CHAPTER 5

CURCUMIN LOADED POLY (2-HYDROXYETHYL METHACRYLATE) NANOPARTICLES FROM GELLED IONIC LIQUID – IN VITRO CYTOTOXICITY AND ANTI-CANCER ACTIVITY IN SKOV-3 CELLS

5.1 INTRODUCTION

Generally, free radical initiators are employed to start HEMA polymerization as discussed in chapter 3. Here, toxic initiators to produce the polymer/hydrogels have not been used but choline based IL was employed as an initiator to produce polymeric gels. The ability of choline based IL (choline formate) to make hydrogels and copolymers was established recently (Jensen et al 2009). In comparison to hydrophobic polymer matrix for drug loading, hydrophilic matrix has a distinct advantage of biocompatibility to the aqueous body environment and does not require surface modifications. Furthermore, the biocompatible polymers prevent monocytes from adhering to their surface (Yamit et al 2009). In literature, there is no report on the use of PHEMA nanoparticles for the delivery of curcumin. The present chapter deals with synthesis of C-PHEMA-NPs (Curcumin loaded PHEMA nanoparticles) from gelled ionic liquid using nanoprecipitation technique. The C-PHEMA-NPs were characterized by different physicochemical techniques and were taken up for in vitro anticancer study to evaluate their potential as drug carriers for the sustained delivery of curcumin on cancer cell lines.
5.2 MATERIALS AND METHODS

Analytical grades of HEMA, choline hydroxide (20 % aqueous solution), formic acid and curcumin were procured from Sigma-Aldrich (Bangalore, India). The choline formate ionic liquid was synthesized as per recorded methods (Jensen et al 2009).

The SKOV-3 ovarian cancer cell line were obtained from the National Centre for Cell Science (NCCS), Pune, India. Antibody to β-actin, PARP, caspases-3 and -9, Bel 2, caspase 8, Bax, Fas, FasL, FADD were purchased from Santa Cruz Biotechnology (Santa cruz, CA, USA), Abcam (Cambridge, MA, USA), and NeoMarkers (Fremont, CA, USA). All other chemicals and reagents were of analytical grade. Double distilled water was used throughout the experiments.

5.2.1 Preparation of PHEMA Nanoparticles

Typically, 0.25 g (1.67 mmol) of CF was dissolved in 0.25 g (1.92 mmol) of HEMA, and the reaction mixture was kept overnight at ambient temperature. The polymer was then washed two to three times with methanol (5 mL) and dried at room temperature overnight in a vacuum oven. The physicochemical properties of synthesized PHEMA are given in chapter 3. The PHEMA-NPs from gelled ionic liquid were synthesized using the nanoprecipitation method (Fessi et al 1989). The stepwise preparation is shown in Figure 5.1. Briefly, a known amount of the polymer from gelled ionic liquid was dissolved in the organic phase (binary solvent solution, acetone (4 mL) and ethanol (1 mL) in the ratio of 4:1 respectively). This polymer solution was added drop wise into an aqueous phase (50 mL) (usually deionized water) with moderate stirring (800 rpm) and the solution left for around 12 h to allow the evaporation of organic solvent, and the nanoparticle suspension was filtered through 1 µm filter paper to remove
aggregated particles. The nanoparticle suspension thus obtained was dispensed into 10 mL vials and transferred to a VirTis freeze-dryer (New York, USA); and the drying process lasted for 48 h at -70 °C, the lyophilized powder was stored for future use. C-PHEMA-NPs were prepared using the same method wherein curcumin was dissolved in choline formate ionic liquid before initiating the polymerization process.

5.2.2 Physicochemical Characterization

5.2.2.1 Particle size and zeta potential

The particle size and the distribution of the polymeric nanoparticles were observed using dynamic laser scattering (Zetasizer ver 6.00, Malvern Instruments Ltd., UK). For light scattering experiments, the samples were measured at a fixed angle of 90 °C at 25 °C. The scattering intensity was adjusted in the range of 50-500 kcps by diluting the sample with deionized water. The zeta potential of polymeric nanoparticles was measured by Zetasizer at 25 °C using the above mentioned protocol. All the measurements were analyzed in triplicate.

