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ApoE3 Mediated Poly(butyl) Cyanoacrylate Nanoparticles Containing Curcumin: Study of Enhanced Activity of Curcumin against Beta Amyloid Induced Cytotoxicity Using *In Vitro* Cell Culture Model

Rohit S. Mulik,[†,‡,§] Jukka Mönkkönen,[‡] Risto O. Juvonen,[§] Kakasaheb R. Mahadik,[†] and Anant R. Paradkar*[,∥]

Department of Pharmaceutics, Poona College of Pharmacy, Bharati Vidyapeeth University, Erandwane, Pune 411038, India, Institute of Pharmaceutical Innovation, University of Bradford, Bradford BD7 1DP, U.K., and Department of Pharmaceutics, and Department of Pharmacology and Toxicology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland

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Abstract: Beta amyloid plays a main role in the pathophysiology of Alzheimer's disease by inducing oxidative stress in the brain. Curcumin, a natural antioxidant, is known to inhibit beta amyloid and beta amyloid induced oxidative stress. However, low bioavailability and photodegradation are the major concerns for the use of curcumin. In the present study, we have formulated apolipoprotein E3 mediated poly(butyl) cyanoacrylate nanoparticles containing curcumin (ApoE3−C-PBCA) to provide photostability and enhanced cell uptake of curcumin by targeting. Prepared nanoparticles were characterized for particle size, zeta potential, entrapment efficiency and *in vitro* drug release. The entrapment of curcumin inside the nanoparticles was confirmed by X-ray diffraction analysis. Physicochemical characterization confirmed the suitability of the method of preparation. The photostability of curcumin was increased significantly in nanoparticles compared to plain curcumin. *In vitro* cell culture study showed enhanced therapeutic efficacy of ApoE3−C-PBCA against beta amyloid induced cytotoxicity in SH-SY5Y neuroblastoma cells compared to plain curcumin solution. Beta amyloid is known to induce apoptosis in neuronal cells, therefore antiapoptotic activity of curcumin was studied using flow cytometry assays. From all the experiments, it was found that the activity of curcumin was enhanced with ApoE3−C-PBCA compared to plain curcumin solution suggesting enhanced cell uptake and a sustained drug release effect. The synergistic effect of ApoE3 and curcumin was also studied, since ApoE3 also possesses both antioxidant and antiamyloidogenic activity. It was found that ApoE3 did indeed have activity against beta amyloid induced cytotoxicity along with curcumin. Hence, ApoE3−C-PBCA offers great advantage in the treatment of beta amyloid induced cytotoxicity in Alzheimer's disease.

Keywords: Beta amyloid; Alzheimer's disease; curcumin; apolipoprotein E3; targeted nanoparticles; apoptosis; cytotoxicity

Introduction

One of the pathophysiological hallmarks of Alzheimer's disease is the progressive accumulation of beta-amyloid (Aβ) in the cerebral vasculature and brain in the form of senile plaques, and Aβ insult to neuronal cells has been identified

* Corresponding author. Mailing address: Institute of Pharmaceutical Innovation, University of Bradford, Bradford BD7 1DP, U.K. Tel: +44-1274-233900. Fax: +44-1274-234679. E-mail: A.Paradkar1@Bradford.ac.uk.

[†] Bharati Vidyapeeth University.

[‡] Department of Pharmaceutics, University of Kuopio.

[§] Department of Pharmacology and Toxicology, University of Kuopio.

[∥] University of Bradford.

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as one of the major causes of onset of the disease.[1,2] Aβ get sequestered by cerebral capillaries and transported across the blood−brain barrier (BBB) as a free peptide and/or complex with apolipoprotein J.[3] The isoforms of apolipoprotein E (Apo E2, E3 and E4) form complexes with Aβ and show different transport behaviors. Complexation with ApoE2 and ApoE3 prevents transport of Aβ across BBB whereas apoE4 promotes the uptake and accumulation of Aβ in the brain. Hence, apoE4 is the major risk factor in Alzheimer's disease (AD) while ApoE2 and E3 have protective action.[3] But, in the case of a complex with apolipoprotein E isoforms (E2, E3, and E4), the transport of Aβ is different. The Aβ peptide induces toxicity to neurons through various mechanisms such as apoptosis,[4] mitochondrial dysfunction, nuclear transcription factor (NF-κB) activation,[5] accumulation of reactive oxygen species[6,7] or caspase-3 activation.[8] There are several options available for the treatment of AD, but every treatment option has some serious side effects. Immunotherapy leads to meningoencephalitis; clioquinol antibiotic, which acts as Aβ disruptor, produces vitamin B_{12} deficiency; NSAIDs, which act by inhibiting COX-1 and COX-2 to suppress inflammatory response in AD, have side effects like gastrointestinal tract (GIT), liver and kidney toxicity; *N*-methyl-D-aspartate (NMDA) inhibitors like memantine have some serious side effects on GIT.[9] Many antioxidants, mainly polyphenolic compounds such as green tea, gingko, fruits, and vegetables rich in antioxidants and various flavanoids, have been tried successfully in various neurodegenerative models both *in vitro* and *in vivo*.[10−13] Curcumin is one of those polyphenols which has very well proven antioxidant and antiapoptotic activity in AD against Aβ induced toxicity without inducing any side effects.[2,7,8,14] However, very short half-life, low bioavailability and photolabile nature are the major concerns with curcumin.[15,16]

We have prepared ApoE3 mediated poly(butyl) cyanoacrylate nanoparticles containing curcumin (ApoE3−C-PBCA) using biodegradable and biocompatible materials for enhanced drug uptake by receptor mediated endocytosis to overcome the aforementioned drawbacks of curcumin. Transport of ApoE across BBB is studied by Martel et al.[3] All three apolipoprotein E isoforms, i.e. ApoE2, ApoE3 and ApoE4, have shown low cerebrovascular uptake with undetectable blood-to-brain transport. But, a significant uptake of ApoE3 and ApoE4 was observed by the choroid plexus. This suggested that ApoE isoforms could enter the cerebrospinal fluid (CSF) by crossing the choroidal epithelium, and ultimately be taken up by brain parenchymal cells by low density lipoprotein receptor (LDL-R) mediated endocytosis.[3] Further, Martel et al.[3] have shown that low capillary sequestration of ApoE2, ApoE3, and ApoE4 may reflect nonspecific cellular adsorption, and/or uptake by receptors such as the LDL-R. Overexpression of LDL-R on the surface of human neuroblastoma cells (SH-SY5Y) is well documented.[17−19] ApoE3 has strong affinity for LDL-R, hence, ApoE3−C-PBCA will bind specifically to these receptors and enter inside the SH-

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SY5Y cells by receptor mediated endocytosis.[20] Apart from the receptor mediated endocytosis, ApoE3 has antioxidant and antiamyloidogenic activity in Aβ induced toxicity[21−23] and competes with Aβ for receptor mediated endocytosis by LDL-R.[20] Hence, by developing ApoE3−C-PBCA, we have tried to provide a more effective and safe treatment option for Aβ induced toxicity in AD. This is the first report highlighting the use of ApoE3 as a ligand for brain targeted drug delivery in the treatment of AD.

In the present study, the protective effect of prepared curcumin nanoparticles on Aβ induced cytotoxicity in SH-SY5Y neuroblastoma cells was studied and compared against plain curcumin solution. We have performed detailed flow cytometric analysis of enhanced antioxidant and antiapoptotic activity of nanoparticles containing curcumin compared to plain curcumin solution.

