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ORIGINAL ARTICLE

Curcumin loaded self assembled lipid-biopolymer nanoparticles for functional food applications

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Abstract The supramolecular nano-assemblies formed by electrostatic interactions of two oppositely charged lipid and polymer have been made and used as nanocarriers for curcumin to address its bioavailability and solubility issues. These curcumin encapsulated nano-supramolecular assemblies were characterized with respect to their size (dynamic light scattering), morphology (TEM, SEM), zeta potential (Laser Doppler Velocimetry), encapsulation efficiency (EE), curcumin loading (CL) etc. Stability of the nano-assemblies was assessed at different storage times as a function of varying pH and temperature. The physicochemical characterization of nano-assemblies was performed using Fourier Transform Infra Red Spectroscopy (FT-IR) and Differential Scanning Calorimetry (DSC). The in-vitro antioxidant lipid peroxidation (TBARS), radical scavenging (DPPH, NO, H_2O_2, reducing power) activity assays of powdered curcumin and nano-encapsulated curcumin were performed. It was found that nano-encapsulated curcumin were roughly spherical in shape, presented high positive zeta potential (>30 mV), monodisperse (polydispersity index <0.3), amorphous in nature, stable in the pH range of 2–6 and have enhanced antioxidant potency in comparison to crystalline curcumin in aqueous media. In conclusion, the curcumin encapsulated nanocarriers system has great potential as functional food ingredient of natural origin.

Keywords Supramolecular assemblies · Nano-encapsulation · Aqueous food · Antioxidant

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Introduction

Curcumin is a major polyphenolic nutraceutical ingredient of Turmeric which forms an indispensible part of South East Asian culinary practices. It is a polyphenolic ingredient which is considered as major antioxidant, coloring and flavoring agent in the balanced diet (Yao et al. 2011). Several studies have been published reporting pluripotent pharmacolological actions of Curcumin e.g., anticancer (Ketron and Osheroff 2014), antiretroviral (Barthelemy et al. 1998), anti-inflammatory (Kant et al. 2014), antidiabetic (Zhang et al. 2013), antidepressant (Pathak et al. 2013), anti-alzheimer (Lazar et al. 2013), antioxidant (Khalil and Ali 2011), antibacterial (Gunes et al. 2013), antifungal (Sharma 2012) etc. These varied medicinal benefits also suggest application of this extraordinary molecule as a functional food (nutraceutical) ingredient. Recently there is growing concern worldwide regarding use of synthetic antioxidants and colorants as food additives (Delgado-vargas and Paredes-López 2003). These artificial food additives have shown unfavorable interactions with co-administered food ingredients, cytotoxicity and allergies in several animal studies (Delgado-vargas and Paredes-López 2003). Therefore it is essential to identify natural alternatives to synthetic food additives. It is also essential to notice their physicochemical attributes and composition at varying food environmental conditions. Curcumin has already presented itself as a potential natural alternative to synthetic food additives as evidenced by its widespread use in cooking practices across the globe (Chattopadhyay et al. 2004). However, outstanding attributes of curcumin as functional foods and therapeutics are restricted in aqueous medium owing to its poor aqueous solubility (Tonnesen and Karlsen 1985). The unmet concentrations for antioxidant action, uniform distribution of yellow color and stability in aqueous medium for sufficient duration are some of the major concerns in using this unique nutraceutical spice as dietary antioxidant in aqueous

medium. Such aqueous material may include fruit juices, beverages, soft drinks, milk etc.

Nanoencapsulation has promised to solve the problem of poor aqueous solubility and bioavailibility issues of many nutraceutical ingredients including curcumin in the past e.g., curcumin encapsulated PLGA nanoparticles (Tiwari et al. 2014), curcumin-bound albumin complex (Van Bracht et al. 2014), curcumin encapsulated dextran-chitosan nanoparticles (Anitha et al. 2011), curcumin solid lipid nanoparticles (Singh et al. 2014) etc. Nanoparticulate systems in these studies have presented one or more benefits in comparison to free curcumin with respect to enhanced pharmacological action In vitro *or* In vivo, greater aqueous solubility, enhanced or modified drug release, site specific drug delivery etc. However focused research directed towards the functional food applications of nanotechnology enhanced curcumin formulations at varying environmental conditions and assessment of their antioxidant potential for functional food applications are rare in the literature. The present study aims at preparation of nanoencapsulated curcumin in self assembled nanocarriers of 'Generally Regarded as Safe' (GRAS) grade ingredients and study the effect of changing environmental conditions e.g., pH, temperature, storage time on their physicochemical attributes. These ingredients were biodegradable, biocompatible anionic lipid (soya lecithin) and cationic biopolymer (chitosan). The components of the nanocarrier system for the functional food applications were carefully selected based on loading of the hydrophobic active ingredient, Generally Regarded as Safe (GRAS) status, biocompatibility and some intrinsic therapeutic activities possessed by these materials e.g., Soy lecithin. Soya lecithin is an amphiphilic lipid component of the nanocarrier and has shown numerous benefits like anti-ageing (Ueda et al. 2011), anti-stress effect (Hellhammer et al. 2004), anti-hypercholesterolemia (Mastellone et al. 2000), immunomodulator effect (Miranda et al. 2008) etc. Further, addition of lecithin in aqueous food system like fruit juices in the concentration range of 0.1–2 % has been advocated by various researchers (Oke et al. 2010). It was thought that, lecithin in the food matrix have stabilized various active components in the food matrix such as lycopene in tomato juice by enabling complex formation (Oke et al. 2010). The accessibility of various metabolizing enzymes to active components in the food matrix would be restricted because of such complex formation (Oke et al. 2010). It was also noticed that organoleptic characters of the food such as color, odor and flavor were not affected significantly by lecithin in the mentioned concentration range (Oke et al. 2010). Chitosan, biodegradable and biocompatible polysaccharide of seafood origin was selected as a cationic component of the self assembled nanocarrier system. The use of chitosan in food industry for food preservation has already been established (Vasilatos and Savvaidis 2013; Lee and Je 2013). Further, chitosan is an effective antimicrobial (Champer et al. 2013), antioxidant (Yen et al. 2008) and antifungal agent (Park et al. 2008) which was an added advantage for intended functional food applications. Hence in our viewpoint, curcumin encapsulated in nanoassemblies generated by interactions of two different functional food ingredients might have offered added advantage to total nutritive quality of food material in addition to its increased preservative effect by antioxidant mechanism in aqueous system.