5.2.2.2 Absorption spectra and standard plot of curcumin

The absorption spectra of the free drug (10 µg/mL) was found to be 426 nm. The standard plot for curcumin was plotted using different concentrations of curcumin (2, 4, 6, 8 & 10 µg/mL). The linear equation from the standard plot was used for further calculation.

5.2.2.3 Loading efficiency

The loading efficiency (%) of curcumin loaded in C-PHEMA-NPs was determined as follows: A known amount of C-PHEMA-NPs was taken and dissolved in absolute ethanol (Analytical Reagent 99.9 % v/v) and the
drug content was analyzed using UV spectrophotometer (Shimadzu UV 2101 PC) at 426 nm after appropriate dilutions. The loading efficiency was calculated from the following Equation (5.1):

\[
\text{Loading efficiency (\%) = } \frac{\text{amount of curcumin incorporated in PHEMA nanoparticles}}{\text{amount of curcumin initially added}} \times 100
\]  

(5.1)

5.2.2.4 Stability study

Existence of curcumin and its stability in C-PHEMA-NPs was examined by UV-spectrophotometer analysis. Curcumin was accurately weighed and dissolved in absolute ethanol to obtain a final concentration of curcumin per mL ethanol at 25 µg/mL. One ml of ethanol solutionised curcumin was then added into a vial containing 9 ml of PBS, pH 7.4. C-PHEMA-NPs equivalent to 25 µg curcumin was weighed and added into the vial containing 1 % tween 80 in 10 ml of PBS, pH 7.4 and the vials were kept under stirring at 37 °C. At definite time intervals, 200 µL of samples were collected and diluted with 800 µL absolute ethanol. The absorption spectra were recorded using UV-vis spectrophotometer at a scanning range of 200-600 nm.

5.2.2.5 Scanning electron microscopy and atomic force microscopy

Morphological studies of C-PHEMA-NPs were performed with a SEM (JEOL SEM model JSM 5610 LV). Lyophilized polymeric nanoparticles were placed on a graphite surface. The sample was then coated with gold by ion sputter. The coating was performed at 20 mA for 4 minutes and then images were recorded. The shape and surface morphology of C-PHEMA-NPs further analyzed by AFM, Park Systems XE-70, South Korea) and image processing was done using XEL software. The nanoparticle suspension was diluted and a drop was placed on freshly cleaved mica. After
1 minute of incubation the surface was gently rinsed with deionized water to remove unbound nanoparticles. The sample was air dried at room temperature (25 °C) and mounted on the microscope scanner.

5.2.2.6 Fourier transform infra-red spectroscopy

Fourier Transform Infrared (FT-IR) spectra were obtained on a FT-IR spectrometer (Paragon 1000), Perkin-Elmer. The spectra were recorded in the region of 400–4000 cm\(^{-1}\) (resolution of 1 cm\(^{-1}\) ) for curcumin, PHEMA nanoparticles and C-PHEMA-NPs, after the respective samples were mixed with dried KBr powder and compressed on a disc by a hydraulic press at 5 T Compression.

5.2.2.7 X-ray diffraction

X-ray diffraction patterns of curcumin, PHEMA nanoparticles and C-PHEMA-NPs were determined using a diffractometer equipped with a rotating target X-ray tube and a wide-angle goniometer. The X-ray source was Kα radiation from a copper target with graphite monochromater. The X-ray tube was operated at a potential of 45 kV and a current of 40 mA. The range (2θ) of scans was performed from 10° to 80° at a speed of 4° per minute at increments of 0.05°.

5.2.2.8 Thermal analysis

The curcumin, PHEMA nanoparticles, and C-PHEMA-NPs were analyzed by thermo gravimetric analyzer (TGA Q50 V 20.6 Build 31, Universal V 3.9 A TA instruments). A sample of 5 mg was placed on a thin platinum pan and the measurement was conducted at a heat flow rate of 10 °C/min under nitrogen purging. The temperature range of measurement was between 30-600 °C. Differential scanning calorimetry was performed
using DSC Q200 V23.10 Build 79, Universal V 4.4A instruments. Test sample of 5 mg was hermetically sealed in a standard aluminum pan. The temperature range of measurement was 0–300 °C, under inert nitrogen atmosphere at a flow rate of 20 mL/min. The thermograms of curcumin, PHEMA nanoparticles and C-PHEMA-NPs were recorded.