Experimental Section

Materials. *n*-Butyl cyanoacrylate was a kind gift from Evotite Corporation, China. Poloxamer 188 and sodium sulfate were purchased from Merck, Germany. Curcumin was a kind gift from Indsaaf Inc., Batala, India. Coomassie brilliant blue G dye was purchased from Himedia, Mumbai, India. Apolipoprotein E3, human, and β-amyloid peptide (1−42), human, were purchased from Calbiochem, Europe. AnnexinV−fluorescein isothiocyanate (AnnexinV−FITC) and propidium iodide were purchased from Biolegend Europe BV, Netherlands. 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCF-DA) was purchased from Sigma Aldrich, USA. CaspGLOW red active caspase-3 staining kit was purchased from Biovision Research Products. Tetramethylrhodamine methyl ester (TMRM) was purchased from Sigma Aldrich, Germany. Growth medium, DMEM (BE 12-604F) was purchased from BioWhittaker Inc., Lonza, Belgium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich, Germany. All aqueous solutions were prepared with distilled and deionized water. All other reagents and chemicals used were of analytical grade.

Methods. *Preparation of Beta Amyloid (Aβ) Solution.* Aβ was preincubated using conditions as per data sheet of the supplier to make them neurotoxic (Calbiochem, Cat. No. PP69). In brief, the lyophilized peptide was dissolved in 50 mM Tris-HCl, pH ≥ 9.0 at 1 mg/mL concentration. Then it was diluted to desired concentration with calcium and magnesium free phosphate buffer solution (PBS) and incubated at 37 °C for 48 h prior to neurotoxicity studies.

Preparation of Nanoparticles. *Curcumin Loaded PBCA Nanoparticles (C-PBCA).* These nanoparticles were prepared by an anionic polymerization method previously reported by us.[24] Briefly, Poloxamer 188 and sodium sulfate were dissolved in a mixture of 0.1 N HCl and ethanol. Curcumin was then added and stirred to dissolve. Finally, *n*-butyl cyanoacrylate was added under continuous stirring in a dropwise manner. After 4 h of stirring, the pH of the suspension was adjusted to 6.0 ± 0.5 and stirring was continued for an additional 1 h to complete the reaction. The nanoparticles were then coated with Tween 80 (0.1% w/v) by stirring for 30 min at 200 rpm.

ApoE3 Mediated PBCA Nanoparticles (ApoE3−C-PBCA). The ApoE3 (20 μg/mL) was finally conjugated to the C-PBCA by stirring for 1 h at room temperature.[25]

Blank Nanoparticles. PBCA nanoparticles without curcumin (B-PBCA) and ApoE3 mediated PBCA nanoparticles without curcumin (ApoE3−B-PBCA) were prepared by the same method as described above.

Preparation of Curcumin Solubilized Surfactant Solution (CSSS). Curcumin was dissolved in an aqueous system containing 0.1% w/v poloxamer 188 by stirring for 15 min at 400 rpm. Aqueous system containing 0.1% w/v poloxamer 188 without curcumin was prepared as a blank system and termed as surfactant solution (SS).

Characterization. The particle size of nanoparticles was determined by dynamic light scattering using Malvern Hydro 2000 SM particle size analyzer (Malvern instruments, U.K.). The uniformity of the size distribution was indicated by the polydispersity index (PdI).[24] The zeta (ζ) potential was measured by laser Doppler velocimetry (Zetasizer 3000, Malvern Instruments, U.K.) at 25 °C.[24] For shape and particle size determination, transmission electron microscopy (TEM) was done using a Hitachi S-7500 transmission electron microscope (Hitachi, Japan). The nanoparticles were dispersed in water, and one drop of the diluted dispersion was placed on a 200-mesh carbon coated copper grid. The photographs were taken at 30000× magnification and 100 kV voltage.[24] The percentage drug entrapment (PDE) in the C-PBCA was determined using the ultracentrifugation method (Beckmann TL-100, MN, USA).[24] The quantification of

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ApoE3 conjugation at the surface of C-PBCA was done by the Bradford assay using coomassie brilliant blue G dye.[26,27] All the values are presented as mean ± SD from three replicate samples. The photostability of curcumin encapsulated in nanoparticles was determined by previously described method using HPLC analysis.[24]

X-ray Diffraction Study (XRD) Analysis of Nanoparticles. The encapsulation of curcumin inside the nanoparticles was confirmed by X-ray diffraction measurements carried out with an X-ray diffractometer (PW 1729, Philips, Netherlands) in the diffraction range of 5−50°. A Cu Kα radiation source was used, and the scanning rate (2θ/min) was 5 °C/min.[28]

Cell Culture Study. SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin G-streptomycin (Gibco BRL, Grand Island, NY) at 37 °C in a humidified, 5% CO_2 atmosphere in a CO_2 incubator.

MTT Assay. SH-SY5Y Cells (2×10^4/well) were seeded and allowed to attach for 24 h in a 96 well plate. Fresh medium was added and the cells were treated with Aβ (0.2 μM) with/without different concentrations of CSSS, C-PBCA, and ApoE3−C-PBCA (25, 50, 100, 200, 400, and 800 nM curcumin/well) using SS, B-PBCA, and ApoE3−B-PBCA as the respective controls and incubated for 24 h at 37 °C in CO_2 incubator.[29] The medium was replaced with fresh medium by washing thrice with PBS after 24 h. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells and kept in a CO_2 incubator for 3 h at 37 °C. In viable cells, MTT forms purple formazan crystals by undergoing reduction with active mitochondrial reductase. Finally, 125 μL of a lysis solution (50% v/v N,N, dimethylformamide, 20% w/v sodium dodecylsulfate, with an adjusted pH of 4.5) was added to dissolve the formazan crystals. The optical density was measured with a Victor 1420 Multilabel Counter (PerkinElmer Life Sci., USA) equipped with a 570 nm filter.

To confirm the LDL-R mediated uptake of ApoE3−C-PBCA, LDL-R on the SH-SY5Y cell surface were blocked by incubating the cells with an excess amount of free ApoE3, 1 h prior to incubation with ApoE3−C-PBCA in a separate experiment. The effect on cell viability was determined after 24 h treatment.[30] For this experiment, a 100 nM dose of curcumin was used. Also, to see the possible protective effect of ApoE3, we treated the cells separately with Aβ with/without ApoE3 (concentrations equivalent to 25, 50, and 100 nM curcumin in ApoE3−C-PBCA). Results were expressed as % cell viability vs dose. All experiments were done in triplicate, and results were expressed as mean ± SD ($n = 3$).

