Carbohydrate Polymers 99 (2014) 499–507

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Conjugation of curcumin onto alginate enhances aqueous solubility and stability of curcumin

Soma Dey, K. Sreenivasan*

Laboratory for Polymer Analysis, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojapura, Trivandrum 695012, India

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 12 August 2013
Accepted 23 August 2013
Available online 31 August 2013

Keywords:
Curcumin
Alginate
Polymer-drug conjugates
CMC
Cytotoxicity

ABSTRACT

Curcumin is a potential drug for various diseases including cancer. Prime limitations associated with curcumin are low water solubility, rapid hydrolytic degradation and poor bioavailability. In order to redress these issues we developed Alginate-Curcumin (Alg-Ccm) conjugate which was characterized by FTIR and ^{1}H NMR spectroscopy. The conjugate self-assembled in aqueous solution forming micelles with an average hydrodynamic diameter of 459 ± 0.32 nm and negative zeta potential. The spherical micelles were visualized by TEM. The critical micelle concentration (CMC) of Alg-Ccm conjugate was determined. A significant enhancement in the aqueous solubility of curcumin was observed upon conjugation with alginate. Formation of micelles improved the stability of curcumin in water at physiological pH. The cytotoxic activity of Alg-Ccm was quantified by MTT assay using L-929 fibroblast cells and it was found to be potentially cytotoxic. Hence, Alg-Ccm could be a promising drug conjugate as well as a nanosized delivery vehicle.

1. Introduction

Curcumin (diferuloylmethane) is the principal active ingredient in the spice turmeric (*curcuma longa*). It is basically a low molecular weight natural polyphenol with versatile medicinal assets which are well documented in ancient Indian literatures (Goel, Kunnumakkara, & Aggarwal, 2008). Over the last couple of decades, there has been tremendous interest in curcumin because of its potent antioxidant, anti-inflammatory, antiproliferative and antiangiogenic activities (Aggarwal, Kumar, & Bharti, 2003; Aggarwal & Sung, 2009; Chattopadhyay, Biswas, Bandyopadhyay, & Banerjee, 2004; Lantz, Chen, Solyom, Jolad, & Timmermann, 2005; Ruby, Kuttan, Babu, Rajasekharan, & Kuttan, 1995; Shi et al., 2006). Extensive research works have proved curcumin to be a highly pleiotropic molecule and it can interact with various molecular targets including cytokines, enzymes, genes, transcription factors, growth factors and their receptors *etc.* to control cell proliferation and apoptosis (Goel et al., 2008; Ravindran, Prasad, & Aggarwal, 2009). Thus, the likelihood of developing resistance to curcumin is very less as it induces apoptosis through multiple cell signaling pathways. Besides, curcumin can play a vital role in controlling multidrug resistance (MDR) in cancer cells by down regulating the levels of Pgp, ABCG2 and MRP-1 (three major ABC drug transporters responsible for the development of MDR) (Bava et al., 2005; Manju & Sreenivasan, 2012). Curcumin has been documented to be highly effective against many cancer cells of human origin (Sandur et al., 2007), an efficient photodynamic therapeutic agent for the treatment of skin cancer (Park & Lee, 2007) and a competent curative agent against Alzheimer's and other incapacitating diseases (Mythri, Harish, Dubey, Misra, & Bharath, 2011; Ringman, Frautschy, Cole, Masterman, & Cummings, 2005). In spite of possessing striking anticancer properties, curcumin is not extensively used for cancer treatments due to its poor aqueous solubility and low bioavailability (Anand, Kunnumakkara, Newman, & Aggarwal, 2007). Hydrophobic nature of this polyphenolic compound along with its rapid metabolism, physicochemical and biological instability contribute to its poor bioavailability. To redress these problems several approaches have been proposed like encapsulation of curcumin in liposomes and polymeric micelles, inclusion complex formation with cyclodextrin, formation of polymer-curcumin conjugates *etc.* (Bisht et al., 2007; Manju & Sreenivasan, 2011a,b, 2012; Thangapazham, Puri, Tele, Blumenthal, & Maheshwari, 2008; Yallapu, Jaggi, & Chauhan, 2010). Among the diverse drug delivery approaches, the "polymer-drug" conjugate is a very popular and positive approach for the delivery of hydrophobic drugs. The concept of covalently bound polymer-drug conjugates, proposed by Ringsdorf (1975), is widely accepted for improved aqueous solubility, controlled delivery of drugs in optimum dosages, enhanced therapeutic efficacy, reduced side effects and improved patient

Abbreviations: (Alg), alginate; (Ccm), curcumin.

