution, and compared (Figure 6.1). It revealed that C-PSA-NPs were completely dissolved and a clear yellow well dispersed liquid could be seen. The average particle size, determined using DLS, was found to be 184 nm (Figure 6.2). Zeta potential measurement showed the surface potential of C-PSA-NPs to be -29.3 mV (Figure 6.3).

Figure 6.1 Photographs showing the comparative solubility of curcumin and C-PSA-NPs in PBS (0.01M, pH 7.4)
Figure 6.2 Mean particle size distribution of C-PSA-NPs

Figure 6.3 Zeta potential of C-PSA-NPs by using dynamic laser scattering
Thus, C-PSA-NPs could be successfully synthesized with the desired particle size and zeta potential. The increased drug loading efficiency in the present chapter (in contrast Chapter 5) could be attributed to the role of the lipid SA. The conjoint effort of incorporating SA with PHEMA and curcumin (C-PSA-NPs) appeared to be more promising due to the potential of long term systemic circulation of the hydrophilic polymer and lipid prepared with the use of nonionic surfactants (pluronic F-68) as emulsifiers. The idea of incorporation of SA with PHEMA to achieve high drug loading of hydrophobic drug curcumin, attempted now is found to be promising.

6.3.2 Thermogravimetric Analysis

TGA studies of curcumin, PSA-NPs, and C-PSA-NPs were carried out to assess their thermal behavior. Figure 6.4 showed the degradation of curcumin, PSA-NPs, C-PSA-NPs with increasing temperature. The study confirmed that PSA-NPs were stable up to a temperature of 150 °C; however, C-PSA-NPs showed an increase in stability up to a temperature of 370 °C. A close observation of the TGA curve (Figure 6.4), revealed that PSA nanoparticles underwent a two step degradation, first in the range of 150 to 280 °C showing a weight loss of 58 %, and the second in range 280 to 400 °C with further degradation of PSA-NPs and a weight loss of 42 %. The first step degradation was presumably due to the loss of SA as confirmed from the TGA curve of SA (Zhang et al 2009). This trend confirmed the incorporation of SA in the PSA-NPs and low stability of PSA-NPs as compared to C-PSA-NPs.
Figure 6.4 A comparative plot of thermogravimetric curves of PSA-NPs, C-PSA-NPs, and curcumin

6.3.3 Morphological Study

The SEM image shown in Figure 6.5[a] showed that the particles were spherical in shape with monodispersed size distribution in the range of ~200 nm size, corroborating DLS studies. Further the morphological information of C-PSA-NPs was investigated using AFM images shown in Figure 6.5[b]. The results confirmed that the particles were spherical in shape, without any aggregation (Mohanty & Sahoo 2010).
Figure 6.5  [a] SEM image of C-PSA-NPs; [b] [i] AFM images of C-PSA-NPs and [ii] its three dimensional view
6.3.4 FTIR Study

FTIR spectra of curcumin, PSA-NPs and C-PSA-NPs are shown in Figure 6.6[a]. For both PSA-NPs and C-PSA-NPs, the characteristic peaks of PHEMA were noticed at 3460 - 3560 cm\(^{-1}\) due to the hydrogen bonded O-H stretching, 1728 cm\(^{-1}\) C=O stretching, 1172 cm\(^{-1}\) O-C-C stretching, 2951 cm\(^{-1}\) asymmetric stretching of methylene group and 1454 cm\(^{-1}\) O-H bending, respectively. The results were in agreement with the previous studies of Chouhan & Bajpai (2009). A close observation of FTIR spectra of the sample PSA-NPs and C-PSA-NPs indicated the presence of stearic acid in the peak range of 2850-2960 cm\(^{-1}\) corresponding to CH stretching of aliphatic hydrocarbon (Karnnet et al 2005). The peak observed at 1280.6 cm\(^{-1}\) in the curcumin loaded C-PSA-NPs confirmed the aromatic C-O stretching vibrations of curcumin and the bands from 1280-1114 cm\(^{-1}\) marked the presence of C-O stretching (Yallapu et al 2012). FTIR spectra confirmed the incorporation of curcumin in the C-PSA-NPs.