Measurement of Reactive Oxygen Species (ROS). The measurement of reactive oxygen species was performed by means of the probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) as per previously described method.[31] Briefly, SH-SY5Y cells (2×10^4/well) were seeded into 96 well plate and allowed to attach for 24 h. After 24 h, the fresh medium was added and cells were treated with Aβ (0.2 μM) with/without different concentrations of CSSS, C-PBCA, and ApoE3−C-PBCA (25, 50, 100, 200, 400, and 800 nM curcumin/well) using surfactant solution (SS), B-PBCA, and ApoE3−B-PBCA as the respective controls and incubated for 24 h at 37 °C in CO_2 incubator. After the treatment, the medium was removed, cells were washed thrice with PBS and fresh medium was added. 2′,7′-Dichlorodihydrofluorescein diacetate (10 μM) was added to the cells and incubated for 2 h at 37 °C. H2DCF-DA, a nonfluorescent, permeant molecule, passively diffuses into the cells and gets deacetylated to form nonfluorescent H2DCF by intracellular esterases. Finally, H2DCF get rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. Fluorescence intensity was measured at an excitation wavelength of 502 nm and an emission wavelength of 520 nm using Envision 2104 Multilabel Reader (PerkinElmer Life Sci, USA). In a similar manner, the protective effect of ApoE3 was observed by treating the cells with Aβ with/without ApoE3 (concentrations equivalent to 25, 50, and 100 nM curcumin in ApoE3−C-PBCA). Results were expressed as relative fluorescence units (RFU) vs dose. All experiments were done in triplicate, and results were expressed as mean ± SD ($n = 3$).

Flow Cytometry. *Antiapoptotic Activity.* Phosphatidylserine (PS), membrane phospholipid, gets translocated from the inner leaflet to the outer leaflet of the cell membrane during early apoptosis. AnnexinV, a phospholipid binding protein, binds specifically to PS with high affinity, while propidium iodide (PI), a membrane nonpermeant dye, selectively stains necrotic cells because of cell membrane disruption.[32,33] Hence, the double staining method

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(AnnexinV−FITC and PI) was performed to detect apoptotic and necrotic cells. SH-SY5Y cells (1×10^6/flask) were seeded in a cell culture flask (Nunc, 75 cm^2) and allowed to adhere for 24 h. Fresh medium was added, and cells were treated with Aβ (0.2 μM) with/without CSSS, C-PBCA, ApoE3−B-PBCA and ApoE3−C-PBCA (100 nM curcumin/well) for 24 h at 37 °C in CO_2 incubator. After 24 h treatment, cells were detached using trypsin−EDTA solution and fresh medium was added. The cells were separated by centrifugation at 3000 rpm for 5 min and resuspended in AnnexinV binding buffer (1×10^6/mL). 100 μL of this cell suspension was taken into a 5 mL FACS tube and stained with 5 μL of AnnexinV−FITC/10 μL PI and kept for incubation (15 min) in the dark at room temperature. After incubation, a further 400 μL of AnnexinV binding buffer was added. To detect the possible autofluorescence of curcumin, treated unstained cells (1×10^6/mL) were also prepared in the same manner. Cells were analyzed using flow cytometer (FACSCantoII, BD Biosciences) in FL1 and FL2 channel for FITC and PI respectively. Green fluorescence (FL1) was collected at more than 535 nm, and red fluorescence (FL2) at more than 550 nm, from 10,000 cells. Untreated cells were taken as negative control. The % of cells was determined by using FACSDiva software. All experiments were performed in triplicate, and results were expressed as mean ± SD ($n = 3$).

Hoechst 33342 Staining. Cells were treated with Aβ and different curcumin formulations as described earlier. After the treatment for 24 h, medium was removed, and cells were washed with double distilled water, stained with Hoechst 33342 (10 μM) and incubated for 20 min at room temperature.[34] Samples were observed under a Nikon Eclipse TE300 fluorescence microscope with Nikon F601 camera under UV illumination (Nikon, Japan).

Cell Cycle Analysis. Cell cycle damage and DNA fragmentation being the main features of apoptotic cells, cell cycle analysis is a parameter of paramount significance in the detection of apoptotic cells.[33] Briefly, SH-SY5Y cells (1×10^6/flask) were seeded in a cell culture flask (Nunc, 75 cm^2) and allowed to adhere for 24 h. Fresh medium was added to the cells. Cells were treated with Aβ (0.2 μM) with/without CSSS, C-PBCA, and ApoE3−C-PBCA (100 nM curcumin/well) for 24 h at 37 °C in a CO_2 incubator. After treatment, cells were harvested by centrifugation, washed thrice with PBS and fixed in 70% ethanol for 2 h. After 2 h, ethanol was removed by centrifugation and cells were resuspended in 500 μL of PBS 7.4 containing RNase (100 μg/mL) at room temperature for 30 min. Finally, the cellular DNA was stained with PI (50 μg/mL) and kept in the dark for 30 min for incubation. The cell cycle was analyzed using a flow cytometer (FACSCantoII flow cytometer, BD Biosciences) in red (FL2) channel at more than 550 nm, from 10000 cells. Untreated cells were taken as negative control. The % of subG1 fraction was determined using FACSDiva software. All experiments were performed in triplicate, and results were expressed as mean ± SD ($n = 3$).

Detection of Caspase-3. Activation of caspases plays a central role in apoptosis. In apoptosis, initiator caspases (caspase 2, 8, 9 and 10) cleave inactive pro-forms of effector caspases (caspase 3, 6, and 7) to produce activated effector caspases which in turn cleave cellular protein substrates to start apoptosis.[33] In the present study, we have detected the induction of caspase-3 using CaspGLOW Red Active Caspase-3 Staining Kit. Red-DEVD-FMK is a cell permeable, nontoxic probe which binds irreversibly to the activated caspase-3 in apoptotic cells. The red fluorescence label allows for direct detection of activated caspase-3 in apoptotic cells by flow cytometry. SH-SY5Y cells (1×10^6/flask) were seeded in cell culture flask (Nunc, 75 cm^2) and allowed to adhere for 24 h. Fresh medium was added, and cells were treated with Aβ (0.2 μM) with/without CSSS, C-PBCA, and ApoE3−C-PBCA (100 nM curcumin/well) for 24 h at 37 °C in CO_2 incubator. After 24 h treatment, cells were detached using trypsin−EDTA solution and fresh medium was added. The cells were separated by centrifugation at 3000 rpm for 5 min and resuspended in wash buffer (1×10^6/mL). 300 μL of this cell suspension was stained with 1 μL of Red-DEVD-FMK and kept for incubation (1 h) at 37 °C in a CO_2 incubator. After incubation, cells were centrifuged at 3000 rpm for 5 min to remove the supernatant and cells were resuspended in 0.3 mL of wash buffer. Cells were analyzed using a flow cytometer (FACSCantoII, BD Biosciences) in FL2 channel. Red fluorescence (FL2) was measured at more than 550 nm, for 10000 cells. Untreated cells were taken as negative control. An additional control was prepared by adding the caspase inhibitor Z-VAD-FMK at 1 μL/mL to inhibit caspase activation and then stained with Red-DEVD-FMK. All experiments were performed in triplicate, and results were expressed as mean ± SD ($n = 3$).

Statistical Analysis. Data are reported as mean ± SD. Statistical analysis was done by performing Student's *t* test. The differences were considered significant for p values of <0.05.