* Corresponding author. Tel.: +91 471 252 0248; fax: +91 471 234 1814.
E-mail address: sreeni@sctimst.ac.in (K. Sreenivasan).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.08.067

compliances (Alexis et al., 2008; Van et al., 2010; Zhou, Li, & Chau, 2010). Another important aspect associated with drug conjugates is that they can promote passive tumor targeting *via* the enhanced permeation and retention (EPR) effect (Maeda, 2010). Choosing the exact polymer in one hand can enhance the safer systemic circulation time of a conjugate and on the other hand, controlling parameters like size, functionality, charge *etc.* can amend the effect of biological half-life of a drug in a conjugate (Li & Huang, 2008). Various types of natural and synthetic polymers have been conjugated with hydrophobic drugs for the development of polymer-drug conjugates. Polyethylene glycol (PEG) with hydrophilic nature and ability to avoid nonspecific protein adsorption (stealth property) is the most widely studied polymer for the development of conjugates with longer blood circulation time. The main demerit associated with PEG is its non-biodegradability. Several hydrophilic, biodegradable, natural polymers have also been used for development of polymer drug conjugates. Alginate is an anionic, polysaccharide copolymer consisting of (1 → 4) linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) residues. The natural block copolymer contains regions of sequential M-units (M blocks), G-units (G blocks) and atactically organized M and G units. Alginate is widely used in biomedical applications owing to its several advantages such as biodegradability, high biocompatibility, non-toxicity, non-immunogenicity and the scope of chemical modification. The two secondary C-2 and C-3 hydroxyl groups and the C-6 carboxylate group are the attractive sites in alginate for the desired chemical modifications to develop derivatives with unique characteristics.

In this study, hydrophobic drug curcumin was covalently conjugated to the C-6 carboxylate functionality of hydrophilic sodium alginate *via* an ester linkage to produce Alg-Ccm conjugate. The covalent conjugation was confirmed by FTIR, ^{1}H NMR, fluorescence spectroscopy *etc*.

The conjugate was developed with the aim to enhance the solubility and stability of curcumin in aqueous medium. It was hypothesized that Alg-Ccm could stabilize curcumin in aqueous solution by self-aggregation to generate micelles with hydrophobic curcumin in the inner core and hydrophilic alginate in the outer shell. The Alg-Ccm conjugate micelles were thoroughly characterized by dynamic light scattering, TEM and the critical micelle concentration was also determined. The *in vitro* cytotoxicity of Alg-Ccm was also assessed using L-929 mouse fibroblast cells and was quantified by MTT assay.

2. Experimental

2.1. Materials

Curcumin (95% total curcuminoid content) from turmeric rhizome was obtained from Alfa Aesar (Bangalore, India). Sodium alginate (average molecular weight 4×10^5) was purchased from Sd fine chemicals (Mumbai, India). 3-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) and 1-Pyrenecarboxaldehyde (1-PyCHO) were purchased from Sigma-Aldrich (Bangalore, India). Dimethyl sulfoxide (DMSO) and ethanol were obtained from Merck (Mumbai, India). Ultra pure water (18.2 mΩ resistivity) was obtained from a Mili-Q water purification system. Deionized water was used all through the reaction and purification steps in this study.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent), fetal bovine serum (FBS) and minimum essential medium (MEM) were obtained from Sigma-Aldrich (Bangalore, India).

2.2. Synthesis of alginate-curcumin conjugate (Alg-Ccm)

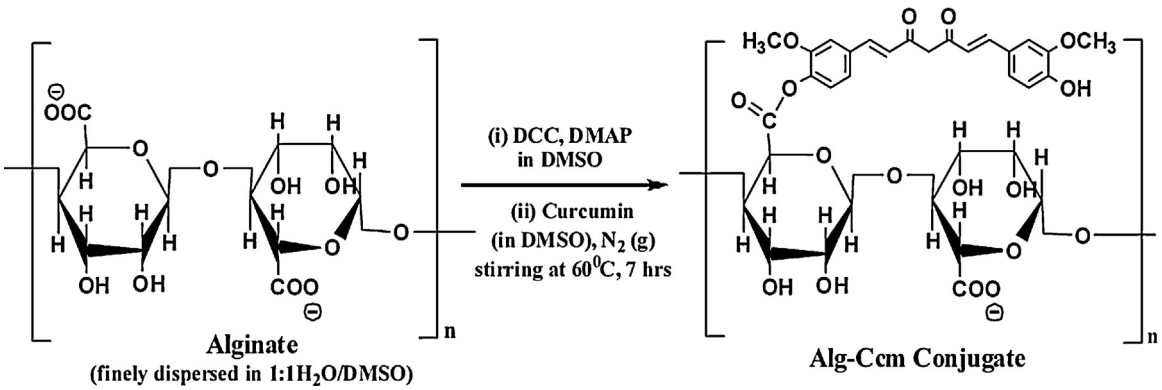
Sodium alginate (500 mg) was slowly added to water/DMSO mixture (50 mL, 1:1 v/v) and stirred vigorously overnight to obtain finely suspended alginate. DCC (40 mg) and DMAP (15 mg) were added to it and the reaction mixture was stirred for one hour at 25 °C to activate the carboxylate groups. To the activated alginate, Curcumin (15 mg dissolved in 10 mL DMSO) was slowly added under N_2 (g) atmosphere. The mixture was stirred well for about 7 h at 60 °C. Resultant solution was then cooled to room temperature and dialyzed for one day against DMSO followed by three days against deionized water using a dialysis membrane of molecular weight cut-off (MWCO) 3500 to remove the unreacted molecules. Finally, Alg-Ccm conjugate was lyophilized and stored in refrigerator for further studies.

