

Chitosan Encapsulation Enhances the Bioavailability and Tissue Retention of Curcumin and Improves its Efficacy in Preventing B[a]P-induced Lung Carcinogenesis



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Abstract

The rate of lung cancer incidence is alarmingly mounting, despite the decline of smoking and tobacco consumption. Recent reports indicate a very high correlation between the growing fast food culture and lung cancer incidence. Benzo[a]pyrene (B[a]P) is a potent carcinogen abundantly present in grilled and deep-fried food and in tobacco smoke. Our previous studies have proved the efficacy of curcumin in curbing B[a]P-induced lung carcinogenesis. However, the poor pharmacokinetic profile of the compound considerably hampers its potential as an effective chemopreventive. This study was intended to evaluate whether encapsulation of curcumin in chitosan nanoparticles can improve the cellular uptake and prolong the tissue retention of curcumin yielding better chemoprevention. The curcumin-loaded chitosan nanoparticles (chitosan nanocurcumin) exhibited a size of 170–200 nm in transmission electron microscopy. *In vitro* drug release

studies showed sustained release of curcumin over a period of approximately 180 hours and excellent intracellular uptake and cytotoxicity in lung cancer cells. Bioavailability studies using healthy Swiss albino mice demonstrated drastic enhancement in lung localization of chitosan nanocurcumin compared with free curcumin. Toxicologic evaluation using chronic toxicity model in Swiss albino mice confirmed the pharmacologic safety of the formulation. Moreover, the formulation, even at a dose equivalent to one fourth that of free curcumin, exhibits better efficacy in reducing tumor incidence and multiplicity than free curcumin, thereby hampering development of B[a]P-induced lung adenocarcinomas in Swiss albino mice. Hence, our study underscores the supremacy of the formulation over free curcumin and establishes it as a potential chemopreventive and oral supplement against environmental carcinogenesis.

Introduction

Lung cancer is the leading cause of cancer-associated mortality world-wide (1). The incidence of lung cancer has always been attributed to the consumption of tobacco and

cigarette smoking (2) and hence, the awareness programs were only focusing on cessation of smoking and consumption of tobacco (3). However, the epidemiologic data indicate that the rate of incidence of lung cancer is increasing despite the low prevalence of smoking or tobacco consumption (4). Several population studies have shown that the recently evolving fast food culture has a very high correlation with cancer incidence, especially that of colon and lung (5).

Benzo[a]pyrene or B[a]P is a polycyclic aromatic hydrocarbon present in deep fried and grilled food (6, 7). It is one of the most potent carcinogens present in tobacco smoke and is a potent embryotoxin and teratogen (8). Studies over the past several years indicate that the amount of B[a]P is dangerously high in deep-fried food, a major attraction in the growing fast food culture. A recent study shows that

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deep-frying methods can generate 10.9 times more B[a]P compared with normal cooking (9). Being both a local and a systemic carcinogen, exposure to B[a]P has been shown to induce cancer, irrespective of the route of administration (10, 11). It increases generation of mitochondrial superoxide and expression of genes such as *Nrf2*, *UCP2*, and *TNF- α* , which are directly associated to oxidative stress and inflammation (12).

B[a]P, being a potent carcinogen associated with food, chemoprevention of lung cancer using dietary phytochemicals will be an interesting attempt to curtail the prevalence of lung cancer in susceptible population. Chemoprevention is the process of preventing the development of cancer, by blocking one or more stages of carcinogenesis using nontoxic, natural, or chemical compounds (13, 14). Curcumin is a chemopreventive agent, which possesses multifaceted potentials to prevent cancer progression (15). There are several studies demonstrating the efficacy of curcumin in preventing cancer progression induced by protumorigenic agents and environmental mutagens (16, 17). Several reports, including those from our laboratory, have shown the chemopreventive efficacy of curcumin against lung cancer (17, 18). Although there are ample reports highlighting the anticancer potential of curcumin, the major hindrance in employing it for cancer therapy and prevention, stems from its poor aqueous solubility and bioavailability (19).

Nanoparticle-mediated delivery of curcumin has been shown to improve its pharmacokinetic profile, thereby improving its chemotherapeutic potential (19–22). Various nanodrug delivery vehicles being employed for curcumin delivery include: poly(lactic-co-glycolic acid) (PLGA; ref. 22), poly(lactide)-vitamin ETPGS copolymer, alginate nanoparticles (23), soy protein nanoparticles (24), poly(vinyl pyrrolidone) conjugate micelle (25), and α -cyclodextrin derivatives (26). Besides the nature of the drug, the choice of material relies largely on its physicochemical properties and route of administration (27). Recent studies from our group have shown that encapsulation of curcumin in PLGA-PEG nanoparticles can greatly improve its aqueous dispersion and bioavailability leading to tremendous augmentation of its therapeutic and chemosensitizing efficacy (22, 28). However, PLGA being very costly, usage of this particle as a drug delivery vehicle for long time use, as in the case of chemoprevention, is not economically feasible. Hence, in this study, we selected chitosan, a more economic and biocompatible nanocarrier, which can also be administered orally in a susceptible population. Moreover, being a mucoadhesive, chitosan is a better nanocarrier of curcumin for chemoprevention, compared with other types of nanocarriers (29, 30). Chitosan is a linear polysaccharide produced by the deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp). Chitosan and its chemically modified variations such as carboxymethyl

chitosan are well-explored nanocarriers (31–34) because of their biodegradable and biocompatible nature, and ability to encapsulate hydrophobic drugs (35).

In this study, we synthesized and characterized chitosan nanoparticles loaded with curcumin by ionic gelation method using tripolyphosphate (TPP) as a cross-linker. We hypothesized that curcumin encapsulated in chitosan nanoparticles may exhibit better bioavailability and tissue retention than free curcumin, so that it may act as a better chemopreventive. To validate this hypothesis, we employed B[a]P-induced lung carcinogenesis model using Swiss albino mice. Our study demonstrated that curcumin-loaded chitosan nanoparticles (chitosan nanocurcumin) exhibited better chemopreventing efficacy compared with that of free curcumin.

Materials and Methods

Cells

Lung cancer cell line, H1299 was a kind gift from Dr. Bharat Aggarwal (MD Anderson Cancer Centre, Houston, TX) and was cultured under standard culture conditions.

