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Purely aqueous PLGA nanoparticulate formulations of curcumin exhibit enhanced anticancer activity with dependence on the combination of the carrier

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ABSTRACT

Curcumin, a yellow pigment present in turmeric, possess potential anti-proliferative and anti-inflammatory activities but poor aqueous solubility limits its applications. In this study we report a novel comparative study of the formulation and characterization of curcumin nanoparticles (nanocurcumin) using two poly (lactide-co-glycolide) (PLGA) combinations, 50:50 and 75:25 having different lactide to glycolide ratios. Nanocurcumin 50:50 showed smaller size with higher encapsulation efficiency. Thermal evaluation suggested the presence of curcumin in molecular dispersion form which supported its sustained release up to a week where nanocurcumin 50:50 showed faster release. Cellular uptake studies in human epithelial cervical cancer cells (HeLa) exhibited enhanced intracellular fluorescence with nanocurcumin when compared to free curcumin, when both given in purely aqueous media. Antiproliferative studies using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Annexin V/propidium iodide staining, poly (ADP-ribose) polymerase (PARP) cleavage and downregulation of clonogenic potential of HeLa cells proved the better antitumor activity of nanocurcumin 50:50 administered in aqueous media. Superior efficacy of nanocurcumin 50:50 in comparison to free curcumin was further demonstrated by electrophoretic mobility shift assay and immunocytochemical analysis. In conclusion, the enhanced aqueous solubility and higher anticancer efficacy of nanocurcumin administered in aqueous media clearly demonstrates its potential against cancer chemotherapy, with dependence on the combination of PLGA.

1. Introduction

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a naturally occurring o-methoxyphenol derivative extracted from the rhizome of turmeric (*Curcuma longa*), having a wide range of biological activities (Aggarwal and Sung, 2009). It is capable of suppressing a number of signaling pathways, inhibits proliferation, angiogenesis and metastasis along with antioxidant (free radical scavenging activity) properties (Goel et al., 2008; Shishodia et al., 2005; Strimpakos and Sharma, 2008). The anti-proliferative, anti-angiogenic, anti-survival and anti-invasive properties of curcumin are a result of the downregulation of various transcription factors like NF-κB (Bharti et al., 2003) and beta-catenin (Jaiswal et al., 2002). From *in vivo* studies it has been proven that it is capable of inhibiting the growth of implanted human tumors and carcinogen-induced tumors (Anand et al., 2008). Moreover, studies in numerous carcinogenesis models (Chuang et al., 2000; Inano et al., 1999; Kawamori et al., 1999; Li et al., 2002; Singh et al., 1998) have confirmed the potential of curcumin as a tool for chemoprevention. However, lack of aqueous solubility, poor tissue absorption, rapid metabolism and quick systemic removal, limits the bioavailability of curcumin making it incompetent for *in vivo* trials. Hence, the development of a delivery system which can facilitate the administration of curcumin in an aqueous phase medium will significantly improve the clinical efficacy of curcumin.

Various studies have shown that encapsulation of curcumin in phospholipids (Sou et al., 2008), cyclodextrins (Salmaso et al., 2007) and liposomes (Li et al., 2005; Takahashi et al., 2009) based microspheres or nanospheres can overcome its solubility problems. Polymeric nanocarriers based formulations provide important and efficient way to enhance therapeutic index of curcumin (Letchford et al., 2008; Ma et al., 2007, 2008; Peer et al., 2007). The advantages of such formulations are their low toxicity, high stability providing longer circulation and smaller size which attribute them increased cellular permeability for passive targeting of solid tumor tissue site with enhanced permeation and retention (EPR) effect (Maeda et al., 2000). Such nanoformulations of anticancer drugs like paclitaxel (Mu and Feng, 2003) and doxorubicin (Vasey et al., 1999) are already under clinical trials. Following the recent advances,

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curcumin based nanoparticle approach has also come up with fresh advances (Sahu et al., 2008).

Therefore, to overcome the solubility problems associated with curcumin and to improve its chemoprevention activity, the present work focus on the formulation of Poly (lactide-co-glycolide) (PLGA) based nanoparticle delivery system for curcumin (hereafter also referred to as nanocurcumin). Also, we report a novel comparative study on the effect of lactide to glycolide ratios of carrier on the biological activity of nanocurcumin. Nanocurcumin with monomeric ratios 50:50 and 75:25 were prepared using solvent evaporation method, characterized and their biological activity was evaluated in human epithelial cervical cancer (HeLa) cells using confocal microscopy, MTT assay, Annexin/PI staining, clonogenic assay and PARP cleavage. The better efficacy of nanocurcumin 50:50 was further confirmed using electrophoretic mobility shift assay (EMSA) and immunocytochemical analysis.

2. Materials and methods

2.1. Materials

Poly (D,L-lactic-co-glycolic acid) (PLGA) with co-polymerization ratios 50:50 and 75:25 (lactic/glycolic), curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagel Medium (DMEM) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Steinheim, Germany). Polyvinyl alcohol (PVA) Mw ~ 1,25,000 was procured from SD fine (Mumbai, India). Mouse monoclonal antibodies against p65, rabbit polyclonal against PARP and the Annexin V apoptosis detection kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rhodamine-conjugated anti-mouse IgG and propidium iodide were provided by Calbiochem (San Diego, CA, USA). Immobilon western reagent was purchased from Millipore (Billerica, MA). All the solvents were of analytical grade.

2.2. Preparation of curcumin loaded PLGA nanoparticles (nanocurcumin)

Curcumin loaded PLGA nanoparticles were prepared by a single emulsion (o/w) method commonly known as solvent evaporation technique. Briefly, 100 mg of PLGA and 10 mg of curcumin were dissolved in an organic mixture of dichloromethane and acetone (w/v, 10:1) to obtain the organic phase. This organic solution was then pre-emulsified with 1% (w/v) PVA aqueous solution. The emulsion was then sonicated and subjected to gentle agitation at room temperature for 12 h to remove the organic phase. The samples were washed 4–5 times with distilled water by centrifugation followed by overnight freeze drying to obtain the dry powder. Nanoparticles with both the polymeric combinations PLGA 50:50 and 75:25 were prepared using the same method. Blank polymer nanoparticles with both the combinations were prepared by the same method exclusive of curcumin.