Results

Characterization of Prepared Nanoparticles. A schematic representation of the relative position of each component in ApoE3−C-PBCA is shown in Figure 1. The mean particle size of C-PBCA was found to be 178 ± 0.59 nm with polydispersity index of 0.24 (Figure 2a). Low polydispersity index indicates the uniformity of particle size distribution. The conjugation of ApoE3 showed much less effect on particle size. The particle size of ApoE3−C-PBCA was found to be 197 ± 2.3 nm with polydispersity index of 0.18 (Figure 2b). The particle size and shape were further confirmed by TEM (Figure 3). Nanoparticles were spherical in shape, while the particle size was supplemented with the particle size measurement by laser diffraction. The ζ potential

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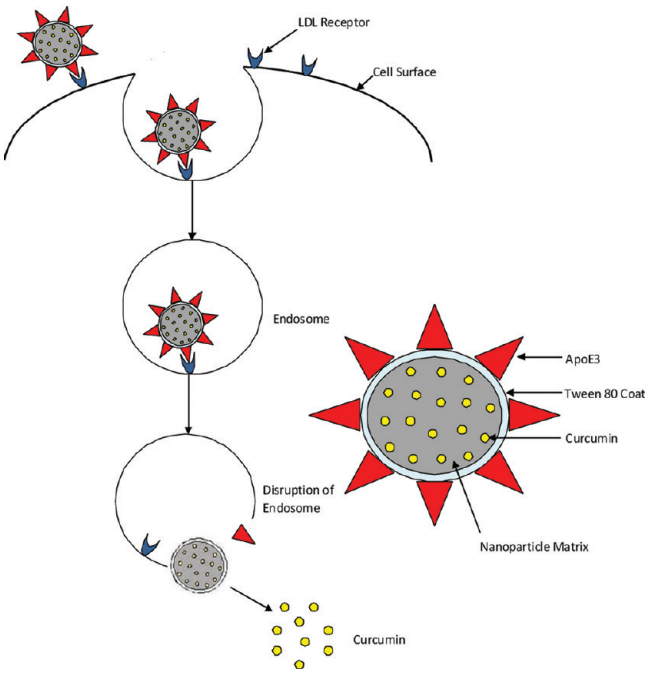

Figure 1. Schematic representation of ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA) and its receptor mediated endocytosis.

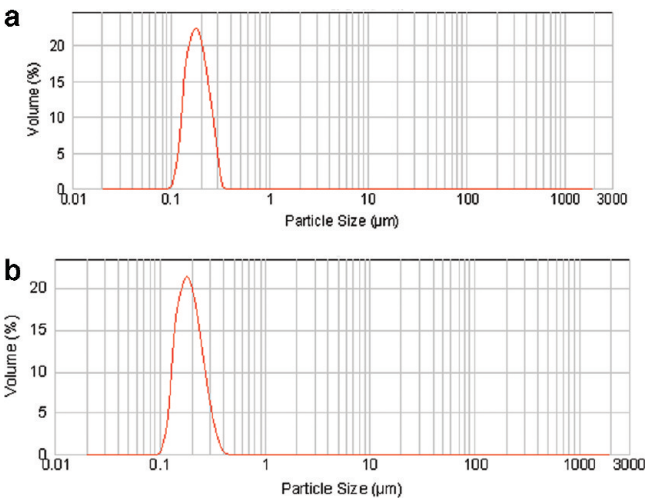

Figure 2. Particle size analysis by dynamic light scattering. (a) Curcumin loaded PBCA nanoparticles (C-PBCA). (b) ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA).

of C-PBCA and ApoE3−C-PBCA was −28.33 ± 0.16 and −22.44 ± 2.3 mV respectively, indicating the stable nature of formed nanoparticles (Figure 4a and 4b). There was slight decrease in the ζ potential of ApoE3−C-PBCA which can be attributed to the conjugation of ApoE3 at the surface of C-PBCA. PDE of C-PBCA was found to be 77.99 ± 0.91%, indicating good encapsulation efficiency of curcumin by the prepared nanoparticles. The conjugation of ApoE3 at the surface showed a very insignificant effect on PDE. The conjugation efficiency of ApoE3 with C-PBCA was expressed as % ApoE3 conjugated, and it was found to be 22.3 ± 1.2%. The stability study showed increased stability of

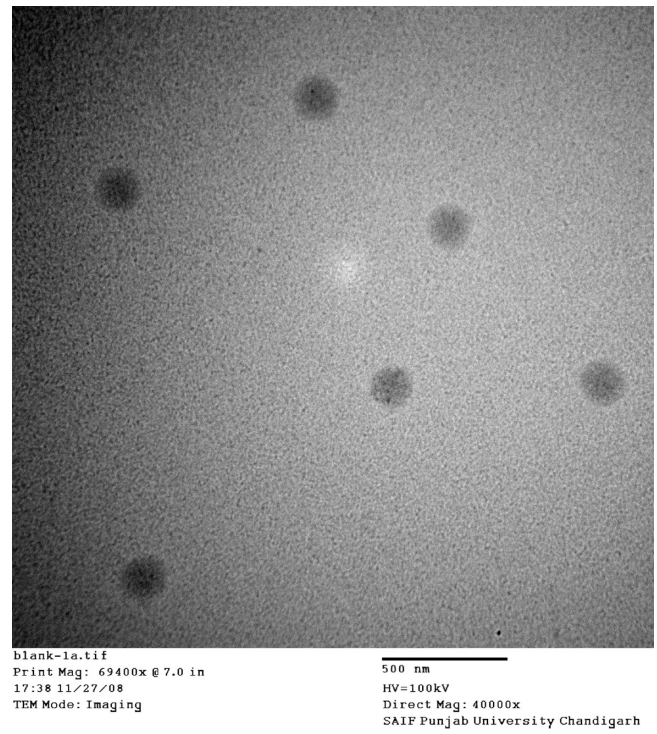

Figure 3. Transmission electron microscopy of ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA). The magnification bar in the image is 500 nm.

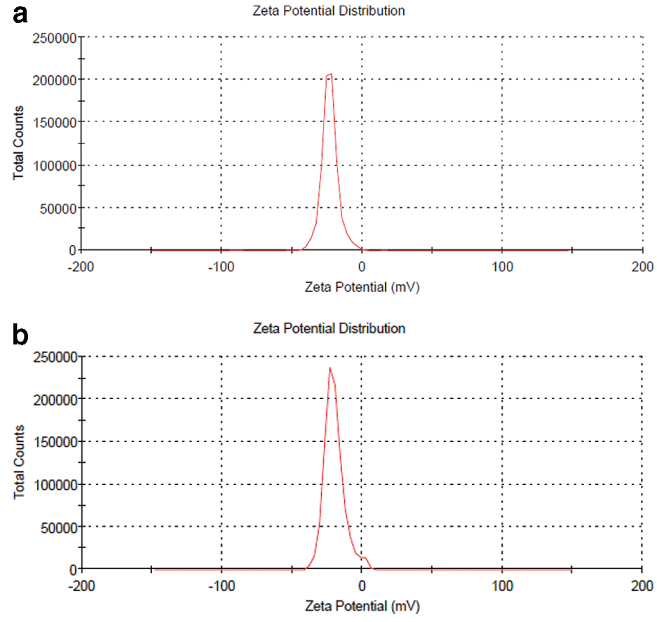

Figure 4. Measurement of zeta potential by laser Doppler velocimetry. (a) Curcumin loaded PBCA nanoparticles (C-PBCA). (b) ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA).

curcumin against photodegradation in C-PBCA compared to CSSS. The percentage curcumin remaining in the absence and presence of light after 3 and 6 months in both the nanoparticles was higher compared to that in CSSS (data not shown).[24]