Materials and methods

Curcumin (purity: 97 %) was purchased from Himedia Labs, Mumbai, India. Soya lecithin (Lipoid s45) was obtained as a gift sample from Lipoid Germany. Chitosan (MW: 110 kD, 90 % deacetylation) was a gift sample from Indian Fisheries, Cochin, India. Glacial acetic acid, ethanol and all other solvents were of analytical grade and used as received without further purification. Deionized water was prepared through a Millipore Q purification system (Millipore Corp., Bedford, MA)

Preparation of self assembled and curcumin encapsulated nanocarriers

The lipid polymer self assembled nanoparticles were formed by ionic gelation technique as previously reported with slight modifications (Sonvico et al. 2006). Briefly, fixed amount of lecithin (5%w/v) was dissolved in 4 ml of ethanol to obtain organic phase. Chitosan 1 % w/v solution was prepared by dissolving chitosan in 1%v/v glacial acetic acid solution in deionized water. For preparation and optimization of nanocarriers, 4 ml of organic phase was slowly injected in 46 ml of aqueous phase previously kept on magnetic stirring at 1000 rpm at room temperature. The ratio of lecithin to chitosan were varied by dissolving different amount of soya lecithin in organic phase to obtain lecithin to chitosan ratio of 40:1, 20:1, 10:1 and 5:1. The supramolecular electrostatic interaction of oppositely charged lipid and polymer was thought to be taking place for sufficient duration of 30 min under magnetic stirring. After sufficient stirring was allowed, temperature of the nanosuspension was slowly raised to 40 °C and stirring continued for further 4 h in order to evaporate ethanol completely. Nanosuspension was allowed to come to room temperature and stored at temperature of −70 °C in deep fridge until its further use. For preparation of curcumin encapsulated nanocarriers all the above steps for nanocarrier preparation were same except for preparation of organic phase where fixed amount (500 μg to 10 mg) of curcumin was added to organic phase.

Physico-chemical characterization of nanoparticles

Hydrodynamic diameter (Size)

The Hydrodynamic diameter (size) and the polydispersity index (PDI) of nanoparticles were measured on a Zetasizer-NanoZS, Malvern Instruments Ltd., U.K. with He-Ne laser source (633 nm). All samples were sufficiently diluted with ultrapure water so that multiple scattering resulting from concentrated suspension is minimized. Photon correlations spectroscopic measurements were carried out at a scattering angle of 173° (NIBS=non-invasive backscatter detection) in 10 mm diameter quartz cells. All measurements were carried out in triplicate at 25 °C. Accordingly, the results are expressed as the z-average diameter and the PDI with Malvern software package using multiple mode analysis.

Zeta potential

Zeta potential is an important parameter of nanoparticles which represents its surface charge. The value of zeta potential determines the stability of the colloidal system. The zeta potential measurements of the resultant nanosuspensions were carried out using Zetasizer-NanoZS (Ma lvern, UK) by Laser Doppler Microelectrophoresis technique. All measurements were carried out in triplicate at 25 °C.

Morphology

Morphology of the nanoassemblies was assessed by Transmission Electron Microscopy (TEM; make: JEOL, JEM 2100) and Scanning Electron Microscopy (SEM; Model EVO 18, Special Edition, Carl Zeiss Inc.) For TEM measurements, nanosuspension was sufficiently diluted with deionized water and immobilized on carbon coated 300 mesh copper grid and allowed to dry for 3–4 h. Acceleration voltage of 200 kV was applied to obtain clear image of the nanoparticles. For SEM analysis nanosuspension was suitably diluted with deionized water and a drop of the nanosuspension was placed on graphene sheet coated on stainless steel holder. The samples were coated with gold particles prior to visualization in order to improve detection of nanoparticles under electron beam.

Encapsulation efficiency (EE) and curcumin loading (CL)

The encapsulation efficiency (EE) and curcumin loading (CL) in nanoassemblies were assessed by UV-visible spectrophotometry (make: Jasco V-600). Prior to measurement, suitable Spectrophotometric method for the determination of curcumin was developed using calibration curve method in the concentration range of 1–10 ppm of curcumin and high correlation coefficient was obtained (r=0.99) at the wavelength of 421.0 nm which corresponds to absorption maxima of curcumin. For determination of encapsulation efficiency and curcumin loading, nanosuspension was centrifuged at a speed of 3,000 rpm in order to settle down free curcumin from the aqueous nanodispersion. The supernatant was separated and further ultracentrifuged at a high speed of 20,000 rpm for 3 h (REMI motor) in order to form pellets of nanoparticles. Pellets were allowed to dry under the stream of nitrogen at −45 °C. The predetermined quantity of pellets and supernatant were dissolved in methanol in order to break nanoparticles and solubilize curcumin in the solvent. EE and CL were determined from the nanoparticles using Eqs. (1) and (2) respectively.

$$\mathrm{EE}(\%) = \frac{\mathrm{Wtotal\text{-}(Wfree} + \mathrm{Wprecipitated)}}{\mathrm{Wtotal}} \times 100 \qquad (1)$$

$$\mathrm{CL}(\%) = \frac{\mathrm{Wtotal-(Wfree} + \mathrm{Wprecipitated)}}{\mathrm{Wnp}} \times 100 \qquad (2)$$

Where Wtotal was the total curcumin weight in nanoparticles suspension; Wfree was the weight of free-curcumin in the nanosuspension and Wnp was the weight of the nanoparticles.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR analysis of curcumin powder and pre-dried nanoassemblies and curcumin encapsulated nanoassemblies were performed on FT-IR spectrometer (JASCO, 4100, Japan) with diffused reflectance technique (DR-41 sample cell).

Thermal characterization

Thermal characterization of the loaded nanoassemblies and curcumin powder were performed on Perkin Elmer DSC 6000 differential scanning calorimeter. Thermal transitions of the particles were checked in the temperature range of 30–200 °C with a heating rate of 20 °C/min.

Antioxidant activity

2, 2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The free radical scavenging activities of equivalent amount of curcumin powder dissolved in methanol (CP), blank

nanoassemblies (Blank Np) and curcumin encapsulated nanoassemblies (CLN) were evaluated by performing DPPH assay according to modified version of the method described by Kumari et al. (2010). Briefly, 200 μL of DPPH (0.2 mM) in methanol was added to volume of test solution containing 20 μL of nanosuspension and same volume of curcumin powder solution prepared in methanol (CP). Thirty minutes later, the absorbance was measured at 517.0 nm. For assessing the antioxidant activity of CLN in apple juice, this protocol was slightly modified. 20 μL of nanosuspension was diluted to 200 μL volume with apple juice which was freshly purchased from the local vendor and filtered through the membrane filter of 0.22 micron pore size prior to addition of any sample solution. 20 μL from this mixture was added to 200 μL of 200 μM DPPH solution in methanol and subsequent steps were similar to previous steps. Low absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as a control. The DPPH scavenging activity of ascorbic acid was calculated at the equal concentration of curcumin and considered as 100 %. The DPPH scavenging activities of blank nanoparticles, CLN and CP were then expressed as ascorbic acid equivalents in percentage as per Eq. (3) given by Stoilova et al. (2005)

$$\text{DPPH scavenged AA equivalent (\%)} = \left(\frac{\text{Ablank}-\text{Asample}}{\text{Ablank}-\text{Acontrol}}\right) \times 100 \quad (3)$$

DPPH scavenged AA equivalent (%) is radical scavenging activity expressed as percentage scavenging activity of curcumin in equal concentration to ascorbic acid. Where Asample is the absorbance obtained by addition of blank nanoparticles, CLN and CP; Ablank is the absorbance obtained by addition of distilled water and Acontrol is the absorbance obtained by addition of equal amount of ascorbic acid to that of curcumin in DPPH solutions respectively (Bursal et al. 2013).