5.2.2.9 In vitro release study

The in vitro release study of curcumin from C-PHEMA-NPs was performed using a dialysis bag method. C-PHEMA-NPs were suspended in 5 mL of PBS (pH 7.4) containing 1 % v/v tween-80 and the dialysis bag (molecular weight cut-off 12 kDa, Sigma) was placed in 35 mL of PBS, and kept under stirring (100rpm) at 37 °C (Sun et al 2013; Lin et al 2012). Commercially procured curcumin from sigma aldrich was used as free curcumin in the release studies. 1 mL of sample was taken out at predetermined intervals for the analysis of released curcumin, and then equal volume of fresh buffer was added to maintain sink conditions. The released curcumin was determined spectrophotometrically at 426 nm. The concentration of released drug was then calculated using standard curve of curcumin. The percentage of curcumin released was determined from the following Equation (5.2):

\[
\text{Release (\%)} = \frac{\text{Released Curcumin}}{\text{Total Curcumin}} \times 100 \quad (5.2)
\]

To investigate the mechanism of release, the data were analyzed with mathematical models such as Zero order kinetic (Equation (5.3)), First order kinetic (Equation (5.4)), Higuchi kinetic (Equation (5.5)), and Korsmeyer-peppas model (Equation (5.6)). The graphs were plotted as follows: percentage of drug release, \(Q_t\) Vs time (zero order kinetic model), log percentage drug remaining Vs time (first-order kinetic model), percentage
of drug release, $Q_t$ Vs square root of time (Higuchi model) and log cumulative percentage drug released Vs Log Time (Korsmeyer-peppas model)

$$Q_t = K_0 t$$  \hspace{1cm} (5.3)

$$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}$$  \hspace{1cm} (5.4)

$$Q_t = K_H t^{1/2}$$  \hspace{1cm} (5.5)

$$\frac{M_t}{M_\alpha} = K_P t^n$$  \hspace{1cm} (5.6)

where $Q_t$ is the percent of drug released at time $t$, $Q_0$ is the initial amount of drug present in the nanoparticles, $M_t$ is the fraction of drug released at time, $t$ and $n$ is the diffusional exponent for drug release, $K_0$, $K_1$, $K_H$ and $K_P$ are the rate constants of the Equations (5.3) to (5.6) respectively.

5.2.3 *In vitro* Cytotoxicity Study

The standard MTT assay was used to determine the *in vitro* cytotoxicity of drug curcumin, PHEMA-NPs, C-PHEMA-NPs using SKOV-3 cells. Cell lines in exponential growth phase were washed, trypsinized and resuspended in DMEM medium, pH 7.4, with 10 % FBS and 1 % antibiotic (penicillin/streptomycin mixture). SKOV-3 cells were seeded on 96 well plates and incubated at 37 °C in a humidified atmosphere of 5 % CO$_2$. For all experiments, cells were seeded to provide experimental stage 80 % confluency in 96 well plates and grown for 48 h. Upon reaching 80 % adherent confluency cells were treated with a various concentrations of drug curcumin (1, 2.5, 5, 7.5, 10, 12.5, 15, 17.25 & 20 µg/mL), PHEMA-NPs and
C-PHEMA-NPs (0.7812, 1.562, 3.125, 6.25, 10, 12.5, 25, 50, 75 & 100 µg/mL). The untreated cells and PHEMA-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 and 90 % humidity for 4 h. The absorbance was read at 570 nm. The cytotoxicity (IC₅₀) was calculated based on the color yield of treated cells which was proportional to the number of metabolically active cells and indirectly approximated cell viability. IC₅₀ is defined as the drug concentration required to inhibit growth of cells by 50 % relative to control (IC₅₀). A graph was drawn using % of cell viability with respect to the concentration of the sample.