2.3. Determination of curcumin content in nanoparticles

Weighed amount of dried curcumin loaded nanoparticles were dissolved in dichloromethane and vigorously vortexed to get a clear solution. The total amount of curcumin in nanoparticles was calculated using a standard obtained by measuring the UV absorbance (Perkin Elmer, USA) of curcumin at different concentrations at a detection wavelength of 430 nm. The drug encapsulation efficiency was expressed as the ratio of curcumin in nanoparticles to the initial amount of curcumin used in formulation. The yield corresponds to the ratio of amount of nanoparticles recovered to the total amount of polymer and curcumin used in formulation.

2.4. Characterization of nanocurcumin

Morphological analysis of nanocurcumin was performed using a transmission electron microscope (TEM, JEOL 1011, Japan). For TEM, the samples of nanoparticle suspension in water milli-Q at 25 °C were dropped on to formvar-coated grids and measurements were taken only after the samples were completely dried. The size distribution of these nanoparticles was analyzed using a particle size analyzer (Beckman Coulter Delsa Nano Particle Analyzer).

Differential scanning calorimetry (DSC) analysis was done to study the state of curcumin inside the polymer matrix. DSC thermograms were obtained using an automatic thermal analyzer system (Pyres 6 DSC, Perkin-Elmer, USA). Samples were crimped in standard aluminum pans and heated from 20 to 250 °C at a heating rate of 10 °C/min under constant purging of N_2 at 10 ml/min. An empty pan, sealed in the same way as the sample, was used as a reference.

2.5. Drug release studies

One of the main aims of this study was to analyze the effect of lactide/glycolide ratio on curcumin release from nanoparticles. Lyophilized curcumin loaded nanoparticles (1 mg/ml) were dispersed in phosphate buffer solution (PBS) of physiological pH (7.4) and incubated at 37 °C under gentle agitation. At specific time intervals, a known volume was withdrawn from the medium and replaced with equal amount of fresh media to maintain sink conditions. The supernatant taken for analysis was extracted with 10% ethanol and then analyzed for the amount of released curcumin using UV at 430 nm. Using a standard curve for curcumin absorption and concentration, the percentage of curcumin released was calculated.

2.6. Cell uptake studies

To visualize the cellular uptake of nanocurcumin 1×10^4 HeLa cells were grown on cover slips placed in 24 well plates. Cells were treated with 25 μM curcumin dissolved in dimethylsulfoxide (DMSO), nanocurcumin/free curcumin suspended in aqueous medium and polymer blanks. After 2 h of incubation the cells were washed and their nuclei were stained with propidium iodide. Cells were examined for intracellular fluorescence of curcumin using a confocal laser scanning microscope.

2.7. MTT assay

Cytotoxicity of curcumin was determined by MTT assay as described earlier (Anto et al., 2003). Briefly, cells (3×10^3/well) seeded in 96-well plates were treated with various concentrations of curcumin (5–50 μM) where free curcumin was dissolved in DMSO while nanocurcumin in purely aqueous media. After required time intervals MTT reagent in DMEM was added and incubated for 2 h. Cells were lysed using MTT lysis buffer and incubated for another 1 h at 37 °C and the optical densities were measured at 570 nm using a plate reader (Bio-Rad, CA, USA). The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated samples) × 100.

2.8. Annexin V–propidium iodide staining

To detect membrane flip flop, an early event in apoptosis, Annexin V–propidium iodide staining was done using Annexin V apoptosis detection kit as per manufacturer's protocol. Briefly, cells (0.6×10^6) seeded in 60 mm culture dishes were treated with 25 μM of curcumin formulations for 24 h, harvested and stained with FITC-labeled annexin. The greenish apoptotic cells viewed using a fluorescent microscope were photographed and counted.

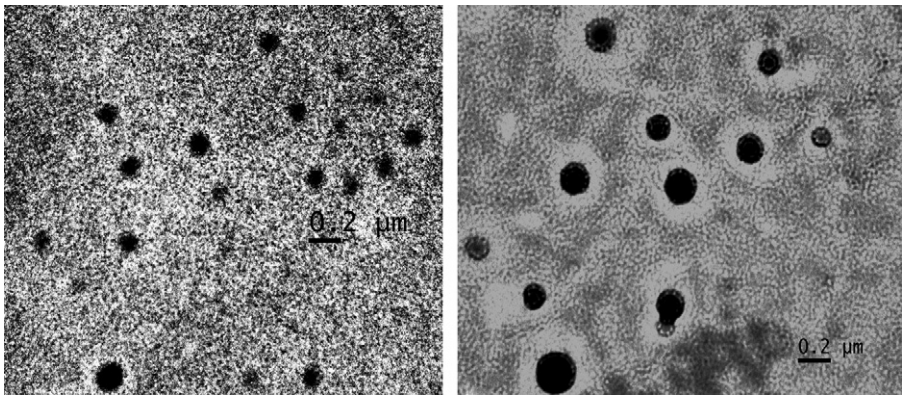

Fig. 1. Transmission electron microscopy mages of PLGA nanocurcumin (A) 50:50 (B) 75:25. The samples of nanocurcumin suspension in aqueous media were dropped on to formvar-coated grids without being negatively stained. These were allowed to dry completely at room temperature and measurements were taken only after the samples were completely dried. Nanocurcumin 50:50 showed smaller size with a mean diameter of 129 nm in comparison to nanocurcumin 75:25 which showed a mean diameter of 191 nm.