Lipid anti-peroxidation assay

The lipid antiperoxidation activity of the CP, Blank Np and CLN were carried out by thiobarbituric acid reacting substances assay (TBARS). The protocol adapted was a modification of the procedure as described by Tarladgis et al. (1960). Briefly, homogenized Salmon tissue preparation (10 %w/v) in distilled water was used as medium for carrying out the study. The extract was filtered and it served as a source of degradation products of the lipid peroxidation. 200 μL of the filtrate was taken and mixed with 200 μL of CLN, CP and Blank Np. This mixture was incubated at 37 °C for 1 h. 400 μL of TBA reagent (200 μM in distilled water) was added and kept aside for 24 h at room temperature. The resultant red colored TBA complex was quantified at 538.0 nm on micro plate reader (ELISA, BIORAD, USA). Absorbance of control sample was measured in the same way except that distilled water was added to homogenate instead of any sample solution. All measurements were carried out in triplicate and results were expressed as mean ± SD. The anti-lipid peroxidation activity was then calculated using Eq. (4).

$$\text{Anti-lipid peroxidation activity (\%)} = \left(\frac{\text{Ablank}-\text{Asample}}{\text{Ablank}-\text{Acontrol}} \times 100\right) \quad (4)$$

Where Ablank is the absorbance of the blank sample containing distilled water instead of curcumin powder and nanosuspension, Asample is the absorbance of sample solutions containing blank nanoassemblies, CLN, and CP solution, Acontrol is the absorbance of sample without Salmon tissue homogenate.

Reducing power assay

It is advocated that effective antioxidant should also act as reducing agent by donating electrons to the oxidants in the system. This activity of the antioxidant is based on the principle of redox reaction in which one compound is reduced at the expense of oxidation of its counterpart. It is suggested that Iron is the most reactive elemental species present in the food which acts as a pro-oxidant of lipid peroxidation process (Love and Pearson 1974). Therefore reducing capacity of the proposed antioxidant for ferric/ferrous redox reaction is the indicator of its antioxidant potential. Reducing power assay was performed for assessment of reducing power capacity of CP and CLN (Oyaizu 1986). CLN, CP and Blank Np in equal concentration were mixed individually with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and potassium ferricyanide $[K_3Fe(CN)_6]$ 2.5 ml, 1 % mixture thus prepared was kept for incubation at 50 °C for half an hour. Further the reaction mixture was acidified with trichloroacetic acid (10 %). Equal volume of this mixture and distilled water were mixed and 0.5 ml of $FeCl_3$ was added to resultant mixture. Absorbance was measured at the wavelength of 700.0 nm in UV spectrophotometer (Jasco V-600). Blank solution was prepared without adding Blank Np, CLN and CP to the mixture, instead distilled water was added and absorbance at the specified wavelength was measured. Increased absorbance is suggestive of greater reducing potential of the antioxidant under question (Gulcin et al. 2012).

Nitric oxide (NO) radical scavenging assay

The method based on Greiss reagent was used to determine the nitric oxide radical scavenging activity of curcumin (Sadzuka et al. 2012). A volume of 2 ml of 10 mM sodium nitroprusside prepared in phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of equal amount of CP and CLN. The mixture was incubated at 37 °C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent (1.0 ml sulfanilic acid reagent, 1 % H_3PO_4, 0.1 % napthylethylenediamine dihydrochloride mixture) was incubated at room temperature for 30 min, followed by the measurement of absorbance at 546.0 nm using microplate reader. The NO radical scavenging activity was expressed in % inhibition and calculated using Eq. (5)

$$\%\ \text{inhibition (NO)} = \left(\frac{\text{Acontrol}-\text{Atest}}{\text{Acontrol}} \times 100\right) \quad (5)$$

Whereas Acontrol is the absorbance of the sample containing distilled water and Atest is the absorbance of the sample containing blank Np, CLN and CP.

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging potential was determined using the method described by Ruch et al. (1989). A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (PBS, pH 7.4). 0.1 ml of CLN, CP and blank Np were added to 1 ml of H_2O_2 solution. After 10 min, the absorbance was measured at 230.0 nm using micro plate reader against a blank solution that contained hydrogen peroxide solution without curcumin. The hydrogen peroxide scavenging activity of the formulations was determined using Eq. (6)

$$\%\ \text{Inhibition H202} = \left(\frac{\text{Acontrol}-\text{Atest}}{\text{Acontrol}} \times 100\right) \quad (6)$$

Whereas Acontrol is the absorbance of the sample containing distilled water and Atest is the absorbance of the sample containing blank Np, CLN and CP.

Stability studies of curcumin encapsulated nanoassemblies

Effect of storage time and temperature

Effect of storage time on physicochemical attributes of CLN has been assessed by keeping CLN in screw capped vials in dark for the period of 3 months. The physicochemical attributes were checked with respect to Z-average size, zeta potential and polydispersity index (PDI) for stipulated time and at two different temperatures i.e., 4 and 25 °C. Triplicate measurements were done and data is expressed as parameter value ± SD.

Effect of pH

The stability of the nanoparticles were checked in the pH range of 2 to 8 adjusting the lower pH with 0.1 M HCl solution and higher pH with 0.1 M NaOH solution. After adjustment of the pH, samples were left in screw capped vial and kept at room temperature for 24 h and subsequently characterized with respect to their hydrodynamic diameter and zeta potential.

Statistical analysis

Data are shown as means ± SE in the measurement (n=3). Statistical data were analyzed by one way ANOVA test at the significance level of P=0.05, 0.01 and 0.001. Further Tukey post test was performed to compare all groups using GraphPad Prism 5 software.

Results and discussion

Size, zeta potential and polydispersity index

In order to optimize the nanocarrier, lecithin weight was varied to that of chitosan and monodisperse blank nanoassemblies were obtained with lecithin: chitosan ratio of 20:1 w/w. This ratio of nanocarriers was selected further for encapsulation of curcumin. The optimized blank nanoparticles presented z-average size of 117.8±2.4 nm, PDI value of 0.236 ±0.008 and zeta potential of 35±0.004 mV. The size, zeta potential and PDI values of the blank nanoparticles formed by varying weight ratios of lecithin to chitosan are presented in Table 1.

Table 1 Size, zeta potential and polydispersity index of the blank nanoparticles with varied ratio of lecithin to chitosan. Bold characters indicate values of optimized batch for lecithin chitosan ratio

LCR	Z average size[a] (nm)	PDI [a]	ZP[a] (mV)
1:0	90.5±2.3	0.199±0.002	−54.5±0.202
5:1	243.0±3.4	0.527±0.027	45±0.005
10:1	233.8±2.2	0.463±0.090	38.7±0.002
20:1	**117.8±2.4**	**0.236±0.008**	**35±0.004**
40:1	126.0±6.5	0.312±0.003	10.4±0.003

[a] mean ± SD, n=3; *ZP*, zeta potential; *PDI*, Polydispersity index; *LCR*, Lecithin to chitosan ratio

An amount of curcumin which could be encapsulated in nanocarrier without significant precipitation was found to be 100 μg/ml of the nanosuspension. It was observed that 5 mg of curcumin could be loaded in the nanoassemblies with z-average size in the range of 143.0±1.55 nm, PDI value 0.253 ±0.020 and zeta potential value of 32.2±1.50 mV. The high positive value of zeta potential suggested that cationic chitosan has coated lecithin core of the nanoassemblies and would act as electrostatic shield to prevent nanoparticle aggregation. The formation of chitosan coated lecithin core nanoparticles was confirmed by preparing uncoated lecithin nanoparticles which presented high negative surface charge. The comparison of blank nanoassemblies and curcumin loaded nanoassemblies with respect to size and surface charge have shown that size of the nanoassemblies have increased upon addition of curcumin. On the contrary, high positive zeta potential value of the loaded nanoassemblies was decreased upon curcumin addition. This could be attributed to electrostatic interaction of phenolic OH and diketonic functionalities of the curcumin with positively charged NH_4^+ groups of chitosan. The values of z-average size, zeta potential and polydispersity index of the chitosan coated and uncoated nanoassemblies are presented in Table 2.