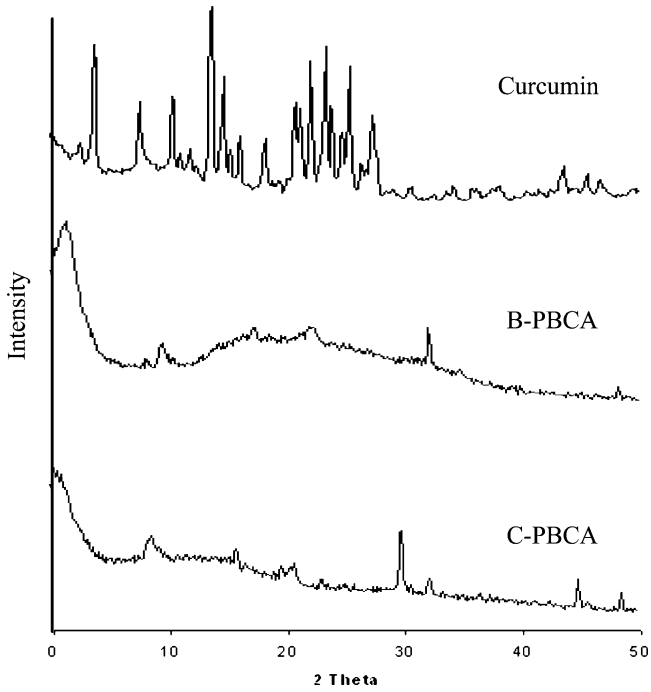

Figure 5. X-ray diffraction (XRD) patterns of curcumin, blank PBCA nanoparticles (B-PBCA), and curcumin loaded PBCA nanoparticles (C-PBCA).

XRD Analysis of Nanoparticles. The X-ray diffraction patterns of curcumin, B-PBCA and C-PBCA were studied (Figure 5). The characteristic peaks of curcumin observed were absent in the diffraction patterns of both B-PBCA and C-PBCA indicating the encapsulation of curcumin inside the nanoparticles in an amorphous form. Also, it was clear from the diffraction patterns of B-PBCA and C-PBCA that curcumin does not affect polymerization and formation of PBCA nanoparticles, since both the diffraction patterns were almost identical.[28]

Cell Culture Study. *Cell Survival Assay.* In this study, the protective effect of curcumin was studied against the Aβ induced cytotoxicity in SH-SY5Y human neuroblastoma cells.[2,35] Initially, the effect on cell viability with different concentrations of Aβ was studied and the sublethal concentration (0.2 μM) was taken for the further study with curcumin treatment. The cell viability was reduced to 36.0% with 0.2 μM Aβ. The treatment with CSSS showed only little increase (45.6% and 49.8%) in cell survival at 50 and 100 nM concentrations (Figure 6). In the case of C-PBCA, the increase in cell survival at 25, 50, and 100 nM concentration was $\geq$10% while with ApoE3−C-PBCA the increase was $\geq$20% (Table 1). At 50 and 100 nM concentration, the increase in cell survival was about 2 times the in case of ApoE3−C-PBCA, while with C-PBCA the increase was $\leq$1.5 times (Figure 6). At 400 and 800 nM concentrations, further increase in cytotoxicity was observed indicating

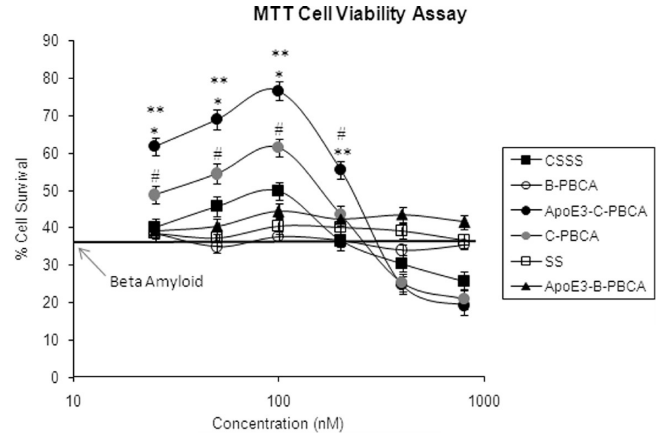

Figure 6. MTT cell survival assay. The effect on % cell survival with treatment with curcumin solubilized surfactant solution (CSSS; black filled squares), curcumin loaded PBCA nanoparticles (C-PBCA; gray filled squares), ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA; black filled circles), blank PBCA nanoparticles (B-PBCA; open circles), surfactant solution (SS; open squares), and ApoE3 mediated blank PBCA nanoparticles (ApoE3−B-PBCA; black filled triangles) was observed against beta amyloid induced cytotoxicity. Data is represented as mean $\pm$ SD, $n = 3$. (*) $p < 0.005$, ApoE3−C-PBCA versus CSSS, (**) $p < 0.05$, ApoE3−C-PBCA versus C-PBCA, (#) $p < 0.05$, ApoE3−C-PBCA or C-PBCA versus CSSS.

Table 1. Protective Effect of Different Curcumin Formulations and ApoE3 against Beta Amyloid Induced Cytotoxicity

	% increase in cell survival			
concn (nM)	CSSS	C-PBCA	ApoE3−C-PBCA	ApoE3 alone
25	4.12	12.7	25.7	3.1
50	9.6	18.3	32.9	4.4
100	13.8	25.4	40.5	8.3

that the concentrations below 200 nM are effective for the protection against Aβ induced cytotoxicity. The increase in cytotoxicity at higher concentrations was due to the pro-oxidant and apoptotic effect of curcumin as reported earlier.[36,37] The control formulations (SS and B-PBCA) showed no effect on cell viability, indicating that they have no role in cell survival. Hence, it was confirmed that the protective effect observed was indeed because of curcumin. Another control (ApoE3−B-PBCA) showed slight protective

(35) Lim, G.; Chu, T.; Yang, F.; Beech, W.; Frautschy, S.; Cole, G. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J. Neurosci.* **2001**, *21*, 8370–8377.

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effect indicating the role of ApoE3. Further, from the receptor blocking study it was observed that there was decrease in the percent cell survival with ApoE3−C-PBCA after LDL-R blocking, indicating the role of LDL-R mediated endocytosis in the uptake of ApoE3−C-PBCA (Figure 1). The cell survival was reduced to 64.2% with ApoE3−C-PBCA in receptor blocking experiment, clearly indicating the reduced cell uptake of ApoE3−C-PBCA and ultimately reduced protective effect. Experiment with free ApoE3 also showed a slight increase in % cell survival, indicating the protective effect of ApoE3. The additional protective (antiamyloidogenic) effect of ApoE3 was determined using the same experiment. It was observed that free ApoE3 in a concentration equivalent to 25, 50, and 100 nM curcumin in ApoE3−C-PBCA is protective against Aβ. The cell survival with ApoE3−B-PBCA was increased to 39.1%, 40.4% and 44.3% from 36.0% (with Aβ).