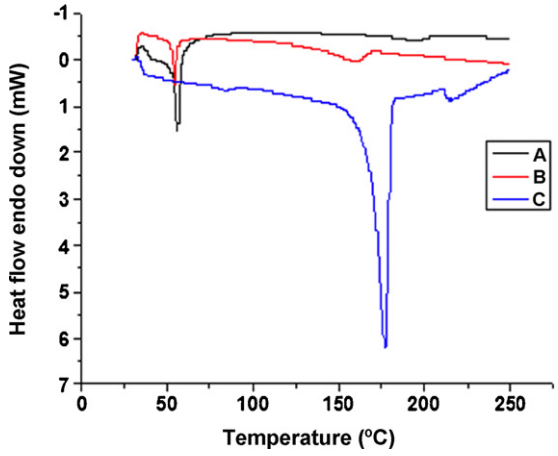

Fig. 2. Differential scanning calorimetry thermograms of: (A) blank PLGA nanoparticles; (B) curcumin entrapped PLGA nanoparticles; and (C) free curcumin. nanoparticles with and without curcumin showed the characteristic peak of PLGA only. The absence of curcumin peak in nanocurcumin thermogram suggests the presence of curcumin in molecular dispersion form inside the polymer matrix.

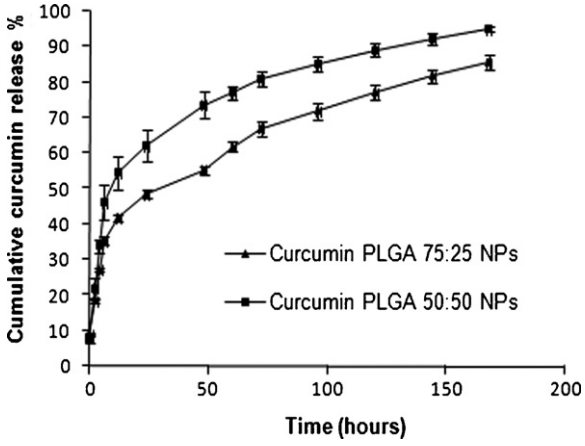

Fig. 3. *In vitro* release of curcumin from nanoparticles with two different combinations of PLGA. Release pattern of curcumin from PLGA 50:50 and 75:25 nanoparticles in phosphate buffer at pH 7.4 and 37°C. All the measurements were done in triplicate and expressed as arithmetic mean ± standard error on the mean (S.E.M.). Nanocurcumin 50:50 showed faster release in comparison to nanocurcumin 75:25.

For quantification, the average number of green fluorescent cells in five different fields were counted and compared with that of the untreated control.

2.9. Western blot analysis

Western blot analysis was done to detect the cleavage of DNA repairing protein, PARP, as described earlier (Anto et al., 2003). Briefly, 0.6×10^6 cells were plated in 60-mm plates and treated with different curcumin formulations (25 μM) for 24 h. The total isolated protein was resolved in an SDS-PAGE, immunoblotted using anti-PARP antibody and detected by enhanced chemiluminescence method.

2.10. Clonogenic assay

The cells were seeded in six well plates and treated with curcumin for 72 h after which, the clonogenic assay was done as mentioned previously (Puliyappadamba et al., 2010). Briefly, 1×10^4 cells in six well plates treated with curcumin (25 μM) for 72 h were replaced with fresh medium and incubated for 1 week. The clones developed were fixed in glutraldehyde and stained using crystal violet. The clones were counted and compared with that of untreated control such that the colony containing more than four cells was counted as one clone.

2.11. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from curcumin treated cells were prepared and electrophoretic mobility shift assay (EMSA) was performed to evaluate DNA binding activity of NF-κB essentially, as described elsewhere (Banerjee et al., 2002). Briefly, the binding reaction

Table 1
Characterization of curcumin entrapped PLGA nanoparticles.

Sample	EE% (encapsulation efficiency)	Yield%	Mean diameter (nm)	PDI (polydispersity index)
PLGA 50:50 nanocurcumin	90.03 ± 1.35	76.2 ± 2.32	129.7 ± 9.6	0.194 ± 0.09
PLGA 75:25 nanocurcumin	74.73 ± 2.71	68.8 ± 3.52	191.1 ± 9.8	0.172 ± 0.02