Morphology

The Curcumin loaded nanoassemblies have shown roughly spherical morphology in TEM analysis and corroborated the size measurement analysis done by dynamic light scattering technique. In TEM analysis nanoparticles have clearly shown outer coat of chitosan which was absent in uncoated lecithin nanoparticles. Curcumin was found to be well dispersed in the lecithin core of the nanoparticles. SEM measurements also corroborated evidence of roughly spherical geometry of the nanoassemblies. However chitosan coating of the nanoparticles could not be established by SEM measurements. This could be attributed to uniform gold coating of the nanoassemblies prior to SEM measurements. The morphology of the nanoparticles is depicted in Fig. 1.

Encapsulation efficiency (EE) and curcumin loading (CL)

Encapsulation efficiency and curcumin loading data have presented interesting observations. EE constantly decreased within the range of 92–82 % when concentration of curcumin was gradually increased from 10 to 100 μg/ml. However it dropped significantly to 60 % when curcumin concentration was increased to 150 μg/ml of the final volume. Hence 100 μg/ml concentration was chosen for performing stability studies and in-vitro antioxidant activities. This type of constant decrease in encapsulation efficiency of active ingredient was also observed in another independent study where hydrophobic nutraceutical ingredient (vitamin E) was encapsulated in wheat gliadin nanoparticles. It was found that 900.0 nm sized nanoencapsulated vitamin E particles were formed with encapsulation efficiency of 95–77 % in increasing order of vitamin E concentration relative to gliadin weight (Duclairoir et al. 2002). The decrease in fraction of spontaneously forming cavities of nanoassemblies to the added curcumin amount could be the reason behind aggregation of the nanoparticles and precipitation of curcumin beyond this concentration. The percentage of encapsulation efficiency and curcumin loading of the nanoassemblies are presented in Table 3.

Fourier transform infra red spectroscopy (FT-IR) analysis

The characteristic peaks of FT-IR spectra of Curcumin loaded nanoassemblies (a), Curcumin-lecithin-chitosan physical mixture (b), Curcumin powder (c), Lecithin (d) and Chitosan (e) are depicted in Fig. 2. In FT-IR analysis curcumin shows characteristic bands at 3509 cm^{-1} (phenolic OH stretch), 1508, 1280, 1029, 1592 cm^{-1} etc (Mangolim et al. 2014). Chitosan shows broad peak at 3655 cm^{-1}which corresponds to its amine group, and peak at 1626 cm^{-1} which corresponds to its amide group (Brugnerotto et al. 2001). Curcumin lecithin chitosan physical mixture for FT-IR analysis was prepared in the ratio corresponding to their amount present in the nanoparticles. FT-IR analysis of physical mixture has shown that

Table 2 Size, zeta potential and polydispersity index of the curcumin loaded lecithin/chitosan nanoassemblies (20:1 ratio) with varied ratio of curcumin. Bold characters indicate data values of optimized batch

Sr. No.	Curcumin (mg)	EE[a] (%)	CL [a] (%)	Z average size [a] (nm)	PDI [a]	ZP [a] (mV)
1	1	92±1.07	0.43±0.05	117.1±0.45	0.242±0.001	35.07±2.03
2	2	87±0.89	0.82±0.03	118.2±0.63	0.234±0.003	33.9±0.25
3	4	88±1.34	1.64±0.09	122±1.22	0.247±0.008	33.5±0.404
4	**5**	**82±1.23**	**1.90±0.02**	**143.0±1.55**	**0.253±0.020**	**32.2±1.50**
5	7.5	60±4.5	2.06±0.03	172.5±0.55	0.357±0.025	21.3±0.30
6	10	40±3.8	1.73±0.05	202.60±0.60	0.574±0.045	21.2±0.28

[a] mean ± SD, n=3; *ZP*, zeta potential; *PDI*, Polydispersity index; *EE*, Encapsulation efficiency; *CL*, Curcumin loading

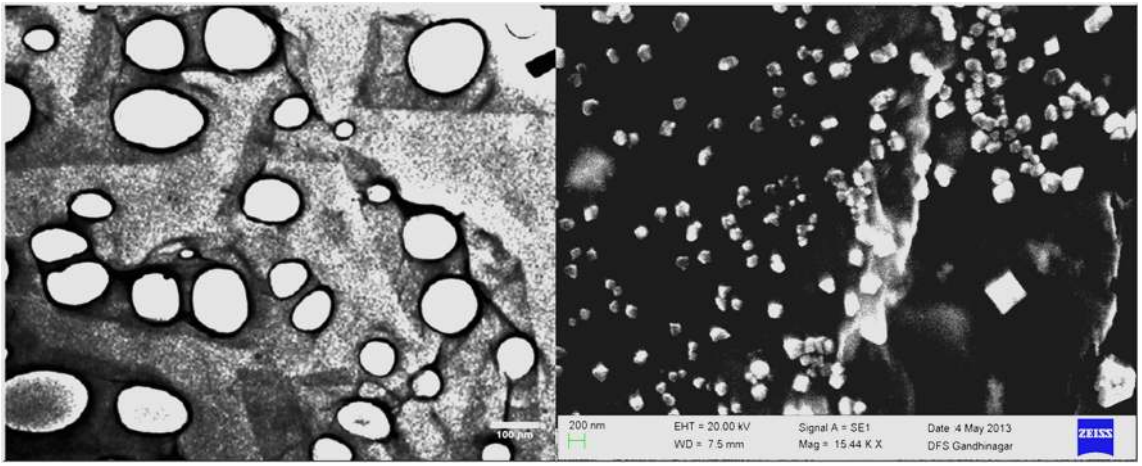

Fig. 1 Morphological characterization of Curcumin loaded self assembled nanoparticles by Transmission electron microscopy (*left*) and Scanning electron microscopy (*right*)

there is no significant interaction among the components in powdered mixture form. Curcumin peak is observed at 3511 cm^{-1} in the physical mixture which confirms there is no significant interaction of phenolic OH group of curcumin with other polar groups in lecithin and chitosan. The physical mixture shows broad OH band at 3313, 1737 cm^{-1} (C=O stretch), 1629 cm^{-1}(C=O stretch, secondary amide), 1601 and 1511 cm^{-1}. Curcumin encapsulated nanoparticles show dramatic reduction in the intensity in the range of 3511–3200 cm^{-1} which corresponds to stretching frequencies of OH and NH_2 groups of lecithin, curcumin and chitosan. This confirms the strong electrostatic interaction between phenolic group of curcumin, OH groups of lecithin and primary amine group of chitosan. FT-IR spectra of curcumin nanoassemblies did not show any characteristic molecular peaks of curcumin which is the indication of complete encapsulation of curcumin inside nanoassemblies. This is further confirmed by the fact that bending vibration of the NH_2 group of chitosan at 1626 cm^{-1} has been reduced in intensity suggesting its participation in the strong non-bonding interactions with other components in the formulation.