Materials

Chitosan, TPP, DMSO, acetone, curcumin, diaminobenzidine, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma-Aldrich. MTT ([3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]) was purchased from Calbiochem. DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride) and antibodies against p65, PCNA, and pERK were purchased from Santa Cruz Biotechnology. Super Sensitive Polymer-HRP IHC Detection System and Mouse-on-Mouse Iso-IHC Kit were purchased from Biogenex. DMEM and streptomycin sulphate were purchased from Invitrogen Corporation. All other reagents were procured from Sigma-Aldrich, unless otherwise mentioned. All solvents used in this study were of analytical grade.

Preparation and characterization of curcumin-loaded chitosan nanoparticles

Chitosan nanocurcumin and blank chitosan nanoparticles were formulated as reported earlier (36). Chitosan (500 mg) was dissolved in 2% v/v acetic acid solution (50 mL) and mixed with curcumin in ethanol (1 mg/mL). TPP solution (15 mL, 1% w/v) was added to it drop by drop, under constant magnetic stirring. The solution was then stirred for further 1 hour and centrifuged at 10,000 rpm for 30 minutes. The pellet obtained was resuspended in water and further lyophilized to obtain curcumin-entrapped chitosan nanoparticles. The same protocol was followed for blank nanoparticles with no addition of curcumin. The size and morphology of nanoparticles formed were

analyzed using transmission electron microscopy (TEM). The nanoparticle suspension was diluted in Milli-Q (Millipore Corporation) water at 25°C and drop-casted onto formvar-coated grids and analyzed using TEM (JEOL 1011).

Administration of curcumin

For all *in vitro* studies, chitosan nanocurcumin was dispersed in aqueous media and free curcumin was dissolved in DMSO. For *in vivo* studies, both chitosan nanocurcumin and free curcumin were administered orally in autoclaved water and corn oil, respectively.

Differential scanning calorimetry analysis of chitosan nanoparticles

Differential scanning calorimetry (DSC) was done to analyze the thermal behavior of the blank chitosan nanoparticles and chitosan nanocurcumin. DSC thermograms obtained were analyzed using an automatic Thermal Analyzer System (Pyres 6 DSC, PerkinElmer). Samples were placed in standard aluminum pans and heated from 20°C to 250°C at a rate of 10°C/minute under constant purging of N₂ at 10 mL/minute. An empty pan, sealed in the same way as that of the sample, was used as a reference.

In vitro release kinetics of curcumin from chitosan nanoparticles

A known amount of chitosan nanocurcumin was dispersed in 10 mL PBS (pH 7.4) and kept in a shaking incubator at 37 ± 0.5°C. A constant volume of PBS was withdrawn at different time intervals, which was later replaced with the same volume of fresh buffer, to maintain total volume. The amount of curcumin released from the nanoparticles was measured using ultra violet spectrophotometer (PerkinElmer) at 420 nm.

Cell uptake studies

Cellular uptake of free curcumin and chitosan nanocurcumin in H1299 cells was assessed using confocal microscopy. Briefly, 2 × 10⁴ cells were grown on cover slips placed in 24-well plates and treated with free curcumin dissolved in DMSO (25 μM) or chitosan nanocurcumin (25 μM), or their corresponding blanks. After 2 hours, the cells were washed with PBS and fixed using 4% paraformaldehyde. The nuclei were stained using DAPI for 5 minutes and mounted using DPX to detect intracellular fluorescence of curcumin using confocal laser-scanning microscope in the FITC channel (488 nm).

MTT assay

MTT assay was used for assessing the viability of cells treated with chitosan nanocurcumin. Briefly, 3 × 10³ cells were treated with different concentrations of curcumin dissolved in DMSO (5–50 μM) or chitosan nanocurcumin (5–50 μM), or their corresponding blanks for 72 hours. The media was then removed and MTT working solution was

added and incubated for 2 hours. The formazan crystals were lysed by adding lysis buffer for 1 hour, and the optical densities were measured at 570 nm. The relative cell viabilities in percentage were calculated as mentioned elsewhere (37).

Clonogenic assay

Clonogenic assay was performed to compare the antitumor potential of free curcumin or chitosan nanocurcumin, or blank chitosan nanoparticles. Briefly, approximately 3 × 10³ cells were seeded in 12-well plates, and treated with the respective samples for 72 hours. Later, fresh medium was added and incubated for 1 week. The clones developed were fixed with glutaraldehyde and stained using crystal violet. The clones were then viewed under microscope (Leica DM 1000) and were photographed.

Toxicity studies

Because the biological safety of curcumin has already been established by several groups (17), we focused on evaluating whether chitosan nanoparticles cause any chronic toxicity on long term use. For toxicity evaluation, we selected the dose, which we used for the chemopreventive study (0.5% diet). Chronic toxicity of chitosan nanoparticles was conducted in healthy Swiss albino mice after obtaining the approval from Institutional Animal Ethics Committee (Reference number: IAEC/224/RUBY/2013). The animals were divided in to two groups of 5 each. Animals of group I were given corn oil before normal diet on alternative days and group II mice were fed with 0.5% dosage of void nanoparticles on alternative days for 4 months. All the mice were sacrificed at the end of the experiment and serum was isolated for biochemical analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), and liver was isolated for histopathologic verification. After sacrificing the animals using CO₂ euthanasia, the liver tissues, which were isolated from mice (toxicity model) were fixed in 4% paraformaldehyde and were transferred to 30% sucrose solution, until the tissues sank. The tissues were then cryo-sectioned after embedding in optimum cutting temperature formulation (OCT). Sections of 7 μm thickness were obtained using Leica CM 1850 UV cryostat on to a gelatin-coated micro-slide and were kept in –80°C until further analyses. The tissue sections were then used for hematoxylin and eosin (H&E) staining.

In vivo bioavailability studies

In vivo bioavailability of curcumin loaded in chitosan nanoparticles in mouse lung tissues was analyzed using confocal microscopy. Aqueous suspension of chitosan nanocurcumin or free curcumin dissolved in corn oil (both 25 mg/kg) was administered orally to Swiss albino mice. Control group was given blank nanoparticles.

The animals were euthanized using CO₂ after the desired time interval and lung tissues were collected. The tissue sections were stained using DAPI and mounted using fluoromount. The fluorescence of curcumin was visualized and images were captured using Nikon A1R confocal microscope at FITC channel (488 nm) and images were analyzed using NIS elements software.