6.3.5 X-Ray Diffraction Study

X-Ray Diffraction studies were carried out for PSA-NPs, C-PSA-NPs and curcumin to understand the nature of curcumin in C-PSA-NPs (Figure 6.6[b]). The characteristic peak of curcumin was observed in the 2\(\theta\) range of 10–30 °C, indicating the high crystalline nature of curcumin. However, the significant peaks of curcumin were absent in C-PSA-NPs. This absence of functional peaks of curcumin in C-PSA-NPs indicated the amorphous nature of curcumin or disordered crystalline phase. A similar phenomenon was reported earlier (Mohanty & Sahoo 2010, Anitha et al 2011a, Yallapu et al 2010b). Thus, the x-ray diffraction studies confirmed the desired amorphous nature of C-PSA-NPs.
Figure 6.6  [a] FTIR spectra of curcumin, PSA-NPs, C-PSA-NPs;  
[b] XRD patterns of curcumin, PSA-NPs, C-PSA-NPs
6.3.6  *In vitro* Release Study

In order to obtain quantitative and qualitative information on drug release from the C-PSA-NPs, the release profile was determined by an *in vitro* dialysis method. Figure 6.7[a] and 6.7[b] demonstrate the release profile of curcumin and C-PSA-NPs. Sustained release of curcumin was observed from C-PSA-NPs over a period of 8 days. A comparative release pattern of the C-PSA-NPs and curcumin in the first 12 h indicated that 10 % release was noticed from C-PSA-NPs, whereas curcumin exhibited a release up to 60 %. Thus, the initial release in the first 12 h period could not be treated as a burst release in comparison to the curcumin release. The sustained release of C-PSA-NPs (40 %) during the period of 8 days indicated its applicability as a drug delivery system (Liu et al 2013).
Figure 6.7  *In vitro* release profile of [a] curcumin, [b] curcumin from C-PSA-NPs; in PBS at pH 7.4 (mean±SD, n=3)
To determine the release model that best described the drug release, the *in vitro* release data were substituted in equations of zero order, first order and Higuchi model and the results noted. Among them the Higuchi model showed a high $R^2$ value 0.9877, indicating that the release of the drug followed Higuchi model release kinetics. To understand the mechanism of drug release, Korsmeyer-Peppas equation was applied and it showed a good linearity ($R^2=0.94259$). The release kinetics results are in Figure 6.8. The release exponent ‘$n$’ was found to be 0.6841 According to this model, if the value of ‘$n$’ was between $> 0.43$ and $< 0.85$, it indicated that drug release followed anomalous transport (Non-Fickian) (Chouhan & Bajpai 2009) and was controlled by more than one process (the coupling of Fickian diffusion and polymer matrix relaxation).

![Figure 6.8 Release Kinetics profiles obtained for C-PSA-NPs following (a) Zero order model, (b) First order model, (c) Higuchi model, and (d) Korsmeyer-peppas]
6.3.7 *In vitro* Cytotoxicity Study (MTT Assay)

MCF-7 cells were used to evaluate the anti-cancer activity of curcumin, PSA nanoparticles and C-PSA-NPs. The cell viability was studied by a standard MTT assay (Tang et al 2010). The untreated cells and PSA-NPs were kept as control and blank respectively. The assay was terminated at 48 h and colorimetric determination of cell viability was performed using ELISA Reader (Qualigens, Bangalore, India). (The results are shown in Figure 6.9[a] and 6.9[b]). The IC$_{50}$ concentrations of curcumin and C-PSA-NPs were 10.72 μg/mL and 7 μg/mL, respectively. The results showed that C-PSA-NPs had better uptake profile than curcumin in MCF-7 cell line (Mohanty & Sahoo 2010).
Figure 6.9  [a] *In vitro* cytotoxicity studies (MTT assay) of curcumin, PSA-NPs and C-PSA-NPs in MCF-7 cell line, [b] Images of MCF-7 Cell line visualized under inverted microscope.
6.3.8 DNA Fragmentation

DNA was extracted from cultured MCF-7 cells treated with IC_{50} concentration of C-PSA-NPs and the presence of necrosis was detected by agarose gel electrophoresis. Fragmented DNA or DNA ladder formation was observed at the time of apoptosis (Qi et al 2005). In this study, C-PSA-NPs treated MCF-7 cells showed that the necrosis was induced and this was confirmed by observing the continuous spectrum of DNA fragments of low molecular mass (Figure 6.10).