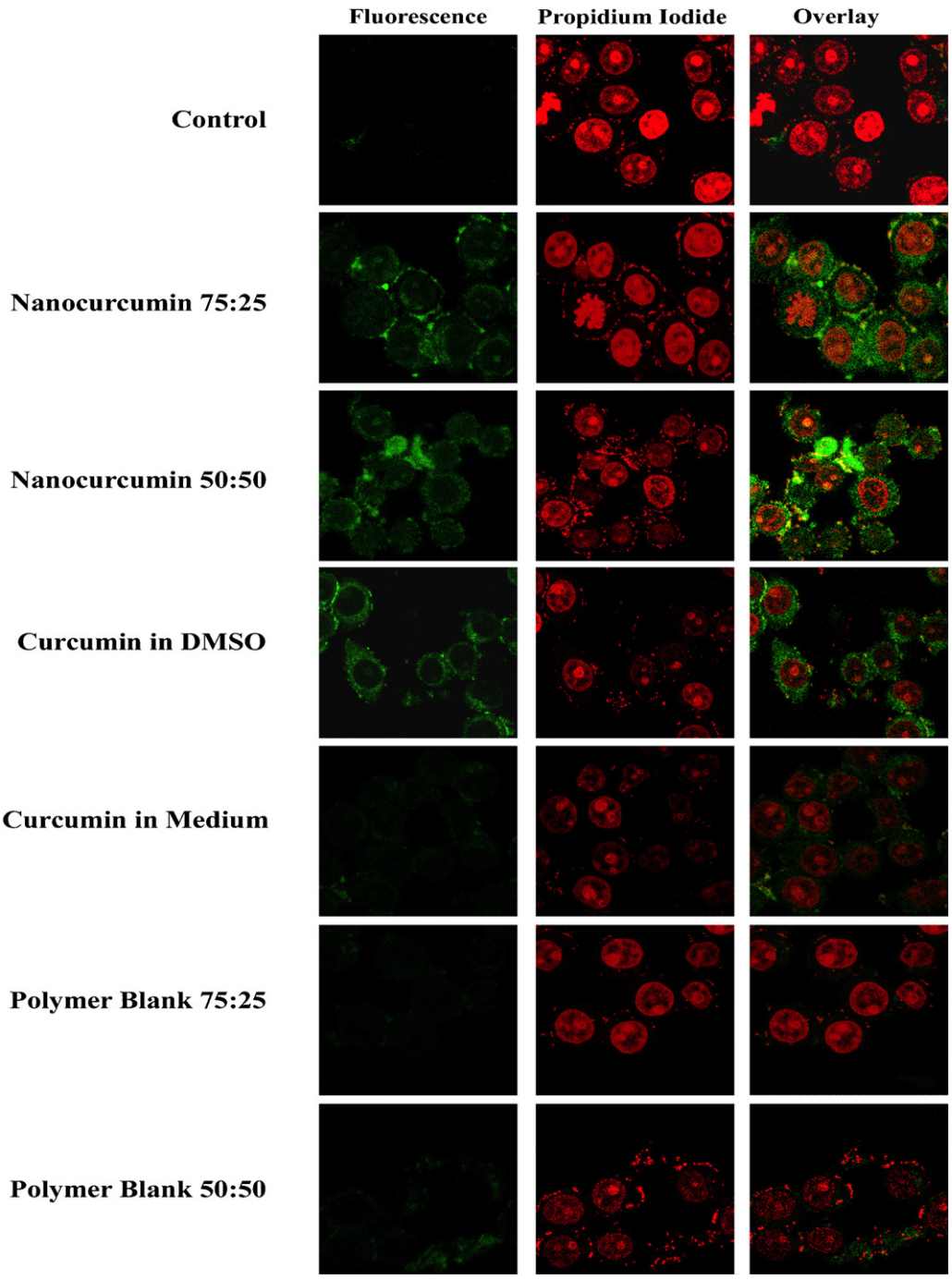

Fig. 4. Cellular uptake of curcumin and nanocurcumin. HeLa cells were treated with equivalent concentrations (25 μM) of curcumin formulations for 2 h, as indicated. Free curcumin was administered in DMSO and medium, whereas nanocurcumin 75:25 and 50:50 was administered in purely aqueous media. Curcumin uptake was shown by confocal images taken at an excitation of 420 nm and magnification 60×.

mixture containing 10 μg of nuclear proteins and 0.5 fmol of double-stranded ^{32}P-labeled NF-κB was incubated at 37 °C for 30 min. The resulting DNA–protein complexes were resolved in 6.6% non-denaturing polyacrylamide gel. The image was captured by Phosphor Imager (Bio-Rad, CA, USA) and analyzed using Personal Molecular Imager FX (Bio-Rad. CA, USA).

2.12. Immunocytochemical analysis for NF-κB p65 localization

The effect of curcumin/nanocurcumin on Phorbol Myristate Acetate (PMA)-induced nuclear translocation of p65 was studied using immunocytochemical analysis as described earlier (Bava et al., 2010). Briefly, the cells were grown on glass cover slips and exposed to curcumin/nanocurcumin and polymer blank. The cells were then washed, fixed, permeabilized and immunostained with PMA for 1 h. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, Steinheim, Germany). The cover slips with cells were mounted in DPX, examined and photographed under a fluorescence microscope.

2.13. Data normalization and analysis

Each individual experiment was carried out in triplicates and in duplicates for apoptosis. The normalization of the data was done by