Measurement of Reactive Oxygen Species. Considerable evidence of involvement of Aβ induced oxidative stress in the pathogenesis of Alzheimer's disease is available.[38] In this study, we induced the oxidative stress in SH-SY5Y human neuroblastoma cells using 0.2 μM Aβ. The Aβ induced oxidative stress was then treated with different formulations of curcumin. Reduction in reactive oxygen species was observed up to 200 nM concentration with maximum reduction at 100 nM concentration (Figure 7). The increase in ROS reduction with ApoE3−C-PBCA was 2−3 times more compared to C-PBCA at concentrations between 25−200 nM and 5−6 times more compared to CSSS (Table 2). Among C-PBCA and ApoE3−C-PBCA, ApoE3−C-PBCA showed more decrease in ROS (at doses less than 200 nM) compared to C-PBCA complementing with the result of cell survival assay, indicating the increased cell uptake by receptor mediated endocytosis (Figure 1) and synergistic antioxidant and antiamyloidogenic effect of ApoE3[21,22] and curcumin.[2,8] The control formulations (SS and ApoE3−B-PBCA) showed no effect on ROS, indicating that they have no role in antioxidant activity. Hence, it was confirmed that the antioxidant effect observed was because of curcumin. Another control (ApoE3−B-PBCA) showed slight antioxidant effect, confirming the role of ApoE3. Further, to support the possible antioxidant activity of ApoE3 alone, the effect of ApoE3 (concentration of ApoE3 was equivalent to 25, 50, and 100 nM curcumin in ApoE3−C-PBCA) on the Aβ induced ROS in SH-SY5Y cells was observed. It was found that ApoE3 was able to reduce the ROS induced by Aβ to a considerable extent (6.78, 10.2 and 12.5%), confirming the antioxidant activity of ApoE3 (Table 2).

Antiapoptotic Activity. Antiapoptotic activity of curcumin was studied against the apoptosis induced by Aβ in SH-SY5Y human neuroblastoma cells (Figure 8). Aβ induced approximately 60% of apoptotic cell population (42.2% early apoptotic and 17.6% late apoptotic/early necrotic), which was reduced to 45.8% with CSSS treatment. The decrease in apoptotic cell population was increased with the treatment of both nanoparticles (C-PBCA and ApoE3−C-PBCA) compared to CSSS. Treatment with C-PBCA reduced the apoptotic population to 37.6%, while with ApoE3−C-PBCA the percentage reduced to 11.6%. It was clear from this study that ApoE3−C-PBCA was highly effective against Aβ induced apoptosis compared to C-PBCA and CSSS. Even C-PBCA also showed considerably increased antiapototic activity compared to CSSS. Hence, increased antiapoptotic activity of ApoE3−C-PBCA compared to C-PBCA further confirmed the increased cell uptake and possible synergistic effect of ApoE3 and curcumin in ApoE3−C-PBCA.[2,21] A slight antiapoptotic effect observed with ApoE3−B-PBCA further confirmed the possible role of ApoE3. Treated,

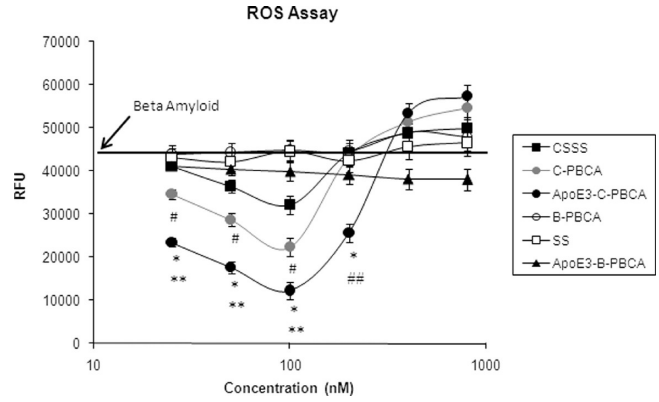

Figure 7. Reactive oxygen species (ROS) assay. The decrease in relative fluorescence unit (RFU) with treatment with curcumin solubilized surfactant solution (CSSS; black filled squares), curcumin loaded PBCA nanoparticles (C-PBCA; gray filled circles), ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA; black filled circles), blank PBCA nanoparticles (B-PBCA; open circles), surfactant solution (SS; open squares), and ApoE3 mediated blank PBCA nanoparticles (ApoE3−B-PBCA; black filled triangles) was observed against beta amyloid induced cytotoxicity. Data is represented as mean ± SD, $n = 3$. (*) $p < 0.005$, ApoE3−C-PBCA versus CSSS, (**) $p < 0.05$, ApoE3−C-PBCA versus C-PBCA, (#) $p < 0.05$, ApoE3−C-PBCA or C-PBCA versus CSSS, (##) $p < 0.005$ ApoE3−C-PBCA versus C-PBCA.

Table 2. Antioxidant Effect of Different Curcumin Formulations and ApoE3 against Beta Amyloid Induced Cytotoxicity

	% decrease in RFU			
concn (nM)	CSSS	C-PBCA	ApoE3−C-PBCA	ApoE3 alone
25	10.9	23.7	48.4	6.78
50	20.3	36.7	61.1	10.2
100	30.84	50.8	72.85	12.5

(38) Nunomura, A.; Perry, G.; Aliev, G.; Hirai, K.; Takeda, A.; Balraj, E.; Jones, P.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R.; Smith, M. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **2001**, *60*, 759–767.

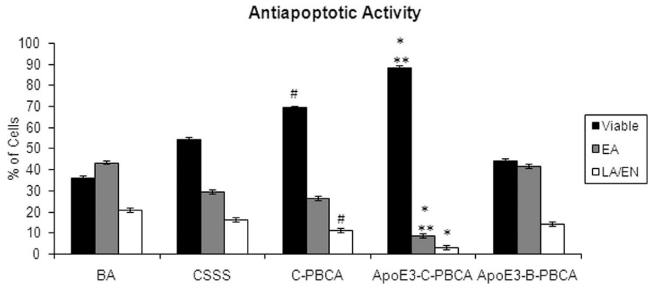

Figure 8. Antiapoptotic activity of curcumin using curcumin solubilized surfactant solution (CSSS), curcumin loaded PBCA nanoparticles (C-PBCA), ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA), and ApoE3 mediated blank PBCA nanoparticles (ApoE3−B-PBCA) against beta amyloid induced apoptosis. Data is represented as mean ± SD, $n = 3$. (*) $p < 0.005$, ApoE3−C-PBCA versus CSSS, (**) $p < 0.05$, ApoE3−C-PBCA versus C-PBCA, (#) $p < 0.05$, ApoE3−C-PBCA or C-PBCA versus CSSS. Black filled bars, viable cells; gray filled bars, early apoptotic cells (EA); open bars, late apoptotic and/or early necrotic (LA/EN).

unstained cells showed no fluorescence in FL1, indicating the absence of possible autofluorescence of curcumin.

Hoechst 33342 Staining. Apoptotic cells, due to the change in membrane permeability, show an increased uptake of the vital dye Hoechst 33342 in the nucleus compared to live cells after a short period of exposure. The nuclei in viable cells were normal and exhibited diffused staining with less intensity. The cells treated with Aβ showed bright nuclear staining with condensed chromatin and shrunken nucleus indicating apoptosis. The decrease in staining intensity in the case of CSSS, C-PBCA and ApoE3−C-PBCA treated cells was observed, indicating the protective action of curcumin against apoptosis. ApoE3−C-PBCA showed much less staining, indicating increased protective action compared to CSSS and C-PBCA (Figure 9).