5.2.4 DNA Fragmentation

DNA fragmentation was performed according to the method described by Moore & Matlashewski (1994). In this study SKOV-3 cells were seeded at 15x10⁵ cells per well and treated with two different concentrations of C-PHEMA-NPs. It was then incubated for 48 h and the cells were collected, washed with PBS, and lysed with a solution containing 10 mM Tris-HCl, pH 7.4; 25 mM EDTA; 0.5 % SDS, 100 mM NaCl. The lysate was incubated with 200 mg/mL proteinase K for 1 h at 55 °C. The cell lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and then centrifuged for 15 min. The supernatant was incubated with RNase A (0.2 mg/mL) at 37°C. After 1 h, the DNA was extracted with phenol and precipitated with one-tenth the volume of 3 M isopropanol and 3 volumes of absolute ethanol. The DNA samples were dissolved in 1 X TE buffer and electrophoresis was carried out on 1.5 % agarose gel at 60 V and DNA fragments were visualized in the gel documentation system.
5.2.5 **Cell Cycle Analysis**

Flow cytometry was employed to determine the apoptotic peaks of the cells (Qi et al 2005). SKOV-3 cells were seeded on 100 mm dishes at 1x10^6 cells per dish and grown in DMEM supplemented with 10 % fetal bovine serum. After being treated with various concentrations of the C-PHEMA-NPs (3.125, 6.25, 10 µg/mL) and DMSO (0.05 %) as control for 48 h, the cells were harvested and centrifuged at 3000 rpm for 5 min, washed with PBS and equal volume of ethanol added. After ethanol (70 %) fixation, the cells were washed, centrifuged and re-suspended in staining solution (50 µg/mL propidium iodide, 200 µg/mL DNase free RNase, 4 mM sodium citrate, 0.1 % triton X-100) and incubated for 10 min at 37 °C. NaCl (1.5 M) was then added to make an isotonic final suspension. The incubated sample was analyzed on a FACS calibur flow cytometer (Becton & Dickinson, San Jose, CA) and cell cycle data were re-analyzed using cell quest software.

5.2.6 **Western Blot Analysis**

Western blot analysis was carried out to study the molecular mechanisms and the concentration of the proteins involved in the inflammatory and intrinsic pathways of apoptosis in cancer cell lines. Exponentially growing (1 X10^5) SKOV-3 cells were seeded into 6 well plates for 24 h followed by treatment with C-PHEMA-NPs (equivalent to IC_{50} concentration) for 48 h. Untreated cells were kept as a control for the experiment. Cells collected by centrifugation was washed once with PBS and lysed using cell lysis buffer containing 0.05 mol/l Tris–HCl (pH 7.5), 0.15 mol/L NaCl, 0.001 mol/L PMSF, 0.001 mol/L EDTA (pH 8.0), 1 % triton X-100, 0.1 % SDS, protease and phosphatase inhibitor, followed by centrifugation at 12,000 rpm for 5 min at 4 °C. The protein assay to measure
protein content was performed by using Bio-Rad protein assay kit. The sample was transferred to a Polyvinylidene Difluoride (PVDF) membrane. The PVDF membrane was treated with PBS containing 5% non-fat dry milk at room temperature for 1 h followed by incubation with the primary antibodies such as PARP monoclonal antibody (dilution 1:2000), (1:1000 dilutions), Caspase-3, -8, -9, their cleaved forms, Bax, Bcl-2, Fas (1:10000), Fas Ligand (1:5000), anti FADD, anti TRAIL (1:5000), NFkB, Survivin, COX-2, anti human VEGF, IκBα and β-Actin. The membrane was washed three times with PBS containing 1% tween-20 and probed with secondary antibody (dilution 1:5000) and incubated at room temperature for 1 h. After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were visualized by chemiluminescence detection system (ECL PLUS, Pierce) using detection reagents (Amersham Bioscience).