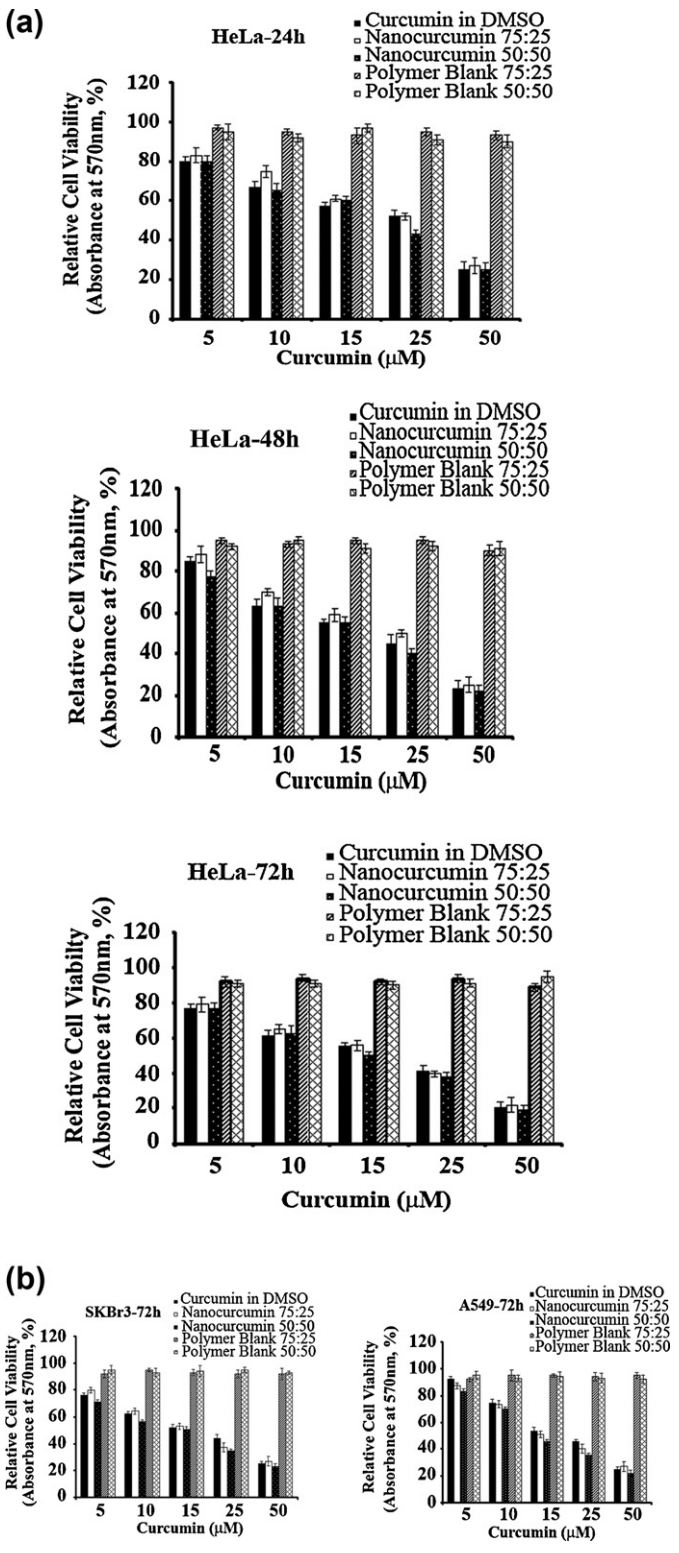

Fig. 5. Nanocurcumin is as effective or slightly more effective in inducing cytotoxicity. Comparison of cell viabilities on treatment with equivalent concentrations of free curcumin in DMSO and nanocurcumin in aqueous medium was conducted by MTT assay in (A) HeLa cells (B) SKBr3 and A549 cells at various concentrations and time intervals as indicated. All the measurements were done in six replicates and results are expressed as arithmetic mean ± standard error on the mean (S.E.M.).

considering the mean value of the untreated samples as 100%. All other data points were expressed as the percentage of the control. The error bars represent ± SD of the experiments. The statistical analysis was carried out using Student's *t*-test.

3. Results

3.1. Characterization of nanocurcumin

Curcumin loaded PLGA nanoparticles with both the polymeric combinations were successfully prepared using solvent evaporation method. The nanoparticles obtained were easily re-dispersible in water and were in the size range of 100–200 nm (Fig. 1). It was seen that >nanocurcumin 50:50 was smaller in size when compared to PLGA 75:25 while other parameters did not show much variation. The variation in size, encapsulation efficiency, polydispersity and yield of the nanoparticles with lactide to glycolide ratio is summarized in Table 1.

To analyze the thermal behavior of nanoparticles, DSC was done on free curcumin, blank PLGA nanoparticles and curcumin entrapped PLGA nanoparticles. As shown in Fig. 2, pure curcumin showed the presence of an endothermic peak at 180 °C whereas vacant as well as drug entrapped nanoparticles showed a peak at 50 °C, which is a characteristic of the polymer (glass transition temperature (T_g) of PLGA). These thermograms suggest the presence of nanocurcumin in molecular dispersion form inside the polymer matrix.

One of the main aims of this study was to generate a nanoparticle based controlled delivery system for curcumin and to compare its release with the variation in carrier combination. The release kinetics of curcumin loaded PLGA nanoparticles showed an initial burst release followed by a sustained release which continued for more than a week (Fig. 3). On comparing the release pattern of curcumin from PLGA 50:50 and 75:25 nanoparticles, it was observed that within 24 h, PLGA 50:50 nanoparticles showed a release of 62% curcumin when compared to 48% of curcumin from PLGA 75:25 nanoparticles. On continuing the study for a week, it was seen that in case of 75:25 nanoparticles, 85% of curcumin was released when compared to 94% curcumin from 50:50 nanoparticles.

3.2. Nanocurcumin exhibit better cellular uptake in aqueous medium

The two different nanoformulations in aqueous media were compared to curcumin in DMSO for cellular uptake. A strong fluorescence in cells treated with nanocurcumin suspended in medium and curcumin dissolved in DMSO was observed indicating that the cellular uptake of nanocurcumin is equivalent to that of curcumin dissolved in DMSO (Fig. 4). Interestingly, the cellular uptake of nanocurcumin was independent of the PLGA combination used. Also, a faint fluorescence was observed in the wells treated with free curcumin suspended in water.

3.3. Nanocurcumin suspended in aqueous medium induces equivalent or better cytotoxicity in cancer cells compared to free curcumin dissolved in DMSO

Evaluation of cytotoxicity induced by nanocurcumin suspended in aqueous medium and curcumin dissolved in DMSO showed the comparable anticancer efficiency of nanocurcumin. Nanocurcumin 50:50 showed consistently better cytotoxicity when compared to other two treatments at all the three time durations. Moreover, blank polymer did not show any cell death (Fig. 5A). The same experiment conducted in A549 (breast cancer) and SKBr3 (lung cancer) cells for 72 h showed a similar trend of results (Fig. 5B).

3.4. Nanocurcumin 50:50 induces significantly better apoptosis

We also compared the apoptosis inducing efficacy of curcumin in DMSO to that of nanocurcumin in HeLa cells by Annexin V/PI staining (Fig. 6). Supporting the data obtained in MTT assay, there

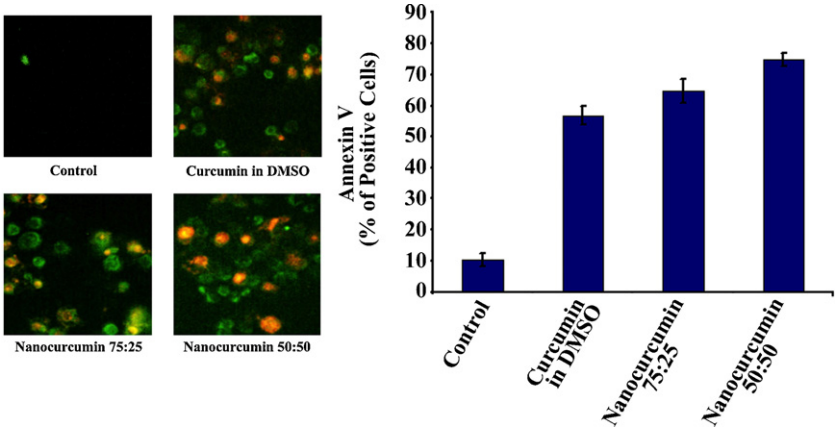

Fig. 6. Curcumin and nanocurcumin induces membrane flip–flop in HeLa cells. HeLa cells were treated with equivalent curcumin formulations (25 μM) for 16 h where free curcumin was administered in DMSO and nanocurcumin in aqueous media and stained for Annexin-V/PI positivity as per manufacturer's protocol. The greenish apoptotic cells were viewed using a fluorescent microscope and photographed. Annexin V positive cells in five various fields were counted and the average was taken.