Table 3 Effect of storage time on the size, zeta potential and polydispersity index of the curcumin loaded lecithin/chitosan nanoassemblies at 4 °C temperature

DOS	Z-average size[a] (nm)	ZP[a] (mV)	PDI [a]
1	143.0±1.55	32.2±1.50	0.242±0.002
2	149.4±1.54	32.9±1.42	0.244±0.008
4	143.6±2.07	32.7±1.21	0.231±0.004
8	144.4±3.05	32.2±1.50	0.222±0.005
12	145.4±1.44	32.1±1.33	0.248±0.007
15	146.3±2.00	33.0±1.41	0.242±0.003
30	146.7±2.76	32.8±1.42	0.243±0.001
45	146.7±1.02	31.0±1.04	0.269±0.002
60	150.5±1.78	28.1±5.60	0.273±0.032
90	168.4±5.60	21.3±0.306	0.36±0.021

[a] mean ± SD, n=3; *DOS*, Days of storage; *ZP*, zeta potential; *PDI*, Polydispersity index

Thermal characterization (DSC)

Differential scanning calorimetric measurements of curcumin, curcumin physical mixture and curcumin loaded nanoparticles have been depicted in Fig. 3. DSC curves of curcumin shows the sharp endothermic transition at 178 °C which corresponds to its melting point temperature (*Tm*) and is suggestive of its highly crystalline nature in free form (Kumar et al. 2008). Curcumin along with lecithin chitosan physical mixture shows slight depression in its *Tm* at 171 °C and broadening of the endothermic peak suggests non-bonding interactions of curcumin with functional groups of lecithin and chitosan in the mixture and defects in its crystalline packing due to presence of lecithin and chitosan. However DSC curve of curcumin loaded nanoassemblies completely depicted new peak in between *Tm* of curcumin and lecithin at 123.37 °C. Endothermic peak at curcumin *Tm* was completely absent in the nanocarrier system. This data suggests that curcumin loaded nanoassemblies present completely new system in terms of its thermal behavior and crystallinity. Melting temperatures of these nanoassemblies above 100 °C and absence of any degradation peaks till melting point transition temperature of curcumin suggests that nanoassemblies would remain stable at the room temperature and would not undergo any unwanted thermal transitions upto this temperature during various stages of food processing. It has been advocated that amorphous and metastable forms of the drugs would show enhanced solubility and dissolution profile as compared to free form (Hancock and Zografi 1997). DSC results have shown that there was either decrease or loss of curcumin crystallinity in CLN owing to absence of any sharp endothermic transition at its *Tm* which would likely enhance its solubility and dissolution profile.

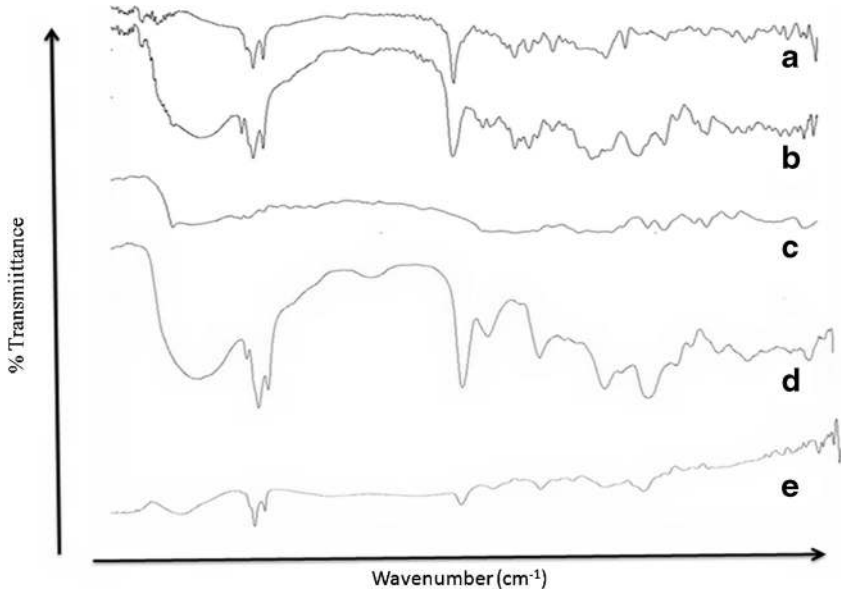

Fig. 2 FT-IR spectra of **a**) Curcumin loaded self assembled nanoparticles **b**) Curcumin, lecithin chitosan physical mixture **c**) Curcumin powder **d**) Lipoid S45 **e**) Chitosan

Antioxidant activities

DPPH radical scavenging assay is an important determinant of hydrogen donating ability of the proposed antioxidant to DPPH free radicals. DPPH is a stable radical showing purple color at 517.0 nm. The presence of any antioxidant which acts a hydrogen donor will degenerate this stable free radical lowering the absorbance at 517.0 nm. The extent of decrease in absorbance at specified wavelength shows potent radical scavenging ability of the antioxidant. Curcumin have already shown free radical scavenging ability against DPPH radical in various In vitro studies (Ak and Gulçin 2008). However, most of the reported literatures have shown effectiveness of curcumin on DPPH scavenging when it is dissolved in organic solvent (Borra et al. 2013). Authors feel that antioxidant potential of curcumin in aqueous food could not be established by this way as food contains no organic solvent to solubilise curcumin. The use of any alkaline buffer system for the dissolution of curcumin is also doubtful as food pH may vary significantly from that of nanosuspension pH (Gulcin 2011). Further, curcumin is unstable at alkaline pH and undergoes degradation. Hence, DPPH scavenging activity shown by

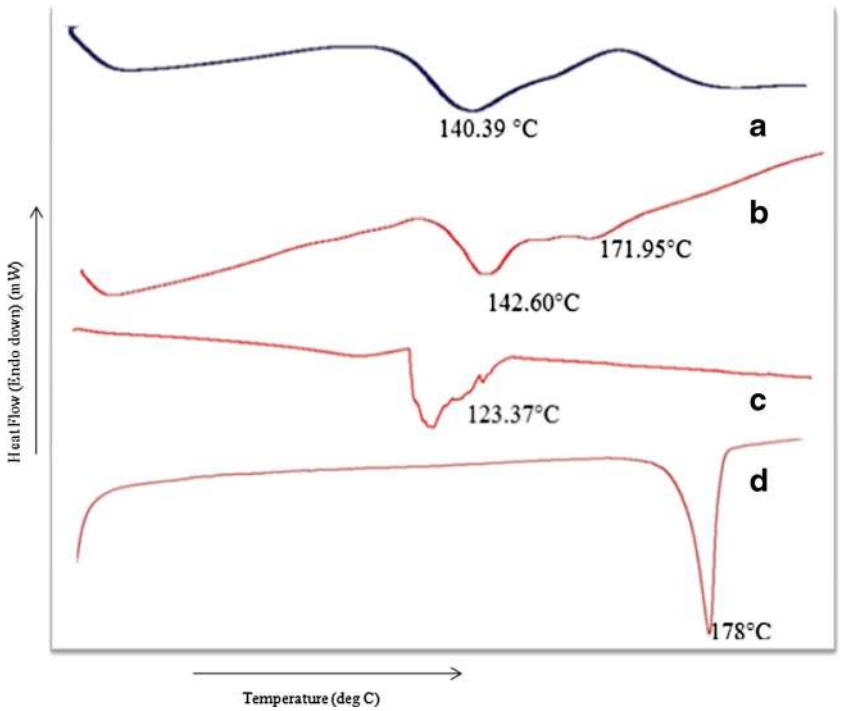

Fig. 3 Thermal characterization of **a**) Lecithin **b**) Physical mixture of curcumin, lecithin and chitosan **c**) Curcumin loaded self assembled nanoparticles and **d**) Curcumin crystalline powder