Evaluation of the chemopreventive efficacy of chitosan nanocurcumin or free curcumin formulations in B[a]P-induced lung carcinogenesis

The efficacy of different formulations of curcumin in downregulating B[a]P-induced lung carcinogenesis was assessed in male Swiss albino mice after obtaining the approval from Institutional Animal Ethics Committee (IAEC/171/RUBY/2012). Briefly, the animals were divided into five groups ($n = 15$). The control animals (group I) received corn oil. Group II animals were given void chitosan nanoparticles (0.5% of diet) dissolved in aqueous media, for 16 weeks. Group III animals received B[a]P (50 mg/kg) dissolved in corn oil, twice weekly, for a period of 4 weeks as oral gavage. Group IV animals were given B[a]P (50 mg/kg) dissolved in corn oil, twice weekly, for a period of 4 weeks along with free curcumin (2.0% of diet), on alternate days for a period of 16 weeks. Group V animals were given B[a]P (50 mg/kg) dissolved in corn oil, twice weekly, for a period of 4 weeks along with chitosan nanocurcumin (0.5% of diet; one fourth the dose of free curcumin) on alternate days for a period of 16 weeks. As the experiment was intended to study the chemopreventive efficacy of curcumin, both free curcumin and chitosan nanocurcumin administration was started 1 week prior to B[a]P treatment. Tumor samples were taken and washed in ice cold PBS, and were fixed using 4% paraformaldehyde. After processing with 30% sucrose, the tissues were cryo-sectioned using Leica CM 1850 UV cryostat. The tissue sections were used for histopathology using H&E staining and IHC using standard protocols.

IHC of lung tissue sections

Immuno-localization of specific proteins in the lung tissue sections was done using the Mouse-on-Mouse Iso-IHC Kit (Biogenex). Paraformaldehyde-fixed OCT-embedded tissue sections that were kept at -80°C were brought to room temperature. They were then kept in PBS or PBS-T (PBS with 0.1% Tween-20) for 15 minutes. Antigen retrieval was done using heat-induced antigen retrieval method using citrate buffer. Nonspecific antibody-binding sites on tissue sections were blocked by appropriate reagents supplied with the kit. The primary antibody prediluted in 5% BSA was added enough to cover the sections and were incubated for 12 hours at 4°C . The unbound primary antibody was washed off with PBS-T. The sections were then covered with streptavidin-horse-radish peroxidase conjugate, incubated for 20 minutes at

room temperature, and rinsed with PBS-T. Immunostaining was visualized using diaminobenzidine chromogen, counterstained with Mayer hematoxylin, and the sections were mounted using SuperMount mounting medium. Photomicrographs were captured using a Leica DM 1000 microscope.

Results

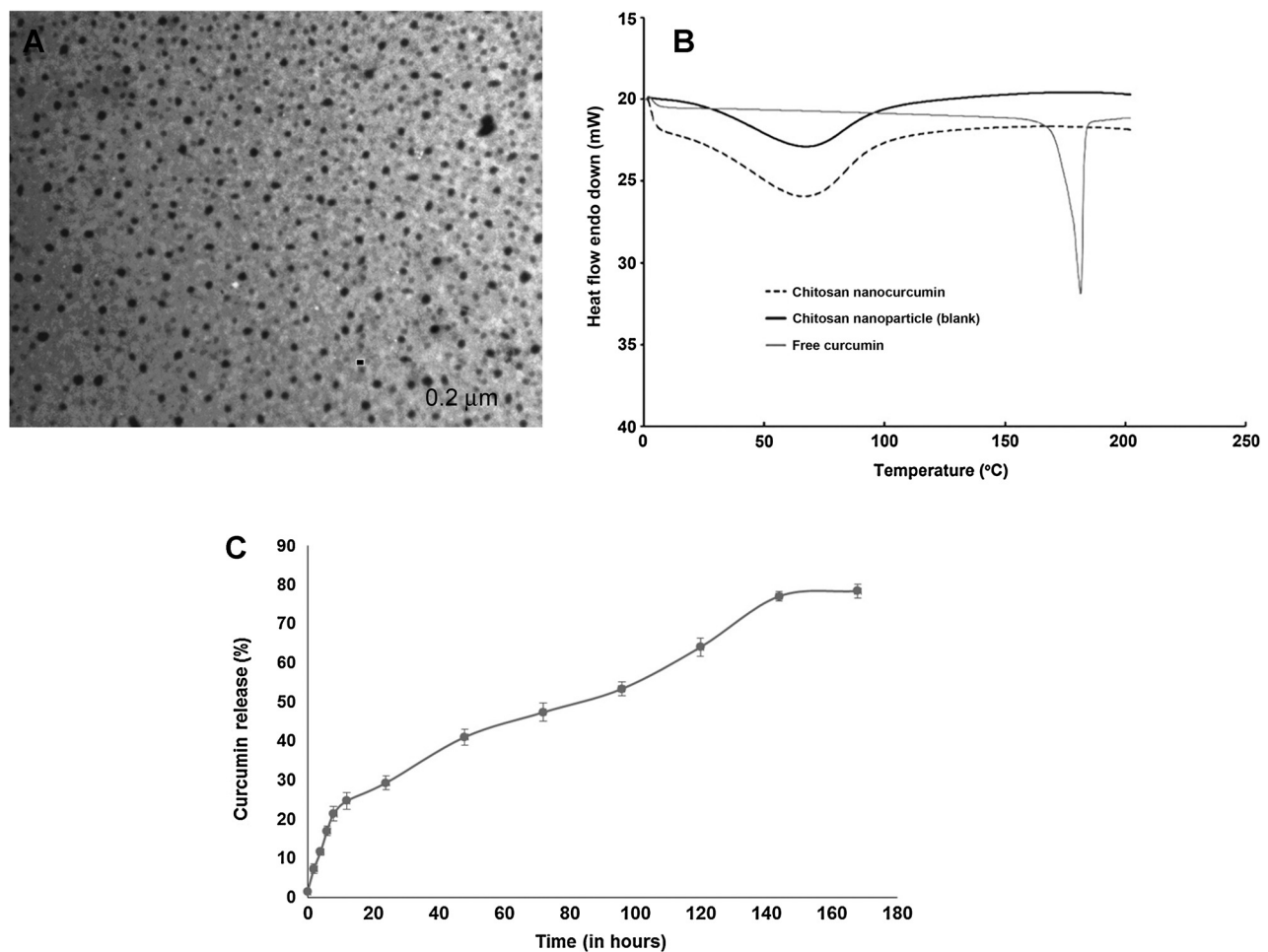
Synthesis and characterization of chitosan nanocurcumin