5.3 RESULTS AND DISCUSSION

5.3.1 Preparation of C-PHEMA-NPs

Polymerization of HEMA is fully dependent on the weight ratio of monomers and initiators. PHEMA gel was synthesized at Room Temperature (RT), without the presence of heat or addition of any initiators, using CF and HEMA for 24 h. The study showed immiscible curcumin was completely solubilized in CF IL (The stepwise preparation is shown in Figure 5.1). The method of gel preparation in the present study is quite novel in the sense that it is free from unwanted toxic residual solvents and initiators. The way of preparation ensured more bio-compatibility for biomedical applications. The basic characterization of the PHEMA synthesized through this method is already reported in chapter 3.
5.3.2 Physicochemical Characterization of PHEMA-NPs and C-PHEMA-NPs

C-PHEMA-NPs were prepared by adopting the nanoprecipitation method. Curcumin loaded PHEMA gel, used in our preparations, was soluble in binary solvent solutions acetone and ethanol in the ratio of 4:1 respectively; here we used water as a nonsolvent phase for the polymer. It was interesting to note that by changing the weight of polymer or nonsolvent: solvent volume ratio, the nanoparticles of different sizes were obtained. In this study satisfactory curcumin loading (as high as 26.4±0.3 %) was achieved.

The UV absorbance spectra of curcumin (Figure 5.2[a]) is at 426 nm, and standard plot (Figure 5.2[b]) was developed using different known concentrations of curcumin and a slope value 0.146 was used to find out the loading efficiency.
The UV absorbance spectra showed peak at 426 nm for both free curcumin (Figure 5.3) and curcumin in nanoparticles (Figure 5.4). In the case of free curcumin, fast degradation was observed (Figure 5.5), however, it was found that curcumin in C-PHEMA-NPs was stable. Further, to clarify, the % curcumin vs time was plotted. It confirmed the stability of C-PHEMA-NPs as compared to free curcumin in phosphate buffer solution.
Figure 5.3  UV-Vis spectra (200-600 nm) of free curcumin at different time interval

Figure 5.4  UV-vis spectra (200-600 nm) of C-PHEMA-NPs at different time interval
Figure 5.5 Stability of free curcumin and C-PHEMA-NPs at 426 nm

was much less in present studies compared to 45-95 % reported in different carrier systems. Most of the reported systems were hydrophobic in nature which required surface modification using hydrophilic emulsifiers to ensure biocompatibility in aqueous body environment, because monocytes adhere to the surface of the hydrophobic carrier molecules (Yamit et al 2009) and prevent entry into cells.

Contrary to the reported systems, the polymer matrix PHEMA employed in the present study had a distinct advantage of high biocompatibility (Chouhan & Bajpai 2009, Ma et al 2012, Hsiue et al 2001, Wu & Brazel 2008) and did not require surface modification. Although maximum drug loading efficiency was considered as a criterion, there were other parameters more vital, such as the In vitro sustained release, size of the nanoparticles, zeta potential and In vitro Cytotoxicity studies. In the following paragraphs, the characteristics of the nanoparticles are discussed elaborately to prove that the carrier molecule PHEMA is more suitable for drug delivery system.

The particle size distribution is given in Figure 5.6[a]. It shows that nanoparticles produced were of submicron size and indicated a relatively narrow particle size distribution (PDI < 0.153). It was also observed that the size of particles was found to be in the range of 220-342 nm with the mean diameter of 300 nm. The dynamic laser scattering (DLS) studies confirmed a zeta potential value of -33.4 mV (Figure 5.6[b]) and this indicated that nanoparticles would repel each other leading to long term stability and avoid particle aggregation (Manju & Sreenivasan 2011).
Figure 5.6  [a] Particle Size Distribution of C-PHEMA-NPs, [b] Zeta potential of C-PHEMA-NPs