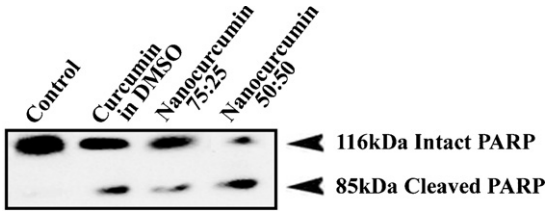

Fig. 7. Nanocurcumin enhances PARP cleavage in HeLa cells. HeLa cells were treated with free curcumin in DMSO/nanocurcumin in aqueous media (25 μM) and incubated for 24 h. The whole cell lysate was immunoblotted against anti-PARP antibody and detected using enhanced chemiluminescence method.

was a significant increase ($p < 0.005$) in the number of apoptotic cells in wells treated with nanocurcumin 50:50 in comparison to nanocurcumin 75:25 and curcumin in DMSO.

To examine PARP cleavage, a clear marker of apoptosis (Simbulan-Rosenthal et al., 1998), western blot was done. Nanocurcumin as well as curcumin in DMSO induced cleavage of 116 kDa form of PARP to the apoptotic fragment of 85 kDa (Fig. 7). In cells treated with nanocurcumin 50:50, the 116 kDa form had almost completely cleaved to 85 kDa form indicating that the extent of caspase activation induced by it is significantly higher ($p < 0.0001$) than other treatments.

3.5. Downregulation of clonogenic potential of HeLa cells by nanocurcumin

Clonogenic assay was done to investigate whether curcumin/nanocurcumin can downregulate the clonogenic potential of HeLa cells. The results clearly indicate that nanocurcumin significantly inhibited ($p < 0.005$) clonogenic potential of HeLa cells like curcumin in DMSO ($p < 0.005$) (Fig. 8). The size and number of clones in curcumin treated wells are much less compared to that of untreated control.

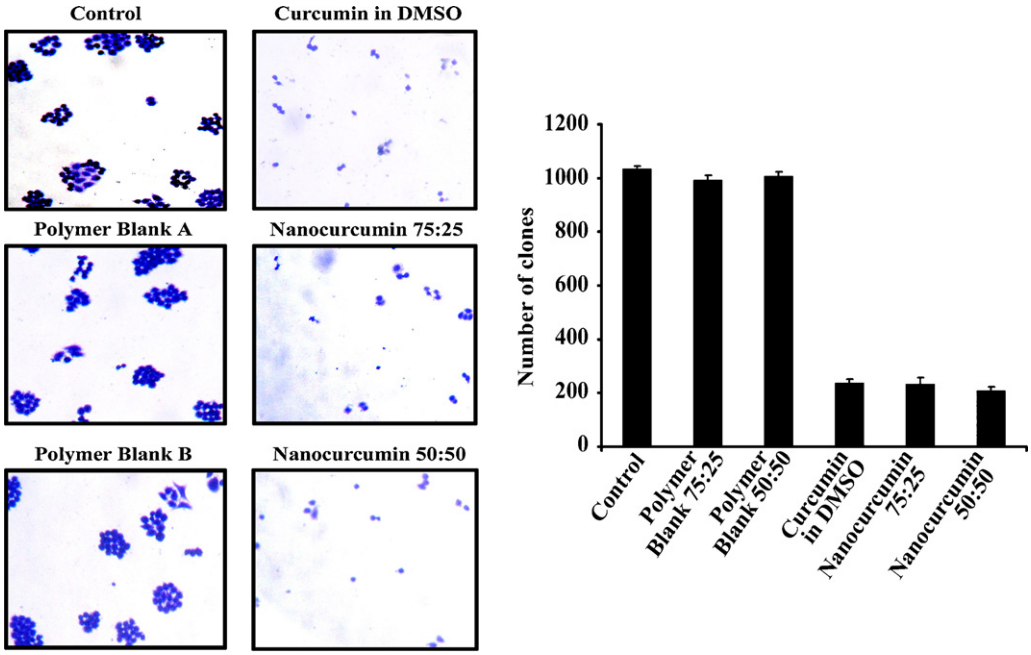

Fig. 8. Curcumin/nanocurcumin inhibits the clonogenic potential of HeLa cells. HeLa cells were treated with curcumin dissolved in DMSO or nanocurcumin formulations in aqueous media (25 μM) for 72 h. Treatments were replaced with fresh medium and incubated for 1 week. The clones developed were fixed in glutraldehyde, stained using crystal violet and counted. All the assays were performed in triplicate and indicated as mean ± standard error on the mean.