Chitosan nanocurcumin was prepared by ionic gelation method using TPP as a cross-linker. TEM analysis revealed that the size of the particles ranges between 170 nm and 200 nm (Fig. 1A). The thermal stability of nanoparticles was assessed by DSC analysis, the result of which is shown in Fig. 1B. Cross-linked chitosan shows an endothermic peak at 68°C and curcumin-entrapped chitosan nanoparticles shows the disappearance of endothermic peak of curcumin indicating that the drug is uniformly dispersed at the molecular level in the polymeric matrix. The *in vitro* release kinetics had shown a sustained and controlled release of curcumin from the nanoparticles (Fig. 1C). An initial release of approximately 12% was shown within 4 hours and approximately 29% within 24 hours, which is indicative of slow and sustained release of curcumin. After 5 days, curcumin release from the nanoparticles became stable and attained a plateau phase.

Curcumin loaded in chitosan nanoparticles exhibited better intracellular uptake of curcumin and cytotoxicity compared with that of free curcumin

Intracellular uptake of aqueous dispersible, chitosan nanocurcumin was compared with that of free curcumin dissolved in DMSO, in the lung cancer cell line, H1299. As clearly visible in the confocal images (Fig. 2A), the aqueous dispersible, chitosan nanocurcumin (25 μM) was readily taken up by the cells and the intracellular fluorescence was much higher compared with that of free curcumin (25 μM) for a period of 2 hours. The enhanced fluorescence can be attributed to the positive charge of chitosan nanoparticles, which aids in better cell interaction with the negatively charged cell membrane and hence better cellular uptake and curcumin localization (31).

We analyzed the cytotoxic effect of chitosan nanocurcumin with that of free curcumin in H1299 cells using MTT assay (Fig. 2B). Void nanoparticles of chitosan were kept as control. Although both free curcumin and chitosan nanocurcumin caused significant cytotoxicity in a concentration-dependent manner, the effect produced by that of chitosan nanocurcumin was slightly better. However, it should be noted that chitosan nanocurcumin was administered as aqueous suspension, whereas free curcumin was dissolved in DMSO, which is not an

**Figure 1.**

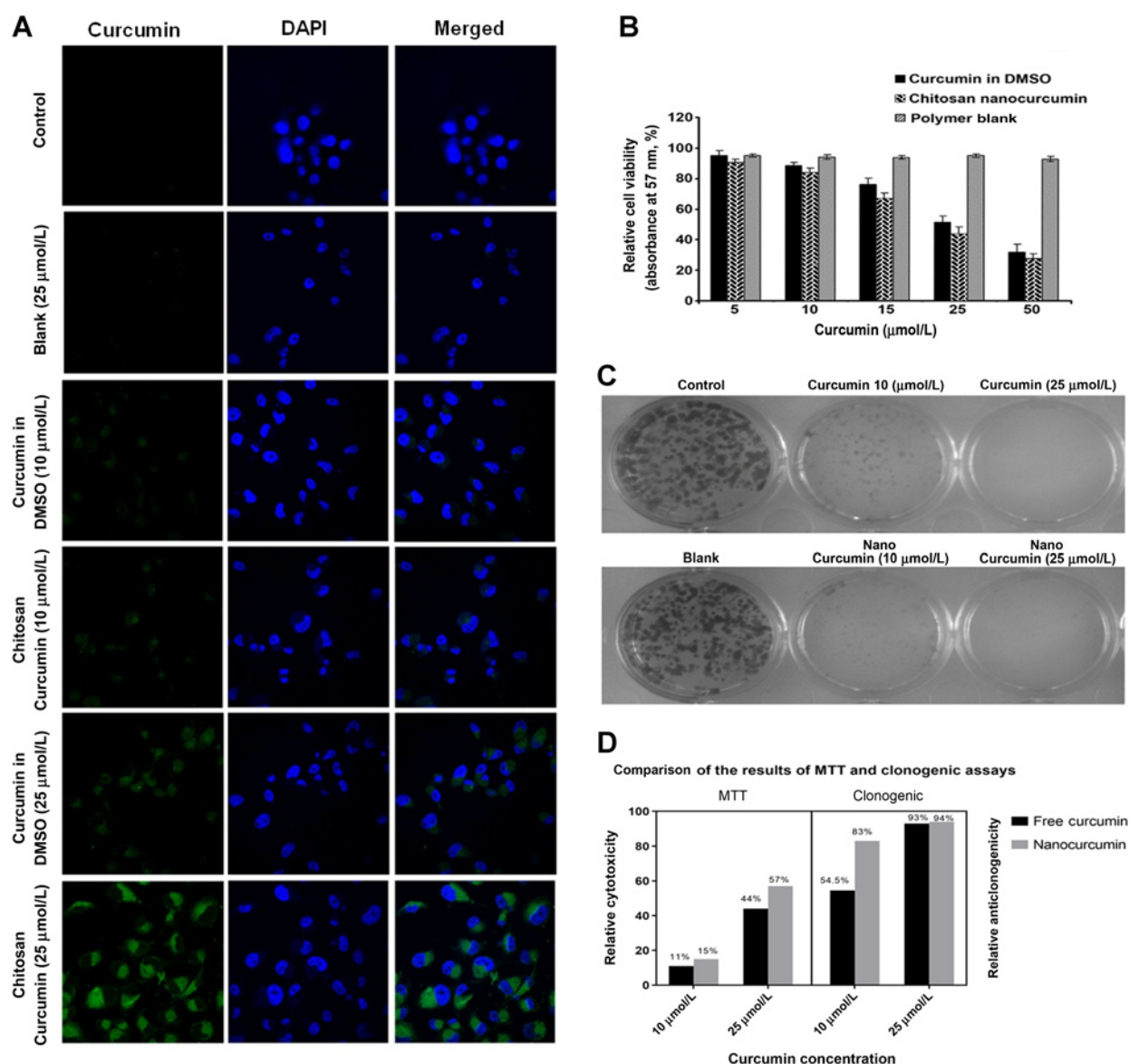
Physicochemical characterization of chitosan nanocurcumin. **A**, TEM image of chitosan nanocurcumin. **B**, DSC analysis of chitosan nanocurcumin and chitosan nanoparticles. **C**, *In vitro* release of curcumin from chitosan nanocurcumin.