Effect on SubG1 Fraction. In cell cycle analysis, the presence of a hypodiploid peak in the subG1 phase indicates apoptosis.[33] This fraction was detected using the flow cytometric method as described earlier using SH-SY5Y human neuroblastoma cells, and the results are shown in Figure 10. After Aβ treatment, the subG1 fraction was increased to 33.4%. This increase in subG1 fraction by Aβ was reduced with the treatment of different curcumin formulations. The subG1 fraction was reduced to 22.1% and 15.4% with the treatment of CSSS and C-PBCA, respectively. But the most significant effect was observed with the treatment of ApoE3−C-PBCA. The subG1 fraction was reduced to 3.4% with ApoE3−C-PBCA, showing increased antiapoptotic activity of curcumin with ApoE3−C-PBCA. Hence, this study further confirmed that the use of ApoE3 as a ligand helps in enhancing the antiapoptotic activity of curcumin very significantly, supplemented with the results of previous experiments.

Effect on Caspase-3. In caspase cascade involved in the induction of apoptosis, caspase-3 is one of the major effector

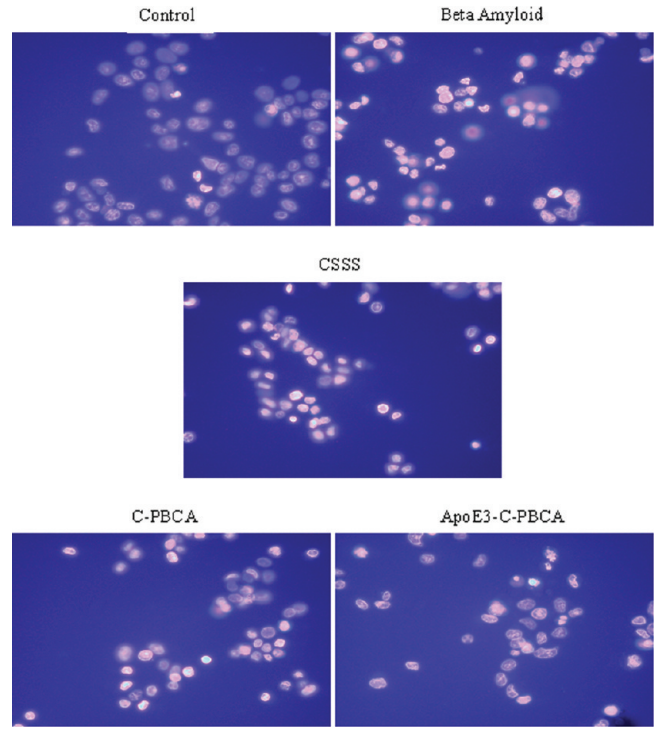

Figure 9. Qualitative analysis of antiapoptotic activity of curcumin solubilized surfactant solution (CSSS), curcumin loaded PBCA nanoparticles (C-PBCA) and ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA) against beta amyloid induced apoptosis by Hoechst 33342 staining. The images were taken using fluorescence microscope under UV illumination.

caspase which ultimately induces apoptosis. Aβ is known to induce apoptosis in neural cells via the caspase pathway, and there are numerous reports of the involvement of caspase-3 in Aβ induced apoptosis in neuroblastoma cells.[35] Hence, we studied the expression of caspase-3 after the addition of Aβ in SH-SY5Y neuroblastoma cells, and then these cells were treated with different curcumin formulations to study the possible inhibition of caspase-3 by curcumin using flow cytometry (Figure 11). With Aβ, caspase-3 positive cell population was 37.6%. This increased caspase-3 positive cell population was reduced to 28.3% and 14.3% with the treatment of CSSS and C-PBCA, respectively. But the most effective reduction was observed with ApoE3−C-PBCA (4.3%). Hence, this study revealed that anti-caspase activity of curcumin was remarkably increased with ApoE3−C-PBCA/C-PBCA compared to CSSS. Also, compared to C-PBCA, ApoE3−C-PBCA showed enhanced anti-caspase and, ultimately, antiapoptotic activity. Hence, the results of anti-caspase activity were also in accordance with all the previous results, suggesting the enhanced activity of curcumin with ApoE3−C-PBCA.

Discussion

Accumulation of the Aβ peptide is a defining feature in all forms of AD, irrespective of the genetic background.[20]

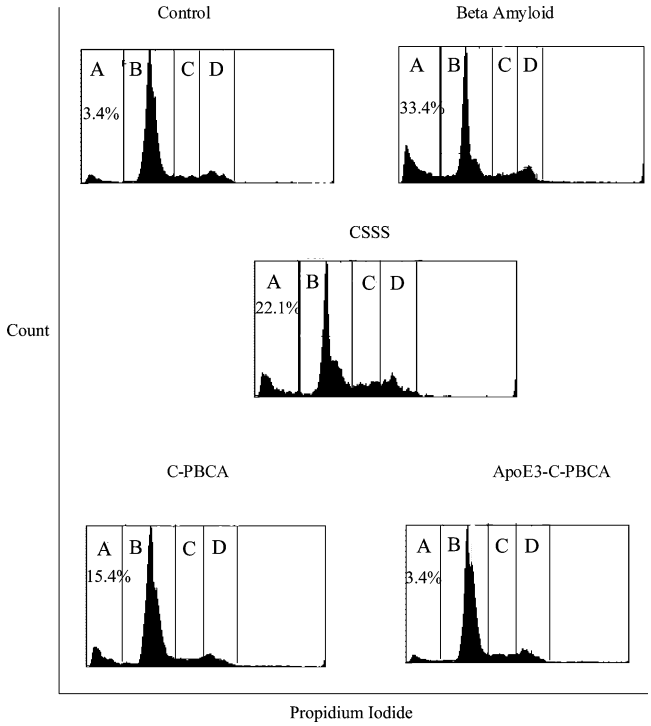

Figure 10. Cell cycle analysis. The decrease in subG1 phase with curcumin solubilized surfactant solution (CSSS), curcumin loaded PBCA nanoparticles (C-PBCA) and ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA) against beta amyloid induced DNA damage.

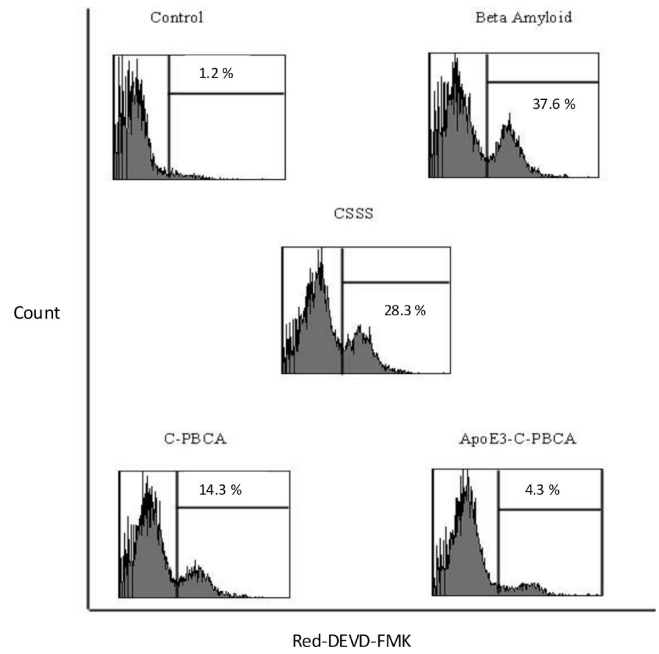

Figure 11. Caspase-3 assay. Reduction of beta amyloid induced caspase-3 by treatment with curcumin solubilized surfactant solution (CSSS), curcumin loaded PBCA nanoparticles (C-PBCA) and ApoE3 mediated curcumin loaded PBCA nanoparticles (ApoE3−C-PBCA) was determined.