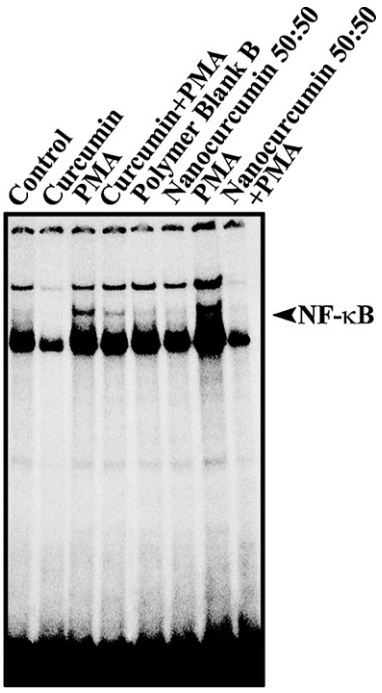

Fig. 9. Nanocurcumin significantly downregulates the activation of NF-κB induced by PMA. Electrophoretic mobility shift assay (EMSA) for assessment of NF-κB inhibition in HeLa cell line. Nuclear extracts were prepared from HeLa cells treated with free curcumin in DMSO and nanocurcumin 50:50 in aqueous media (25 μM) for 2 h followed by incubation with PMA (50 nM) for 1 h. Faster migration of radio-labeled kappa-binding oligonucleotide by nanocurcumin 50:50 showed better inhibition of NF-κB in comparison to curcumin in DMSO.

3.6. Nanocurcumin 50:50 improves the efficacy of curcumin to downregulate PMA induced NF-κB activation

To investigate whether curcumin formulations can downregulate NF-κB, one of the key regulators of apoptosis, EMSA was done in HeLa cells treated with PMA, a well-known inducer of NF-κB. Since nanocurcumin 50:50 was found to be more effective than nanocurcumin 75:25 in inducing apoptosis, curcumin entrapped PLGA 50:50 nanoparticles were selected for this study. Inhibition of NF-κB function is gauged by faster migration (the absence of NF-κB binding) of the radio-labeled kappa-binding oligonucleotide. We observed that inhibition of NF-κB by nanocurcumin 50:50 is significantly higher than curcumin dissolved in DMSO (Fig. 9).

The modulation of PMA-induced nuclear translocation of NF-κB-p65 by nanocurcumin 50:50 was compared with that of curcumin in DMSO by immunocytochemistry. As expected from the results of apoptosis assays and EMSA, the inhibition of nuclear translocation of p65 subunit by nanocurcumin was higher or comparable to that of curcumin in DMSO (Fig. 10).

Altogether, our results indicate that nanoparticle encapsulation improves the aqueous solubility of curcumin which further enhances its efficacy to induce apoptosis in cancer cells.

4. Discussion

Curcumin (diferulyl methane), a polyphenol isolated from the roots of *C. longa* (turmeric) is reported to exhibit a wide spectrum of biological activities (Aggarwal et al., 2005; Bava et al., 2005). Despite these advantages curcumin has the major disadvantages of poor bioavailability and negligible solubility in aqueous medium which limit the use of this phytochemical as a potential chemotherapeutic drug. Nanoparticle mediated drug delivery system has been proven as one of the finest solution for these problems, most of them entering in their clinical trials (Moses et al., 2003). There are reports stating nanoparticle encapsulation improves oral bioavailability of curcumin up to 9-folds compared to that of free curcumin (Shaikh et al., 2009).

In this novel study we report the superior efficacy of PLGA based curcumin nanoparticle (with no modification with stabilizers like PEG) dispersed in aqueous medium than curcumin dissolved in DMSO and its dependence on the composition of carrier. PLGA is a widely used biocompatible polymer which facilitates the slow and sustained release of drugs encapsulated within (Lao et al., 2008). Nanocurcumin prepared from PLGA 50:50 and 75: 25 using modified solvent evaporation method was found to overcome the aqueous solubility limitation of free curcumin (Desgouilles et al., 2003). Physicochemical characterization of nanocurcumin showed spherical shape with mean diameter in the range of 100–200 nm (Fig. 1) and a narrow size distribution (Table 1). However, nanocurcumin 50:50 showed smaller size which might be one of the reasons for its better anticancer activity. The absence of drug peak in DSC (Fig. 2) suggested the presence of nanocurcumin in molecular dispersion form (Jain and Jain, 2008). DSC information is important because the solid dispersion state of drug inside the polymer promotes its sustained release (Panyam et al., 2004). Since the degradation rate of PLGA depends on the lactide:glycolide ratio (Duvvuri et al., 2006; Yoo et al., 2004), the drug release kinetics was expected to be copolymer ratio dependent. The initial burst release may be due to curcumin that was present on the surface or poorly entrapped in the polymer matrix, while the slow and continuous release may be attributed to the diffusion of the curcumin localized in the PLGA core of the nanoparticles. It is important to note that nanocurcumin 50:50 showed a faster release (Janoria and Mitra, 2007) which is due to the presence of higher glycolide content in 50:50 which accounts for its faster degradability (Fig. 3).

To start with biological activity of nanocurcumin, confocal images of cell uptake showed that intracellular fluorescence of nanocurcumin dispersed in aqueous media was comparable to free curcumin in DMSO (Fig. 4). However, free curcumin in media did not show cellular uptake, and a faint fluorescence seen might be due to the slight dispersibility of curcumin in media. This data confirms that nanocurcumin overcomes the aqueous dispersibility limitation of curcumin which opens its possibility for administration *in vivo*, avoiding the usage of toxic solvents like DMSO. Cell viability assays in different cell lines at various time durations showed cytotoxicity in a concentration and time dependent manner, nanocurcumin showing comparable toxicity to free curcumin (Fig. 5). In fact, nanocurcumin 50:50 showed more cytotoxicity and apoptotic effects (Fig. 6) toward HeLa cells than curcumin dissolved in DMSO, while nanocurcumin 75:25 has almost comparable or lesser effect as evidenced by PARP cleavage (Fig. 7) and inhibition of colony formation (Fig. 8). The better efficacy of nanocurcumin 50:50 in comparison to 75:25 might be due to its smaller size and faster release. It is important to note here that unlike recent reports which require additional modification, our nanocurcumin was completely dispersible in aqueous media without any stabilizer (Das et al., 2010).