advisable solvent for *in vivo* studies. Furthermore, chitosan nanocurcumin also inhibited the clonogenic potential of H1299 cells in a dose-dependent manner (Fig. 2C). Here we noted a significant difference in the anticlonogenic potential of free curcumin and chitosan nanocurcumin, although the cytotoxicity results of both were just comparable at 25 μ M (44% and 57%). The inhibition in clonogenicity produced by 10 μ M nanocurcumin (83%) is considerably higher than that produced by the same amount of free curcumin (55%; Fig. 2D) and the percentage difference in cytotoxicity produced by both forms of curcumin, as assessed by MTT assay (72 hours exposure) is much less compared with that assessed by clonogenic assay, although the incubation time with the drug is 72 hours in both the experiments, indicating that chitosan nanoparticles, which were internalized by the cells release the encapsulated curcumin very slowly, so that curcumin is available to the cells for 7 more days to produce better efficacy.

Chitosan nanoparticles was found to be pharmacologically safe as assessed by *in vivo* toxicologic studies

To evaluate the biological safety of chitosan nanoparticles, chronic toxicity study was conducted in Swiss albino mice. Briefly, the animals were divided into two groups of 5 each. Animals of group I were given corn oil in normal diet on alternative days and those of group II were fed with 0.5% dosage of void nanoparticles in water on alternative days for 4 months. All the mice were sacrificed at the end of the experiment, and serum and liver were isolated for biochemical and histopathologic analysis, respectively. The serum levels of the enzymes, ALT, AST, and ALP, elevation which are indicative of drug-induced liver damage, were well within the normal range, in the animals treated with chitosan nanoparticles, confirming the pharmacologic safety of the particles (Fig. 3A). Histopathologic verification of the liver sections also illustrated the biocompatibility of chitosan nanoparticles (Fig. 3B).

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**Figure 2.**

Intracellular uptake and cytotoxicity of chitosan nanocurcumin. **A**, H1299 cells were incubated with free curcumin and chitosan nanocurcumin (equivalent to 25 μmol/L curcumin) for 4 hours and images were captured using confocal laser scanning microscopy. **B**, Cell viability of H1299 cells treated with varying concentrations of free curcumin/chitosan nanocurcumin/blank formulations for 72 hours and MTT assay was performed. OD was measured at 570 nm and relative cell viabilities were plotted. **C**, Chitosan nanocurcumin inhibits the clonogenic potential of H1299 cells more efficiently than free curcumin. H1299 cells were treated with different formulations of curcumin as indicated, for 72 hours and clonogenic assay was performed. **D**, Graph comparing the efficacy of chitosan nanocurcumin and free curcumin using MTT and clonogenic assays.

Chitosan nanocurcumin exhibited enhanced lung localization compared with free curcumin

We evaluated and compared the lung localization capacity of curcumin when administered orally as aqueous suspension of chitosan nanocurcumin and as free curcumin in corn oil (equivalent to 25 mg/kg curcumin), by collecting the lungs after 1 or 2 hours. The lung sections were stained with DAPI and observed under confocal laser-scanning microscope. It was interesting to

see that, curcumin retention in the lungs of mice fed with chitosan nanocurcumin was much better than that fed with free curcumin. Moreover, the fluorescence of curcumin in the lung tissues of mice fed with chitosan nanocurcumin increased from 1 to 2 hours, while in mice fed with free curcumin, only feeble fluorescence of curcumin could be recorded, which decreased over a period of time, and almost completely faded by 2 hours (Fig. 4A and B).

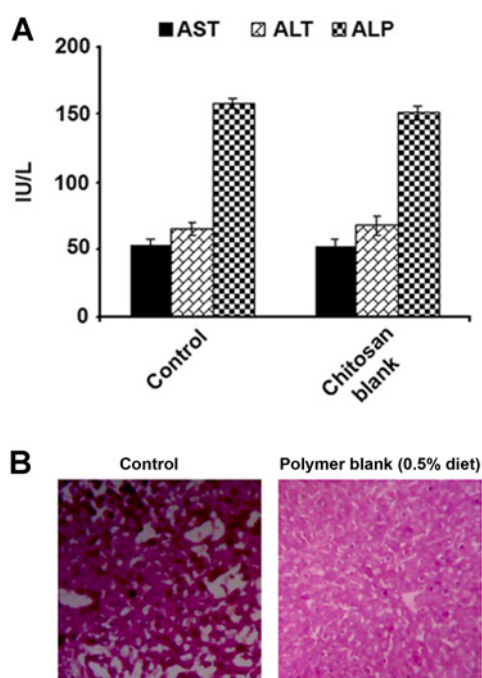


Figure 3. Chitosan nanoparticles do not induce liver toxicity in mice. Biochemical and histopathologic analyses of serum and liver sections of mice injected with chitosan nanoparticles biochemical analysis (A) and histopathology (B).

Chitosan nanocurcumin is more potent than free curcumin in reducing B[a]P-induced lung carcinogenesis in Swiss albino mice

The chemopreventive potential of chitosan nanocurcumin against B[a]P-induced lung carcinogenesis was evaluated in Swiss albino mice. The schematic representation of the experimental pattern is given in Fig. 5A. Because the

study was intended to evaluate the chemopreventive potential of chitosan nanocurcumin/curcumin, both forms of curcumin were administered 1 week prior to B[a]P treatment, on alternative days, and continued for up to 4 months. Free curcumin was administered as 2.0% of diet and chitosan nanocurcumin was administered as 0.5% of diet orally as gavage. B[a]P was dissolved in corn oil and administered orally as gavage, twice weekly for a period of 1 month. After the treatment period, the lungs were excised to visualize the nodules formed. There were no nodules developed in the control and blank groups, as expected. The number of nodules in the lungs of B[a]P-treated groups were compared with that of groups treated with B[a]P and free curcumin or chitosan nanocurcumin. While 73% of mice, which were given only B[a]P-developed lung tumors, 57% of mice treated with free curcumin and 35% of mice treated with chitosan nanocurcumin, along with B[a]P, developed lung tumors. As indicated by the arrows, the number of nodules in the B[a]P-treated group (29.4) were significantly high compared with that of treatment groups. Interestingly, the average number of nodules per mice in chitosan nanocurcumin-treated group (8.4) was found to be significantly less compared with that of free curcumin-treated group (15.6; Table 1). While free curcumin produced 22% reduction in tumor incidence and 46.8% reduction in tumor multiplicity, one fourth the dose of chitosan nanocurcumin produced 52% reduction in tumor incidence and 71.4% reduction in tumor multiplicity compared with the group administered with B[a]P only (Table 1.) This reduction in number of nodules in chitosan nanocurcumin-treated group may be attributed to the increased bioavailability of curcumin to the lungs. It was really exciting to note that even one fourth the dose of chitosan nanocurcumin exhibited better efficacy than free curcumin in inhibiting B[a]P-induced lung