The FT-IR spectroscopy of curcumin, PHEMA-NPs and C-PHEMA-NPs was carried out to investigate the incorporation of curcumin in these polymeric nanoparticles. The spectra are given in Figure 5.7[a]. A major peak in all the three spectra at around 3400 cm\(^{-1}\) was observed and this was due to \(-\text{OH}\) vibrations of intermolecularly bonded OH groups. The strong peak observed at 1720 cm\(^{-1}\) was due to C=O absorption. The C=C functional group appeared at 1600 cm\(^{-1}\) in C-PHEMA-NPs indicating the loading of
curcumin and further suggested that curcumin was present in a dispersed state of PHEMA-NPs (Mohanty & Sahoo 2010). The XRD patterns of curcumin, PHEMA nanoparticles, and C-PHEMA-NPs are given in Figure 5.7[b]. In XRD data of curcumin, a number of peaks were observed (in the 2 Θ range of 10-30°), implying its crystalline nature and the XRD patterns were in good agreement with the results of Donsi et al (2010), while in the C-PHEMA-NPs, there were no such sharp crystalline peaks. This confirmed the formation of amorphous complex or disordered-crystalline phase of curcumin in the PHEMA-NPs (Shaikh et al 2009).
Figure 5.7  [a] FTIR study of i) PHEMA-NPs, ii) Curcumin, and iii) C-PHEMA-NPs; [b] Powder X-ray diffraction pattern of i) Curcumin, ii) PHEMA-NPs, and iii) C-PHEMA-NPs
Thermal characterization of PHEMA nanoparticles, drug curcumin and C-PHEMA-NPs was carried out by employing DSC and TGA techniques. The results are in Figure 5.8[a] and 5.8[b].

Figure 5.8 [a] DSC thermogram of (i) Curcumin, (ii) PHEMA-NPs, and (iii) C-PHEMA-NPs, [b] TGA thermogram of (i) Curcumin, (ii) PHEMA-NPs, and (iii) C-PHEMA-NPs
DSC of curcumin showed a single well-defined peak at 180 °C corresponding to the melting of the curcumin. In contrast, PHEMA-NPs showed a melting transition at about 71 °C and is fairly stable in the temperature range studied. The DSC of C-PHEMA-NPs showed a shift in the endothermic transition at 84 °C indicating the presence of curcumin in PHEMA. Drug curcumin, PHEMA-NPs and C-PHEMA-NPs were examined by TGA. Drug curcumin was fairly stable up to 370 °C and in the case of PHEMA-NPs the thermal stability was around 262 °C whereas in the case of C-PHEMA-NPs, the thermal stability increased (335 °C). This indicated that the introduction of curcumin into the core of PHEMA-NPs led to increase of the thermal stability of the sample.

The surface morphology of C-PHEMA-NPs was examined by both SEM and AFM. The respective images are given in the Figure 5.9 and 5.10. The results indicated that the surface of the drug loaded PHEMA-NPs were nearly spherical in shape with a smooth surface, as reported (Lin et al 2012). The nanoparticles were found to be in the range of 300 nm, comparatively close to the results obtained by dynamic laser scattering. AFM studies also confirmed that the C-PHEMA-NPs were spherical in shape and showed that the prepared nanoparticles had no aggregation.
Figure 5.9 SEM images of C-PHEMA-NPs
Figure 5.10 [i] AFM images of C-PHEMA-NPs, [ii] three dimensional view of AFM images
Thus C-PHEMA-NPs of size 300 nm could be synthesized without the need for a peroxide based initiator and hydrophilic emulsifier. For the first time, a hydrophilic carrier matrix (PHEMA from gelled ionic liquid) is used to load a hydrophobic drug (curcumin) successfully, although the loading yields are less; yet there is a distinct advantage of this carrier matrix in that it is more biocompatible compared to the studied systems.

5.3.3  

\textit{In vitro} Release Studies and its Kinetics

The \textit{in vitro} release pattern of free curcumin and that of C-PHEMA-NPs is shown in Figure 5.1. The insert in Figure 5.1 shows the release of free curcumin studied over a period of 12 h. A comparison of \textit{in vitro} release pattern shown in Figure 5.1 reveals that the free curcumin release is faster as compared to curcumin into nanoparticles. A slow release of \textasciitilde{}10 \% was observed in the first 12 h from C-PHEMA-NPs while a sustained release pattern of entrapped curcumin from C-PHEMA-NPs could be observed later. The slow release observed as compared to free curcumin is perhaps due to the slow diffusion of curcumin through the polymer matrix. The sustained release observed after 12 h over the period of 10 days, was due to diffusion followed by degradation of the polymer matrix (Sun et al 2013; Ma et al 2012). The tween usage in the release studies is reported to enhance the release ability and complete solubility of released curcumin (Ratanajaijaroen et al 2012).