Several types of cancer cells constitutively express NF-κB, a transcription factor which is the key regulator of apoptosis and contribute to the resistance of cancer cells to apoptosis (Cusack et al., 1999; Huang et al., 2001). Analysis of nanocurcumin 50:50 by EMSA (Fig. 9) (Yadav et al., 2010) and immunocytochemistry (Fig. 10) proved that nano encapsulation improves the efficacy of curcumin in downregulating NF-κB (Mohanty and Sahoo, 2010). Our results of apoptosis assays and NF-κB nuclear translocation studies implicate that polymer encapsulation maintains the regulatory role of curcumin, while being soluble in aqueous media.

Hence, the present study clearly indicates that nanocurcumin solves the poor aqueous solubility problem of curcumin which is

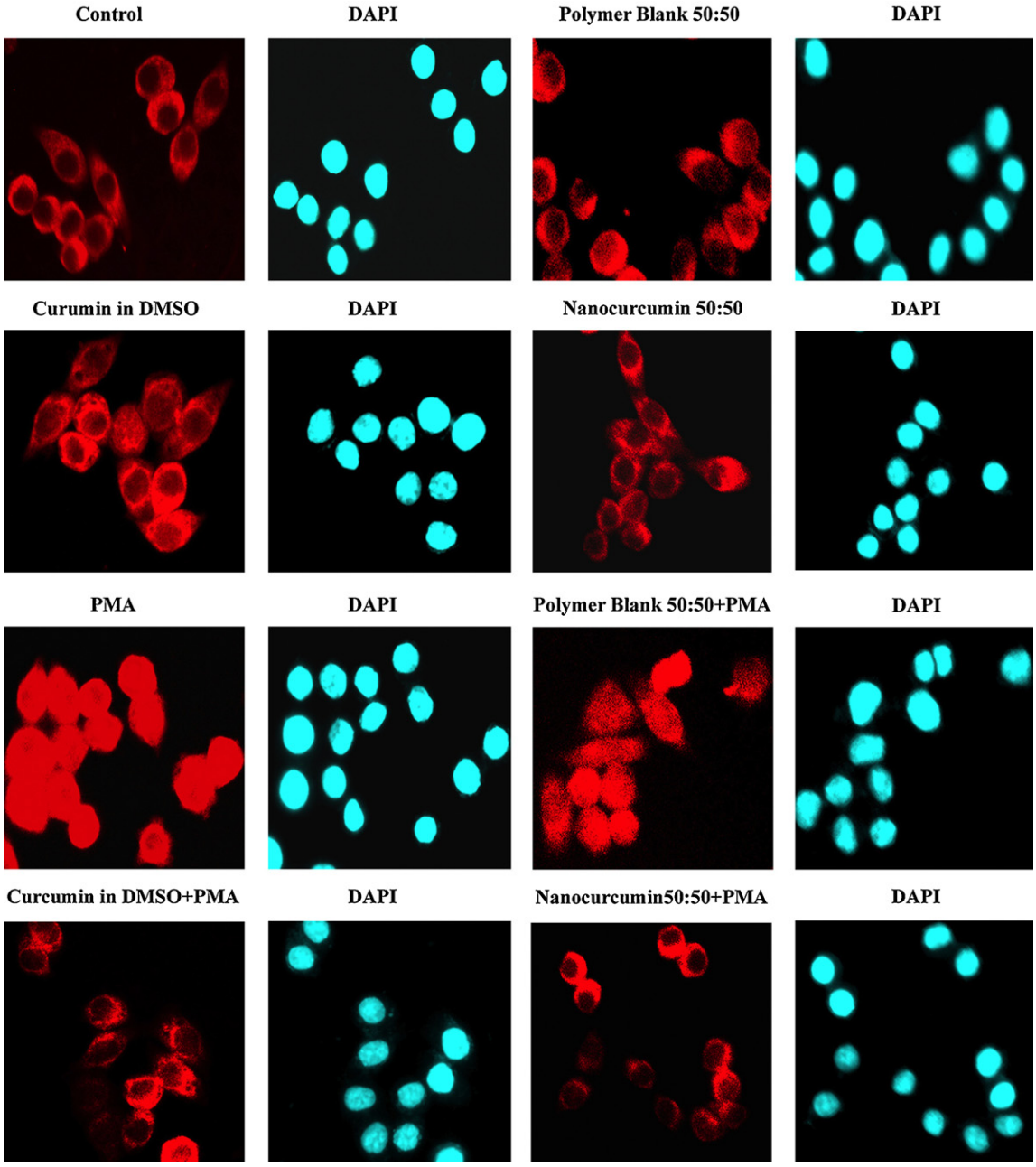

Fig. 10. Immunocytochemical analysis of PMA-induced p65 nuclear translocation. HeLa cells were treated with curcumin in DMSO and nanocurcumin 50:50 in aqueous media (25 μM) for 2 h followed by PMA (50 nM) incubation. Staining with DAPI was followed by immunocytochemical analysis using a fluorescence microscope. Comparable inhibition of nuclear translocation of p65 subunit by nanocurcumin 50:50 was observed.

expected to enhance its *in vivo* bioavailability. Better anticancer activity of nanocurcumin 50:50 than 75:25 shows its dependence on PLGA combination. Although, further studies are required to prove the *in vivo* efficacy of this formulation, successful implementation of nanocurcumin may prove very advantageous to assign curcumin as a chemotherapeutic rather than a chemopreventive.

5. Conclusions

Improved aqueous solubility and enhanced anticancer activity will certainly bring curcumin among one of the leading anticancer therapeutic agents. For this purpose, encapsulation of curcumin with in polymer nanoparticles using two PLGA combinations, 50:50 and 75:25 was done. The results of small size, sustained release, greater cellular uptake and enhanced antiproliferative activity justified the potential of nanocurcumin in aqueous media to induce apoptosis when compared to free curcumin in DMSO. Moreover, NF-κB downregulation and inhibition of nuclear translocation of p65 subunit proved the superior efficiency of nanocurcumin 50:50. In conclusion, our results show that nanocurcumin can be a promising candidate for sustained delivery with better aqueous solubility and anticancer efficacy.

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