CLN is consequence of its increased dissolution profile in aqueous media. The percentage of DPPH scavenged in terms of ascorbic acid equivalents by various curcumin incorporated systems are shown in Fig. 4.

Results have shown that CP and CLN are better scavengers of DPPH radicals as compared to ascorbic acid at the equal concentrations used. Both CP and CLN have shown comparable scavenging activity. It is observed that DPPH scavenging activity of CLN is more potent than that of CP in the confidence interval of 99 % (P value <0.01). This shows the retention and enhancement of antioxidant activity of curcumin when incorporated in nanoassemblies. Similar observations were noted when CLN and CP were mixed with apple juice. CLN-apple juice mixture has DPPH scavenging activity more potent than apple juice-CP mixture and apple juice alone in the confidence interval of 99 and 99.99 % (P value <0.01 and <0.001) respectively. It should be noticed that the scavenging activity of the CP is determined in its completely soluble form in methanol whereas in CLN curcumin is solubilized in nanoassemblies dispersed in aqueous system. Hence it can be concluded that CLN are better free radical scavengers in an aqueous system. The scavenging activity of blank nanoparticles may be attributed to chitosan present in the nanoparticles and cationic charges of the blank nanoassemblies which may donate hydrogen to the DPPH radicals and hence lower the absorbance. The DPPH scavenging activity of different molecular wt chitosan has already been proved in another independent study (Chien et al. 2007). Similar results were obtained in the apple juice which acted as an aqueous food model for the study. Apple juice itself shows potential radical scavenging activities (Santini et al. 2014). Addition of CP and CLN enhance the total radical scavenging effect of the apple juice. CLN enhances antioxidant effect of apple juice by more than 50 % and curcumin powder by 40 %. This also confirms that antioxidant activity of curcumin when incorporated in CLN has been retained. Further, the assessment of its antioxidant activity in fruit juice matrix shows antioxidant activity did not get influenced by matrix effect. Previous studies have also shown that DPPH radical scavenging activity of curcumin nanoparticulate systems was superior to its free form (Yen et al. 2010)

Anti-lipid peroxidation (TBARS) assay

The Salmon tissue homogenate contains iron and other metals bound to various proteins like myoglobin, ferritin, transferrin etc. (St Angelo 1996) which gets released upon the storage and blending procedure. It causes peroxidation of various lipids in the fish tissues resulting in formation of malondialdehyde and other aldehydes as peroxides which further reacts with thiobarbituric acid to form red colored complex. In addition the fish tissues have high percentage of unsaturated fatty acids which act as substrate material for peroxide formation (Hsieh and Kinsella 1989; Shahidi 1997). The anti-lipid peroxidation activity of the proposed antioxidant in such medium could be attributed to several mechanisms e.g., chelation of metals released upon storage of food, binding of the compounds to lipids etc. (Dutta et al. 2004). An effective anti-lipid peroxidation substance will reduce the intensity of colored complex. The results have shown that CLN and CP both have inhibited lipid peroxidation by more than 40 % in Salmon tissue homogenates whereas blank nanoparticles have inhibited lipid peroxidation by less than 10 % as depicted in Fig. 5. Statistical ANOVA test have shown means of all groups were different with P value <0.05 at 95 % confidence interval. This shows that CLN have enhanced anti-lipid

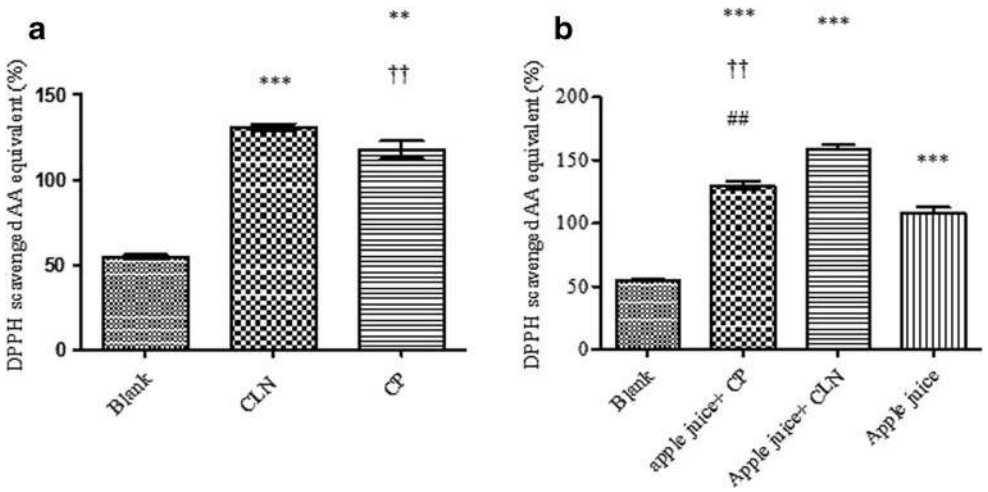

Fig. 4 **a**) The DPPH scavenging activities of blank nanoparticles (Blank), curcumin loaded nanoassemblies (CLM) and curcumin powder (CP) expressed in the terms of activity of ascorbic acid (AA) at the same concentration of curcumin in both the system **b**) DPPH scavenging activities of apple juice, CLN added to apple juice and CP added to apple juice expressed in terms of ascorbic acid equivalents. Data are expressed as mean ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Blank; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. CLN group and $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. apple juice

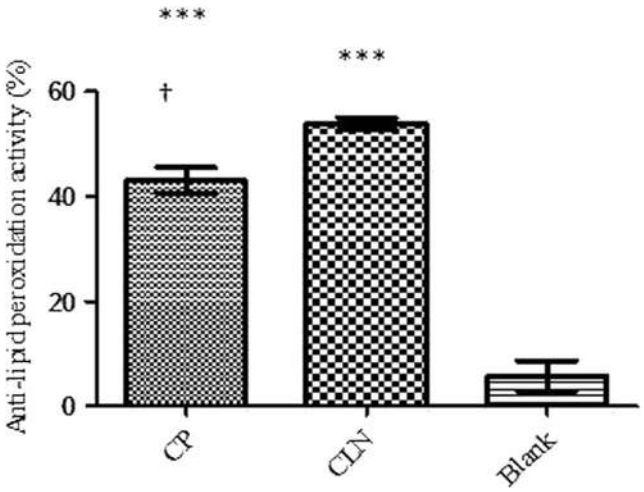

Fig. 5 An anti-lipid peroxidation activity in blank nanoparticles (Blank), Curcumin powder (CP) and Curcumin loaded nanoassemblies (CLN). Data are expressed as mean ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Blank; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. CLN group