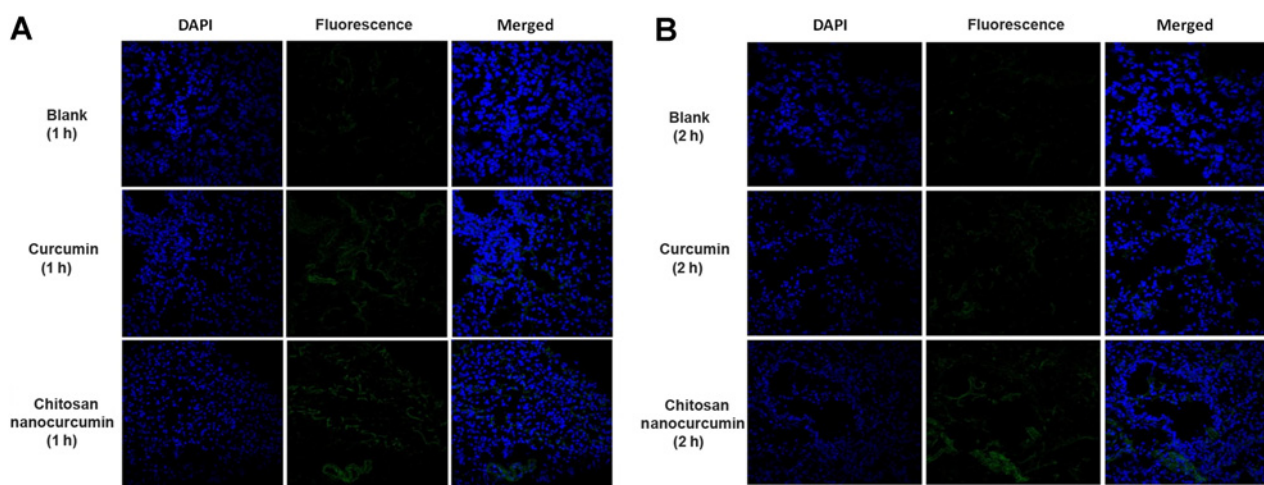


Figure 4. Bioavailability of curcumin/chitosan nanocurcumin in lungs. Confocal microscopy images showing curcumin fluorescence in the lung tissue sections of mice, which were orally administered with free curcumin or chitosan nanocurcumin for different time durations 1 hours (A) and 2 hours (B).