To determine the release model that best described the drug release, the \textit{in vitro} release data were substituted in equations of zero order, first order and Higuchi model and the results noted. Among them the zero order model showed a high $R^2$ value 0.96432, indicating that the release of the drug followed zero order release kinetics. To understand the mechanism of drug
release, Korsmeyer-Peppas equation was applied and it showed a good linearity ($R^2=0.97542$). The release kinetics results are showed in Figure 5.12. The release exponent ‘n’ was found to be 0.49318. 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).

![Graph of Drug Release](image)

**Figure 5.11** *In vitro* drug release of C-PHEMA-NPs and free curcumin (Inset) in PBS at pH 7.4 (mean ± SD, n=3)
Figure 5.12 Release Kinetics profiles obtained for C-PHEMA-NPs following [a] Zero order model, [b] First order model, [c] Higuchi model, and [d] Korsmeyer-peppas

5.3.4 In vitro Cytotoxicity Study

SKOV-3 cells were used to study the cell viability of curcumin and C-PHEMA-NPs by a standard MTT assay (Tang et al 2010). The assay was terminated at 48 h and colorimetric determination of cell viability was performed using ELISA Reader (Qualigens, Bangalore, India). The results of MTT assay for free and encapsulated curcumin on SKOV-3 cell line are shown in the Figure 5.13 [a] and 5.13 [b]. The IC$_{50}$ value for C-PHEMA-NPs was found to be around 3.78 µg/mL while that for free curcumin ranged from 17.25 µg/mL on the ovarian cancer cells. These results indicated that the C-PHEMA-NPs produced here showed better cytotoxic activity than what free curcumin would. Our results confirmed that C-PHEMA-NPs showed a lower IC$_{50}$ value than of free curcumin in SKOV-3 cell line studies. This
could be due to the differences in uptake profile leading to better activity of C-PHEMA-NPs as suggested by Mohanty & Sahoo (2010).

Figure 5.13  [a] *In vitro* cytotoxicity studies of drug curcumin and C-PHEMA-NPs in SKOV-3 cell line. All assays were performed in triplicate and the mean±standard deviations are shown, [b] Images of SKOV-3 cell line visualized under inverted microscope.
5.3.5 DNA Fragmentation and Cell Cycle Analysis

DNA was extracted from cultured SKOV-3 cells treated with two different concentrations of C-PHEMA-NPs and the presence of necrosis was detected by agarose gel electrophoresis. During apoptosis, fragmented DNA produces a series of bands which are described as “DNA ladders” (Qi et al. 2005). C-PHEMA-NPs treated SKOV-3 cells yielded a continuous spectrum of DNA fragments with low molecular mass (Figure 5.14), indicating that necrosis was induced.

![DNA Fragmentation](image)

Figure 5.14 DNA fragmentation studies by agarose gel electrophoresis.
Curcumin is a fluorescent compound which can be analyzed using flow cytometry. The effect of different concentrations of C-PHEMA-NPs (3.125, 6.25 & 10 µg/mL) on cell cycle and apoptosis in SKOV-3 cells was determined by FACS analysis. The Flow cytometry results are in Figure 5.15. Cells in G₀/G₁ phase were significantly reduced after treatment with C-PHEMA-NPs. Similarly C-PHEMA-NPs induced apoptosis in SKOV-3 cells in dose dependent manner (Duan et al 2010, Qi et al 2005).

Figure 5.15  The effect of various concentrations of C-PHEMA-NPs on the cell cycle analysis in SKOV-3 cell line.
5.3.6 Western Blot Analysis

Anticancer chemotherapy drugs eradicate cancer cells by triggering different apoptosis pathways (Fulda & Debatin 2006). C-PHEMA-NPs exhibited potent cytotoxic activity through both inflammatory and intrinsic pathways in SKOV-3 cells.