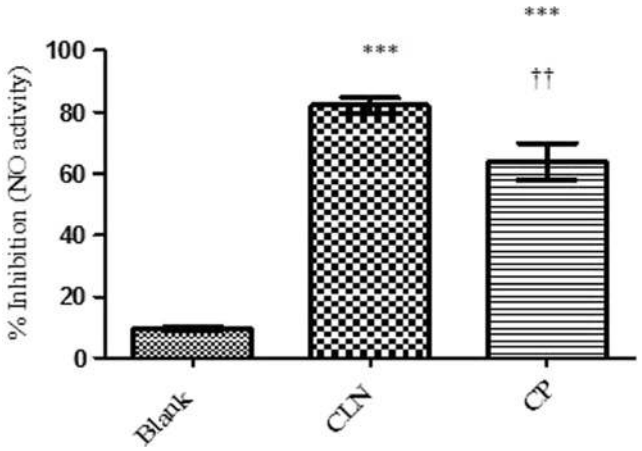

Fig. 6 The % inhibition of Nitric oxide (NO) activity in blank nanoparticles (Blank), Curcumin powder (CP) and Curcumin loaded nanoassemblies (CLN). Data are expressed as mean ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Blank; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. CLN group

peroxidation effect in aqueous media. It should be noticed that some activity has also been shown by Blank Np. The antioxidant activity shown by Blank Np could be attributed to cationic chitosan coating as suggested earlier (Xie et al. 2001). An another study have also reported enhancement in anti-lipid peroxidation activity of melatonin, a hydrophobic molecule upon its nanoencapsulation in different nanocarriers in the range of 8–51 % as compared to its free form (Schaffazick et al. 2005). Similar observation was noted in anti-lipid peroxidation effect of CLN vs. CP in the current study.

Nitric oxide (NO) radical scavenging assay

Nitric oxide (NO) is an important mediator of many physiological reactions in the body (Moncada et al. 1991). The functional food ingredients are supposed to provide some health benefits beyond basic nutrition. Hence, NO scavenging activity of CLN would offer some health benefits and disease prevention in diseases where NO radicals mediate disease progression. The method employed here was based on formation of chromophore owing to reaction between spontaneously formed nitrite/nitrate ions from sodium nitroprusside at physiological pH and Greiss reagent (Sreejayan and Rao 1997). The intensity of chromophore thus formed is then used for quantitation of $NO^{\cdot}$ radical in the reaction. Although the role of NO in food spoilage is not yet clearly reported but $NO^{\cdot}$ radicals on reaction with oxygen radicals generate peroxynitrites (ONOO-) which may cause lipid peroxidation. Therefore, inhibition of excess $NO^{\cdot}$ radicals can indirectly inhibit lipid peroxidation process of the food materials (Brannan et al. 2001). The Results have shown that CLN were almost as effective as CP in inhibiting generation of $NO^{\cdot}$ radicals as shown in Fig. 6. CLN and CP activities in this assay were significantly different at confidence interval of 99 % (*P* value<0.01).

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a nonradical compound which is present in minimal quantity in foods; however it undergoes rapid decomposition to generate hydroxyl radicals ($OH^{\bullet}$) which in turn leads to lipid peroxidation in food and DNA damage when incorporated in cells (Kanner et al. 1987). Hence scavenging of H_2O_2 is an important attribute of any functional food. The hydrogen peroxide scavenging activity of CLN, CP, Blank Np was determined in comparison to solution containing equivalent volume of distilled water without curcumin. Figure 7 shows that 0.1 ml of CP and CLN have inhibited almost 35 %, 46 % of 20 mM of H_2O_2. Blank Np on the other part has shown minimal inhibition upto 7 %. Previous study has shown that curcumin acts as OH• radical generator at lower concentration and scavenger at higher concentration (Kunchandy and Rao 1990). However higher concentration of curcumin in aqueous food could only be achieved by its increased dissolution and solubility profile. Hence, the increased H_2O_2 scavenging activity of CLN could be attributed to higher concentration of curcumin in aqueous system which was achieved through its nanoencapsulation in supramolecular assemblies.

Reducing power assay

The method employed here was based on the potassium ferricyanide reduction method. Figure 8 shows that absorbance measured at 700.0 nm is greater for CLN as compared to CP and Blank Np in the confidence interval of 99.99 % (*P* value <0.001). Enhanced reducing potential of CLN may be

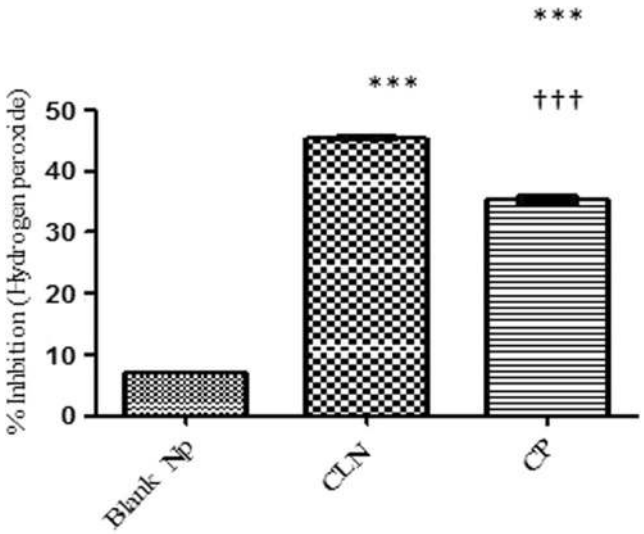

Fig. 7 The % inhibition of H_2O_2 activity in blank nanoparticles (Blank), Curcumin powder (CP) and Curcumin loaded nanoassemblies (CLN). Data are expressed as mean ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Blank; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. CLN group

attributed to positively charged chitosan coating of the nanoassemblies which also acts as reducing agent. This is further confirmed by increased absorbance values of Blank Np as compared to blank solution containing distilled water. The results confirm that electron donor property of the curcumin to neutralize free radicals has not been affected by its nanoencapsulation. Moreover, the reducing power of curcumin in CLN has been enhanced as absorbance of CLN added system has been increased to 1.48 as compared to 1.21 of CP (Fig. 8). This could again be attributed to increased dissolution profile of CLN in assay medium as compared to CP.