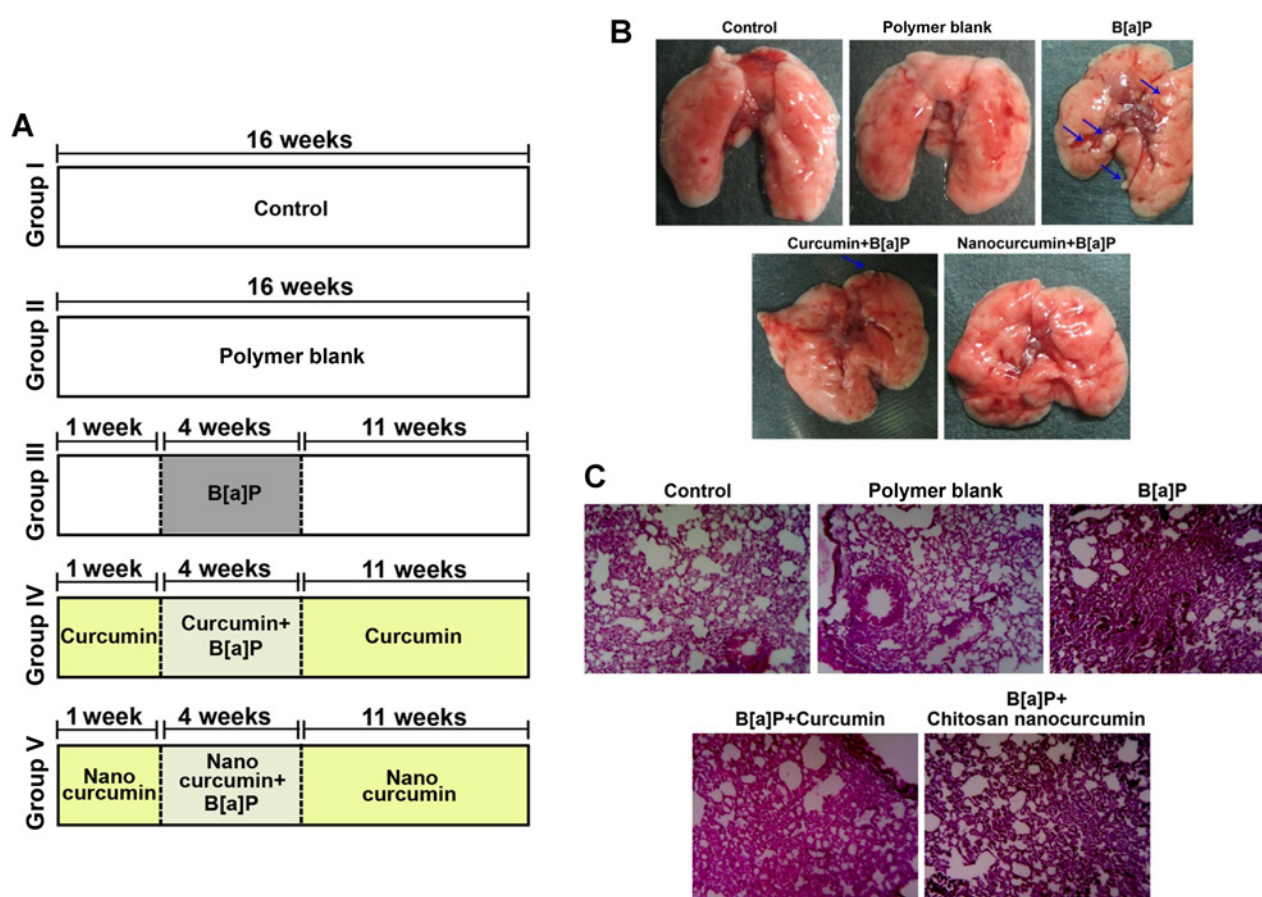


Figure 5. *In vivo* chemoprevention studies. **A**, Schematic representation of chemoprevention study using curcumin chitosan nanoparticles in B[a]P-induced lung carcinogenesis model. **B**, Curcumin or chitosan nanocurcumin inhibits B[a]P-induced lung carcinogenesis. Representative images of lungs from different treatment groups. **C**, H&E staining of lung tissue sections from various treatment groups.

carcinogenesis. Figure 5B shows the representative images of lungs of animals from various treatment groups. The nodules developed on the lungs were confirmed as adenocarcinomas histopathologically, by H&E staining. The nodules show typical features of adenocarcinoma; size larger than 5 mm, atypia in cells and occasional mitoses

Table 1. Comparative analysis of tumor incidence in various treatment groups

Group (N = 15)	Tumor incidence		Tumor multiplicity	
	% Incidence	% Reduction	Average number of tumors/mice	% Reduction
Control	NA	NA	NA	NA
Chitosan blank	NA	NA	NA	NA
B[a]P	73.3	NA	29.36 ± 3.4	NA
B[a]P+ Free curcumin (2% diet)	57%	22%	15.62 ± 3.6	46.8%
B[a]P+ Chitosan nanocurcumin (0.5% diet)	37.5%	52%	8.4 ± 1.5	71.4%

NOTE: Table showing tumor incidence and tumor multiplicity. The percentage reduction was calculated taking B[a]P group as positive control. One fourth the dose of chitosan nanocurcumin downregulates B[a]P-induced lung tumorigenesis more efficiently than free curcumin.

with infiltrative borders. Histopathologic comparison of the lung tissue sections from different groups illustrates better reduction in the number of mitoses, size of nodules, and better differentiation of tumor cells in the group treated with chitosan nanocurcumin confirming the enhancement in chemoprevention, compared with free curcumin (Fig. 5C), which may be further attributed to the enhanced tissue retention and lung localization of chitosan nanocurcumin (Fig. 4), which in turn may be responsible for its enhanced bioavailability.

Chitosan encapsulation enhances the efficacy of curcumin in preventing B[a]P-induced upregulation of survival signals in lung cancer tissues

The improved efficacy of chitosan nanocurcumin inhibiting the cell proliferation in B[a]P-induced lung cancer, was examined by comparing the expression level of proliferating cell nuclear antigen (PCNA) in the lung tissue sections from different groups of mice. PCNA is a crucial player in DNA replication of eukaryotes and its overexpression is a marker of tumor progression (38). IHC

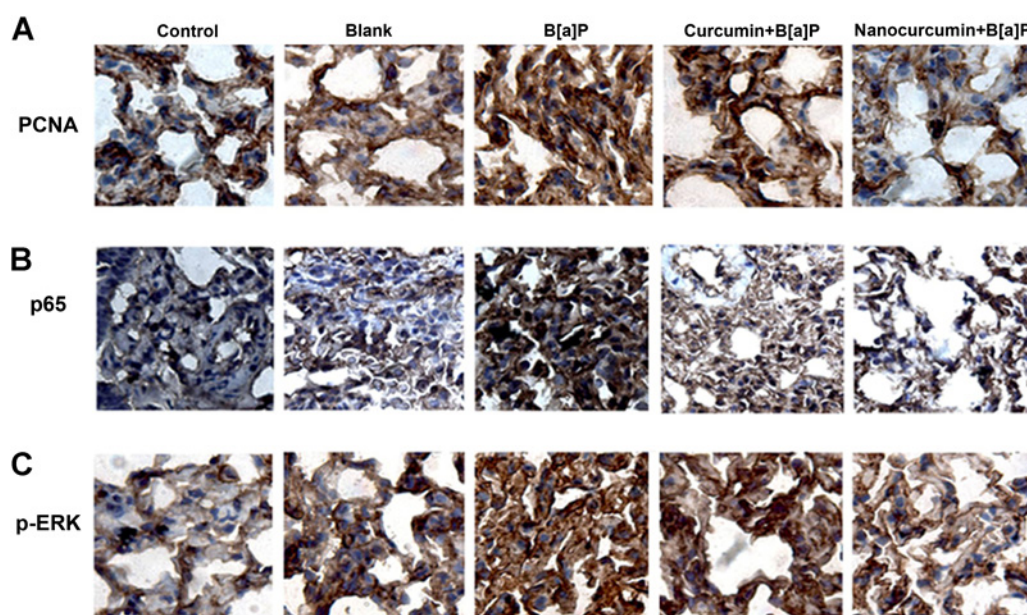


Figure 6.

Downregulation of B[a]P-induced survival and proliferative signals by curcumin/chitosan nanocurcumin: IHC staining of the proliferation marker PCNA (A), NFκB subunit p65 (B), and phosphorylated ERK (p-ERK; C) in the lung tissues of mice in different treatment groups.

analysis revealed that, B[a]P induces overexpression of PCNA in the lung tissue. Treatment of both free and chitosan nanocurcumin efficiently reduced B[a]P-induced overexpression of PCNA, where chitosan nanocurcumin produced considerably better reduction compared with free curcumin (Fig. 6A). It is a well-known fact that curcumin can considerably downregulate the activation of the transcription factor, NFκB (39). Intracellular localization of the NFκB subunit, p65, is an indicator of NFκB activation. As expected, IHC analysis of the normal lung tissue sections from control group and void blank group showed p65 positivity in the cytoplasm whereas, the group treated with B[a]P alone exhibited strong expression of p65 in the nuclei. It is very evident from Fig. 6B that chitosan nanocurcumin is more efficient in inhibiting B[a]P-induced nuclear translocation of p65, than free curcumin. The phosphorylation of ERK, which belongs to MAPKs family of proteins offers immense survival advantage to the cancer cells, and has been reported as a critical player in lung carcinogenesis and progression (39). As observed in the case of NFκB, B[a]P-induced phosphorylation of ERK in the lung tissues is efficiently downregulated by curcumin and the extent of downregulation brought about by chitosan nanocurcumin is extensively high compared with that by free curcumin (Fig. 6C). Taken together, the results of this study clearly demonstrate that, encapsulation of curcumin in chitosan nanoparticles extensively enhances its efficacy as a chemopreventive, probably due to the enhanced cellular uptake and tissue retention of curcumin *in vivo*, thereby enhancing its availability at the target site, for chemopreventive action.