![DNA fragmentation studies by Agarose gel electrophoresis.](image)

6.3.9 Cell Cycle Analysis by Flow Cytometry

In order to evaluate the cell cycle effect of prepared formulations on MCF-7 cell line, experiments were conducted using the IC_{50} value of
curcumin and C-PSA-NPs (Figure 6.11). Curcumin, PSA, and C-PSA-NPs treated MCF-7 had an increased percentage of apoptotic cells in G\textsubscript{1} phase compared to untreated control. In particular, a significantly higher percentage of apoptotic cells was observed following C-PSA–NPs treatment (77.3 %) and drug alone (76.52 %) as compared to control (71.01 %). The observed molecular mechanism of curcumin in cell cycle was in agreement with the earlier reports of Srivastava et al (2007).

![Figure 6.11](image)

**Figure 6.11** The effect of [a] control, [b] Curcumin and [c] C-PSA-NPs on the cell cycle analysis in MCF-7 cell line
6.3.10 Apoptosis Analysis by Flow Cytometry

The apoptotic profile of curcumin and C-PSA-NPs on MCF-7 cell line was evaluated using flow cytometry after 48 h of exposure. The apoptosis inducing efficiency on MCF-7 cell line was investigated. (The percentage population of live cells (bottom left), early apoptotic cells (bottom right), necrotic cells (top right) and late apoptotic cells (top left) are shown in a quadrant in Figure 6.12). Cells treated with curcumin showed 1.5 %, 13.7 % and 44.2 % in necrotic stage, late apoptotic stage and early apoptotic stage respectively. However the C-PSA-NPs treated cells showed less number of cells (43.1 %) in early apoptotic stage and higher number of cells in both necrotic (1.8 %) and late apoptotic stages (16 %). The IC$_{50}$ value of curcumin (10.72 µg/mL) and C-PSA-NPs (7 µg/mL) was found with MTT assay. The same IC$_{50}$ concentration was adopted to perform the apoptosis analysis using flow cytometer. So, 7 µg/mL of C-PSA-NPs was able to produce equal percentage of apoptosis as curcumin (10.72 µg/mL). Thus, C-PSA-NPs showed a better apoptotic activity than what was shown by curcumin. The improved activity of C-PSA-NPs could be due to the better uptake and greater accumulation of nanoparticulate curcumin inside the tumor cells (Mohanty & Sahoo 2010).
Figure 6.12 Detection of necrotic, early, and late apoptosis by flow cytometry with PSA-NPs, Curcumin and C-PSA-NPs in MCF-7 cell line

6.3.11 High Content Imaging

The cellular uptake study was evaluated in MCF-7 cell line for curcumin, PSA-NPs and C-PSA-NPs using high content imaging techniques. The results are shown in Figure 6.13. C-PSA-NPs localization into the cell was compared with that of curcumin and the image was taken in bright field
and fluorescent microscopy. So that merger of both images would help predict the localization of C-PSA-NPs. Control cells were kept without the exposure of curcumin and PSA-NPs and showed there was no fluorescence. The C-PSA-NPs treated cells showed a maximum localization of C-PSA-NPs into cells, more than that of curcumin.

Figure 6.13 Drug localization studies of curcumin, PSA-NPs and C-PSA-NPs in MCF-7 cell line
6.4 CONCLUSIONS

- Polymer lipid based drug delivery is seen as one of the advancements in drug delivery systems. Through the present study, a novel polymer lipid based nanocarrier delivery system loaded with curcumin has been demonstrated as an effective and potential alternative method for tumor treatment in MCF-7 cell line.

- The C-PSA-NPs were successfully synthesized using emulsification-solvent evaporation method and 53.2 % of curcumin loading was achieved. The size and zeta potential of prepared C-PSA-NPs were about 184 nm and -29.3 mV respectively.

- Characterization of the nanocomposite through techniques such as DLS, SEM, FTIR, XRD, and TGA confirmed the ability of C-PSA-NPs as a potential carrier for hydrophobic drug curcumin. A slow and sustained release (% release over a period of 8 days) of curcumin was noted through in vitro release studies.

- The MTT assay indicated higher anticancer properties and flow cytometry studies revealed that there were better apoptotic activity and maximum localization of C-PSA-NPs than curcumin.

- Overall, these findings suggest that C-PSA-NPs may be useful for the treatment of cancer with an improved therapeutic activity of curcumin.