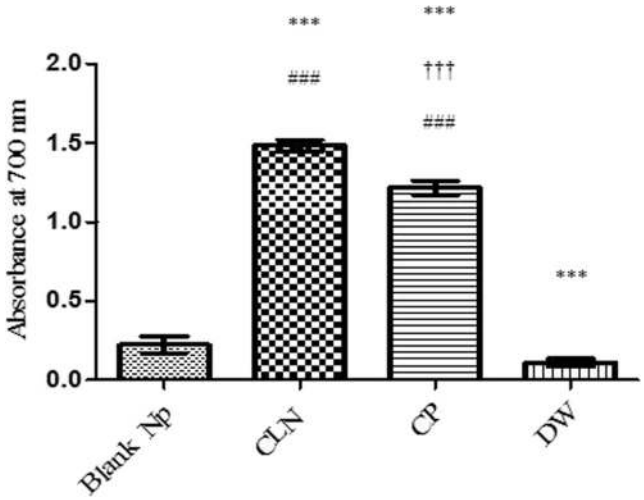

Fig. 8 The absorbance values of ferrous-ferric complex formed in reducing assays performed in blank nanoparticles (Blank), Curcumin powder (CP) and Curcumin loaded nanoassemblies (CLN) and in distilled water (DW). Data are expressed as mean ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Blank; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. CLN group and $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. distilled water (DW)

Table 4 Effect of storage time on the size, zeta potential and polydispersity index of the curcumin loaded lecithin/chitosan nanoassemblies at 25 °C temperature

DOS	Z-average size[a] (nm)	ZP[a] (mV)	PDI [a]
1	143.0±1.55	32.2±1.50	0.242±0.002
2	143.1±1.67	32.5±0.44	0.243±0.002
4	145±4.26	33.1±1.59	0.223±0.006
8	145.8±3.32	32.8±1.43	0.222±0.001
12	144.8±4.32	31.3±2.03	0.277±0.001
15	167.8±1.49	28.7±2.33	0.302±0.003
30	202.7±0.60	23.9±0.51	0.369±0.087
45	292.5±4.86	21.3±0.23	0.426±1.020
60	334.8±3.43	19.1±0.200	0.518±0.1048
90	403±14.18	18.3±0.49	0.623±0.127

[a] :mean ± SD, $n=3$; *DOS*, Days of storage; *ZP*, zeta potential; *PDI*, Polydispersity index

Effect of storage time and temperature

The tremendous increase in effective surface area of the nanoparticles makes them highly unstable system. Such systems try to attain stability by lowering the surface area which could be achieved by particle aggregation. The attractive forces between nanoparticles could be prevented by means of either electrostatic or steric repulsion. The requirement of nanoparticulate system to be effective as food additive is that it should remain in its nano form for sufficient duration. Z-average size and zeta potential are the important determinants of nanoparticle stability as a function of time. The assessment of stability of the nanoparticles at two different storage conditions i.e., 4 and 25 °C has shown that CLN has maintained its average size and surface charge for the period of 90 d when stored at 4 °C. However at room temperature (25 °C), CLN

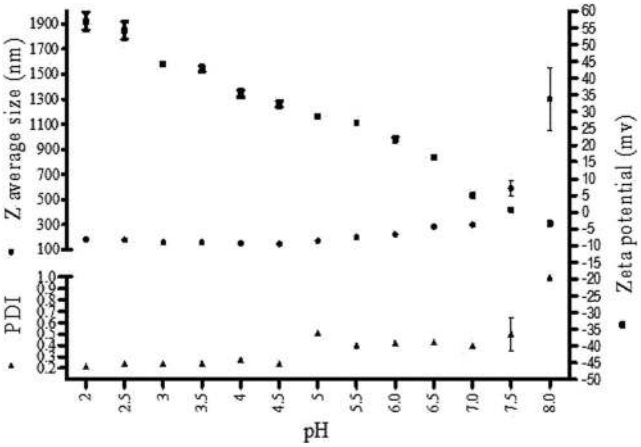

Fig. 9 Effect of pH on zeta potential and polydispersity index and z-average size of the curcumin loaded nanoassemblies in the range of pH 2 (acidic) to pH 8 (alkaline)

could achieve stability only upto the period of 15 d. CLN have shown significant increase in the particle size and decrease in zeta potential after 15 d of storage at room temperature. This data has shown that temperature is an important governing factor for the storage of CLN. Temperature may increase kinetic energy of the nanoparticles resulting into particle aggregation. This observation put an important limitation on CLN to be stored at room temperature for longer duration. Tables 3 and 4 shows that CLN size and surface charge are not affected at two temperature conditions till 15 d. After 15 d, nanoparticles stored at 25 °C have shown increased aggregation behavior which could be attributed to thermal kinetic energy of the nanoparticles.

Effect of pH

Foods and food products represent wide range of pH values from acidic to alkaline (Anon 1962). In this case, conservation of nanoparticulate form of the CLN under the influence of wide range of food pH is an important determinant of its stability and thus, its usefulness as food antioxidant. The prepared CLN have already shown its pH at 4.3±0.1 as recorded by pH meter (ELICO, India). The behavior of CLN under the influence of acidic pH (pH 2) to alkaline pH (pH 8) has been shown in the Fig. 9. The data shows that CLN behaved quite well with respect to small size and high zeta potential in the pH range of 2 to 7. Size was well below 300.0 nm in this range but increased significantly to more than 500.0 nm at higher pH range. PDI, an important determinant of the stability of nanoparticle presented values well below 0.3 upto pH value of five. After this pH range sample became too polydisperse to consider it good for maintaining stability for longer duration. Zeta potential measurement has presented high positive values in the acidic pH range of 2 to 6.5 suggesting that no perturbing effect of acidic food material on surface charge of the nanoparticles and therefore its stability have occurred. The data could be justified by the fact that cationic chitosan and anionic soy lecithin present their pKa values at pH range of 6.3–7 and 1.5 (anionic phosphate group of lecithin) respectively (Ogawa et al. 2004). However there may arise a question that at more acidic pH range values no pronounced effect of possible neutralization of lecithin phosphate groups (pKa 1.5) has been shown on the z-average size, zeta potential and PDI. On the contrary, more pronounced effects on these parameters were shown in the pH range of 6.5 to 8. This observation could be attributed to protection of soy lecithin at the core of nanoparticles by external chitosan coating. Hence, behavior of chitosan is important determinant of nanoparticle stability as compared to lecithin in the given pH range. In conclusion the prepared self assembled particles are most likely to retain their physicochemical characteristics in acidic pH as compared to alkaline conditions.

Conclusion

Curcumin, a prototype polyphenolic ingredient of Turmeric suffers from many issues like low aqueous solubility and bioavailability which preclude its use as an effective functional food ingredient for nutraceutical applications. To overcome these barriers Curcumin was effectively nanoencapsulated in self assembled nanocarriers of biodegradable lipid and polymer. Results have shown that curcumin was effectively nanoencapsulated and has retained its physicochemical attributes like PDI, Zeta potential and average size under the influence of varying environmental conditions which could affect integrity of curcumin loaded nanoparticles. The physicochemical parameters like size, zeta potential and PDI were maintained for average 90 d of storage at 4 °C. Further, the enhancement and retention in the antioxidant potential of nanoencapsulated curcumin in comparison to powdered curcumin have demonstrated efficacy of this nanocarrier system to be used for functional food applications. The research has proved that curcumin could effectively be loaded in self assembled nanoparticles of GRAS status lipid and biopolymer. The process of preparation is neither energy demanding nor requires high expertise in the field of nanoformulations. In conclusion, nanoencapsulation of nutraceutical ingredients opens new gateway to the technology domain of food and nutraceutical industry to address various issues related to food ingredient stability, bioavailability and solubility.

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