Inflammatory pathways of western blot results showed that considerable change occurred in protein concentration directly responsible to apoptotic or programmed cell death. NFkB is a dimeric DNA binding protein and acts as a major transcription factor. The results as seen in Figure 5.16[a], showed that C-PHEMA-NPs were able to inhibit the function of NFkB and expression was dramatically diminished and IKBa showed a high intense band as compared to native curcumin treated cell. Curcumin had the ability to inhibit the activated NFkB to restrict the tumor cell lines (Mukerjee & Vishwanantha 2009) and it was reported that the presence of more IKBa in cells facilitated more inhibition of NFkB pathway (Mohanty & Sahoo 2010). VEGF is a specific stimulator of endothelial cell proliferation in many human cancers (Duan et al 2010), COX-2 is known to play a crucial role in the progress of the disease (Carlson et al 2003) and Survivin acts as an important apoptosis regulatory protein (Watson et al 2010). C-PHEMA-NPs induced apoptosis in cancer cells and it was evident from reduced expression of antiapoptotic survivin and decreased expression of VEGF and COX-2 in SKOV-3 cells. In this study, β-actin was used as a loading control and it showed similar expression in all lanes.
Intrinsic pathways of western blot studies are shown in Figure 5.16[b]. The Bax protein is a pro-apoptotic member and bcl-2 is an anti-apoptotic member in Bcl-2 protein family. The formation of heterodimers among these pro-apoptotic and anti-apoptotic proteins of the Bcl-2 protein family may turn the apoptosis process on and off. Remarkably, the effects of apoptosis inductions are more dependent on the ratio between bcl-2 and bax than on the quantity of bcl-2 alone. In the present investigation C-PHEMA-NPs induced down-regulation of Bcl-2 proteins and up-regulation of Bax proteins in SKOV-3 cells. Caspases, a family of cysteine acid proteases, can be regarded as the key factors in apoptosis (Wang et al 2010; Fan et al 2005). Figure 5.16[b] revealed that C-PHEMA-NPs treated cells induced cleavage of caspase 9, caspase 8 and caspase 3. Similarly the cleaved form of PARP is used as a diagnostic tool for the detection of apoptosis (Dhule et al 2012); Figure 5.16[b] also showed that 116 kDa PARP proteins cleaved into an 85 kDa fragment after treatment with C-PHEMA-NPs. Fas is a membrane protein and Fas L is a natural ligand and both belong to Tumor Necrosis Factor (TNF) family.

TNF-Related Apoptosis-Induced Ligand (TRAIL) is a member of death receptor family. FADD acts as an adaptor protein used to connect both Fas receptor and caspase family and this complex helps to activate other caspases. The Fas system provides an important mechanism for killing of tumor cells (Micheau et al 1999; Jiang et al 2000). C-PHEMA-NPs contributed to obvious decrease in Fas Ligand (37 KDa) protein expression and notable increase in TRAIL (32 KDa), Fas (43 KDa) and FADD (26 KDa) proteins.
Figure 5.16 [a] and [b] Western blot illustrating the different protein expression in apoptosis (extrinsic and intrinsic pathways) after treatment with curcumin and C-PHEMA-NPs. Lane 1: Control, Lane 2: Curcumin, Lane 3: C-PHEMA-NPs
5.4 CONCLUSIONS

- Following a novel method of synthesis PHEMA gels using choline based ionic liquid such as choline formate. C-PHEMA-NPs were prepared by nanoprecipitation method.

- The size and zeta potential of prepared C-PHEMA-NPs were about 300 nm and -33.4 mV respectively.

- Maximum of 26.4% of curcumin loading could be achieved.

- The nanoparticles obtained were spherical in shape with uniform size distribution. FT-IR, TGA, DSC, XRD studies indicated the incorporation of curcumin in PHEMA-NPs.

- *In vitro* release studies confirmed the sustained drug release characteristics of C-PHEMA-NPs. *In vitro* cytotoxicity (MTT assay) study showed that C-PHEMA-NPs effectively inhibited the proliferation of cancer cells.

- DNA fragmentation study revealed that necrosis in cells was induced by C-PHEMA-NPs. Western blotting and flow cytometry techniques indicated that the C-PHEMA-NPs induced higher percentage of cell death in SKOV-3 cells.

- These results showed that C-PHEMA-NPs could be more promising carrier material for drug delivery in anti-tumor therapy.