ApoE is one of the major constituents of amyloid plaques in the brain of patients with AD. There are three common alleles of human apolipoprotein E, namely, E2, E3 and E4. Out of this, E4 is most frequently found in the brains of AD and considered to be responsible for the aggravation of oxidative stress.[21,22] On the other hand, ApoE2 and ApoE3 possess antioxidant and antiamyloidogenic activity with ApoE2 being the strongest.[19−22] But, ApoE2 has much less affinity to the LDL-R compared to ApoE3.[18,19] Hence, considering the antioxidant, antiamyloidogenic potential and high affinity to the LDL-R, ApoE3 was selected as ligand. Since curcumin also possesses very strong antioxidant and antiamyloidogenic activity, our basic idea in designing an ApoE3 mediated drug delivery system was to explore the possible synergistic effect of both ApoE3 and nanoencapsulated curcumin against Aβ induced cytotoxicity along with the targeting. Our attempt is to maximize the uptake of curcumin through targeted nanoparticles which also have ability to protect photodegradation of curcumin and sustained drug release.[2,7,14,39]

The results of physical characterization of C-PBCA and ApoE3−C-PBCA were found to be satisfactory and effective, indicating an efficient drug delivery system. The stability of curcumin was increased significantly with nanoparticles compared to plain curcumin solution. The *in vitro* drug release study showed the capability of nanoparticles to provide sustained drug release[24] (data not shown). The method employed for the conjugation of ApoE3 with C-PBCA was found to be effective and efficient. Some of the previous reports suggest that ApoE gets adsorbed on the surface of nanoparticles coated with Tween 80, though the exact mechanism is not yet fully understood.[36,40,41] We have studied the adsorption of ApoE3 on the surface of C-PBCA with and without Tween 80. It was observed that the adsorption of ApoE3 on C-PBCA was more in the case of Tween 80 coated C-PBCA. The conjugation of ApoE3 to C-PBCA was quantified using the Bradford assay, which confirmed the efficiency of conjugation.

The protective effect of C-PBCA and ApoE3−C-PBCA against Aβ induced cytotoxicity was studied using a cell survival assay. The results suggest that both C-PBCA and ApoE3−C-PBCA were more protective against Aβ compared to CSSS. Further, the therapeutic efficacy of curcumin with

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(41) Petri, B.; Bootz, A.; Khalansky, A.; Hekmatara, T.; Müller, R.; Uhl, R.; Kreuter, J.; Gelperina, S. Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: Revisiting the role of surfactants. *J. Controlled Rel.* **2007**, *117*, 51–58.

ApoE3−C-PBCA compared to C-PBCA was markedly enhanced, suggesting the increased uptake of curcumin with ApoE3−C-PBCA by receptor mediated endocytosis[20] and the possible synergistic antiamyloidogenic effect of ApoE3 and curcumin.[2,21] To support the antiamyloidogenic activity of ApoE3, we performed a separate experiment by treating the cells with ApoE3 alone. An increase in cell survival with ApoE3 alone was observed from this experiment, confirming the possible synergistic activity of ApoE3 and curcumin in ApoE3−C-PBCA. Over the entire dose range used in the study, 25, 50, and 100 nM concentrations showed significant difference ($p < 0.05$) in the protective effect among all the formulations. Moreover, at higher dose range, i.e. at 400 and 800 nM, an increase in cytotoxicity was observed, owing to the apoptosis inducing property of curcumin. Hence, the results of our cell survival assay supported the dose dependent antioxidant and pro-oxidant effect of curcumin on SH-SY5Y cells.[36] The most possible reason behind the cytotoxicity at higher doses is induction of apoptosis by various mechanisms such as, caspase-3 activation, ROS induction, DNA damage etc.[36,37] We have already studied this apoptotic effect using our curcumin formulations on SH-SY5Y cells (not included). The protective effect of curcumin at doses lower than 25 nM was almost stagnant, hence not included (data not shown). The cytotoxicity of B-PBCA and SS was also studied, and it was observed that the blank systems are not toxic to the cells over the entire dose range. Hence, the observed protective effect was indeed because of curcumin. Also, the receptor blocking experiment revealed that the cell uptake of ApoE3−C-PBCA is through receptor mediated endocytosis.[20]

The effect of all the formulations on ROS was studied, since induction of ROS is one of the major events in Aβ induced cytotoxicity.[6,7] The dose dependent effect on reduction of ROS induced by Aβ treatment was observed using the same dose range as in the cell survival assay. The results were complementing to the cell survival study. ApoE3−C-PBCA showed significantly increased effectiveness in ROS reduction compared to C-PBCA and CSSS. The study of antioxidant activity of ApoE3 alone on Aβ induced ROS in SH-SY5Y cells further confirmed the role of ApoE3 in ApoE3−C-PBCA mediated antioxidant activity. Hence, the possible synergistic effect of ApoE3 and curcumin was further substantiated.

Apoptosis is the major event in Aβ induced cytotoxicity.[4−8] We studied the antiapoptotic effect of prepared formulations on Aβ induced apoptosis using flow cytometric analysis. From the AnnexinV−FITC/PI staining, it was observed that the antiapoptotic activity of ApoE3−C-PBCA was increased 2−3-fold compared to C-PBCA and almost 4-fold compared to CSSS, signifying the enhanced uptake of curcumin with ApoE3−C-PBCA. The qualitative analysis of reduction of Aβ induced apoptosis was studied by Hoechst 33342 staining. It was clearly observed that ApoE3−C-PBCA was the most effective in reducing the Aβ induced apoptosis compared to CSSS and C-PBCA. Involvement of caspase-3 in Aβ induced apoptosis is well documented.[8] In the present study, we have observed the inhibition of caspase-3 induction by Aβ treatment using different curcumin formulations. As expected, more effective caspase-3 inhibition was observed with ApoE3−C-PBCA compared to C-PBCA and CSSS. On similar grounds, the effect on cell cycle was studied using all curcumin formulations. The results were supplemented with the previous observations. ApoE3−C-PBCA was more effective in reducing the subG1 fraction (apoptotic) induced by Aβ compared to C-PBCA and CSSS. In all the assays, ApoE3−C-PBCA was found to be more effective compared to C-PBCA, which might be because of possible synergistic antioxidant and antiamyloidogenic effects of ApoE3 and curcumin and increased cell uptake.

Hence, the present study clearly signifies the need for development of nanoparticulate formulations of curcumin and also the targeting of curcumin to specific target sites.

Conclusion

From the present study we conclude that the prepared nanoparticles are biocompatible and stable with enhanced activity against Aβ induced cytotoxicity. The attachment of ApoE3 to C-PBCA nanoparticles clearly increased the uptake of curcumin into the SH-SY5Y human neuroblastoma cells, hence, enhanced activity compared to plain solution or nontargeted nanoparticles. From the present study, we conclude that the ApoE3 mediated drug delivery system offers great future prospects for antioxidant therapy in neurodegenerative and cardiovascular diseases. In our future studies, we are focusing on the effect of prepared formulations *in vivo* using animal models which will further highlight the effectiveness of proposed targeted drug delivery system.

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