Discussion

Studies indicate that despite the decline of tobacco use, lung cancer incidence is alarmingly rising world-wide, presumably due to the consumption of deep-fried food, which contains B[a]P, one of the major carcinogens present in cigarette smoke. Ample evidences are available regarding the carcinogenic potential of B[a]P (8). B[a]P can cause oxidative stress due to reactive oxygen species generation, which can further generate DNA mutations and genome instability (12). Oxidative stress can also induce cell survival, proliferation, angiogenesis, and metastasis (40). Several studies have shown that the consumption of polyphenol-rich fruits and vegetables can prevent cancer (41, 42) due to their antioxidant and anti-inflammatory properties (43). We have published a review, which has compiled several studies on the mechanistic evaluation of the therapeutic efficacy of various phytochemicals (44). There are ample evidences regarding the *in vitro* antitumor efficacy of curcumin, a dietary polyphenol isolated from *Curcuma longa* (45, 46). Being a pharmacologically non-toxic molecule, the chemotherapeutic potential of curcumin seems attractive. However, the efficacy of this molecule is hindered by its poor solubility in water, rapid intestinal metabolism, and short half-life in the circulation making its bioavailability below the threshold level. Commendable efforts have been made to overcome these limitations of curcumin, to exploit its anticancer benefits, among which, nanoencapsulation is one of the most promising approaches (47–49). Even though, nanoencapsulation of curcumin is found very effective, its translation

into clinics is largely affected by the cost-associated burden involved in the large scale production. It is against this backdrop of issues that the properties of chitosan nanoparticle assume significance, with its enhanced cost effectiveness, low toxicity, and enhanced biodegradability (50). Moreover, it can be well suited for oral administration due to its mucoadhesive property, which renders a higher resident time, which in turn enables a better cellular uptake (51).

Similarly, the positive charge of chitosan causes its selective attachment on tumor surfaces (52). The observed slow release kinetics of curcumin from curcumin-entrapped chitosan nanoparticles suggests that, it can be used as an effective drug delivery system for sustained and controlled release of curcumin. According to the cell viability data obtained in this study, void chitosan nanoparticles used for delivering even double the amount of IC₅₀ concentration of curcumin did not exhibit any antiproliferative effect authenticating its non-toxic nature. Moreover, chronic toxicity studies conducted in mouse model attest the pharmacologically safety of these nanoparticles. Results of cytotoxicity and clonogenicity assays conducted in lung cancer cells confirmed the supremacy of chitosan encapsulation. This may be due to the enhanced uptake of chitosan nanocurcumin enabled by the positive charge of chitosan. Corroborating this hypothesis, significant amount of chitosan nanocurcumin was localized in the lungs upon oral administration. The significance of using B[a]P to induce carcinogenesis in Swiss albino mice is worth mentioning. B[a]P has a strong ability to induce tumorigenesis in animal models. As reported earlier, a concentration of 50 mg/kg body weight could induce lung tumor in Swiss albino mice (53). Moreover, B[a]P, is a poly aromatic hydrocarbon present in cigarette smoke, grilled food, and fuel combust and is considered as an environmental factor causing lung cancer. B[a]P-induced carcinogenesis model may, to a certain extent, mimic the actual scenario of lung carcinogenesis. The chemopreventive efficacy of curcumin against B[a]P is attributed to its ability to induce antioxidant and phase II-metabolizing enzymes, which have key protective roles against chemical carcinogenesis and the associated toxicity (54).

Khan and colleagues, have demonstrated that B[a]P administration induces NFκB activation in mice (55). At the molecular level, chitosan nanocurcumin caused significant inhibition of B[a]P-induced upregulation and activation of various prosurvival signals such as PCNA, ERK1/2, and NF-κB in the lung tissues of Swiss albino mice. Chitosan encapsulation, as shown in the results, permits only a slow release of curcumin, which might cause its prolonged retention in tissues. It is safe to assume that chitosan, with such appropriate releasing kinetics, along with its above-mentioned mucoadhesive and charge-based tumor affinity properties, renders the curcu-

min encapsulated in it efficacious in inhibiting the prosurvival signals induced by B[a]P, eventually blocking tumorigenesis. Moreover, it was very exciting to see that, even one fourth of chitosan nanocurcumin (0.5% of diet) exhibited better efficacy than free curcumin (2.0% of diet) in inhibiting the B[a]P-induced lung carcinogenesis. Free curcumin might have undergone fast clearance from circulation as indicated by less curcumin fluorescence in lung tissue sections. Even though this study has selected B[a]P-induced lung carcinogenesis as the model system, curcumin being a universal chemopreventive against cancers induced by various carcinogens, administration of chitosan nanocurcumin as a food supplement may prevent almost all types of environmental carcinogenesis. Taken together, the results of this study demonstrate that chitosan nanocurcumin as an oral chemopreventive against environmental carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: V. Vijayakurup, V.B. Liju, R.J. Anto
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Vijayakurup, V.V. Alex, V.B. Liju, R.J. Anto
Writing, review, and/or revision of the manuscript: V. Vijayakurup, A.P. Retnakumari, V.V. Alex, V.B. Liju, R.J. Anto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.T. Thulasidasan, J. Antony, V.B. Liju
Study supervision: R.J. Anto
Other (synthesis of chitosan nanocurcumin): J. Somaraj, G.S.V. Kumar
Other (performed histopathology): S. Sundaram

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Chitosan Encapsulation Enhances the Bioavailability and Tissue Retention of Curcumin and Improves its Efficacy in Preventing B[a]P-induced Lung Carcinogenesis

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