2.3. Physicochemical characterizations of Alg-Ccm conjugate

Alg-Ccm conjugate was characterized by recording Fourier Transform Infrared (FTIR) Spectrum in a Nicolet 5700 FTIR spectrometer in the range of 4000–500 cm^{-1} using KBr pellets. The formation of the conjugate was further confirmed by ^{1}H NMR spectra recorded on a Nuclear Magnetic Resonance Spectrometer (Bruker Avance DPX 300) using D_2O as the solvent and tetramethylsilane as the internal standard. To scrutinize the possibility of self-assembly of Alg-Ccm, aqueous solution of the conjugate was characterized by dynamic light scattering instrument (DLS, Malvern Zetasizer Nano ZS, UK) with a He-Ne laser beam at a wavelength of 633.8 nm. The hydrodynamic diameter of the aggregated structures formed by the conjugate was determined by using an aqueous solution of Alg-Ccm (1 mg/mL) in deionized water at 25 °C and the zeta potential (ζ) of Alg-Ccm was determined (at pH 7.4) using the same instrument. The size and morphology of the micelles formed by Alg-Ccm in aqueous solution were monitored by Transmission Electron Microscopy (TEM; Hitachi H-7650; Tokyo, Japan). Sample for TEM analysis was prepared by depositing a drop of Alg-Ccm solution (in water, 1 mg/mL) on a 200 mesh copper TEM grid with formver film and air dried at room temperature. The absorbance range of Alg-Ccm was monitored using Ultraviolet-visible (UV-vis) Spectrophotometer (Carry model 100 bio UV-vis Spectrophotometer, Melbourne, Australia). The emission spectrum of Alg-Ccm was recorded using a Spectrofluorometer (Carry Eclipse model EL 0507, Melbourne, Australia) after dissolving the conjugate in water/EtOH mixture (1:1 v/v) and exciting it at 427 nm.

2.4. Dtermination of critical micelle concentration of the conjugate

The critical micelle concentration (CMC) of the conjugate was determined to find the concentration above which Alg-Ccm can form aggregated structure in aqueous solution. The self-aggregation behavior of Alg-Ccm was investigated by both absorption and emission spectroscopy using 1-PyCHO as a probe molecule. The absorption characteristic of 1-PyCHO was determined by using UV-vis Spectrophotometer at 25 °C in the range of 200–700 nm. The emission spectra of 1-PyCHO were recorded in a Spectrofluorometer using slit openings (both excitation and emission) of 5 nm at 25 °C from 400 to 600 nm after exciting the fluorescent probe at 368 nm. A stock solution of Alg-Ccm in water (1 mg/mL) was prepared and the concentration was varied from 10^{-3} mg/mL to 1 mg/mL by diluting the stock solution of Alg-Ccm with distilled water. 8 μL of the stock solution of 1-PyCHO was mixed with 5 mL of each diluted aqueous solution of Alg-Ccm to maintain a final concentration of the probe at 2.4×10^{-6} (M) in each of the solutions. The solutions were incubated for 45 min in the dark at 25 °C before measuring the absorbance. Similar procedure was



Scheme 1. Schematic presentation of the synthetic strategy of Alg-Ccm conjugates.

followed to determine the CMC of the conjugate by fluorescence spectroscopic technique.

2.5. Evaluation of curcumin content and aqueous solubility of the conjuagte

The amount of curcumin content in Alg-Ccm conjugate was evaluated using UV–visible absorption spectroscopy. Free curcumin in H_2O/EtOH mixture (1:1 v/v) was used to generate a calibration curve ($R^2 = 0.99$). Concentration of curcumin in Alg-Ccm was estimated from the absorption intensity of the conjugate solution (in water/EtOH, 1:1 v/v) in the standard calibration curve at 427 nm. To evaluate the aqueous solubility of Alg-Ccm conjugate, an excess amount of Alg-Ccm was added to an aqueous buffer solution (pH 7.4). The mixture was vortexed for few minutes and then centrifuged at 14,000 rpm for 5 min. Water layer was separated and diluted with EtOH. Thus concentration of curcumin was determined from the optical density calculation as mentioned above.

2.6. Determination of stability of Alg-Ccm in aqueous medium

The hydrolytic stability of both free curcumin and Alg-Ccm conjugate was determined in phosphate buffered saline (PBS) at physiological pH (pH = 7.4) by determining the change in the absorbance of curcumin. Solutions of free curcumin and Alg-Ccm conjugate in PBS were incubated at 37 °C for 6 h and in 1 h interval certain portion was taken from each solution to check the absorbance using UV-vis Spectrophotometer.

2.7. Cytotoxicity studies

The evaluation of *in vitro* cytotoxicity of Alg-Ccm conjugate was carried out according to the ISO standards (ISO 10993–5, 2009) by using a monolayer of L-929 mouse fibroblast cells. In brief, cells were subcultured from stock culture (ATCC, Bangalore, India) by trypsinization and seeded into multiwall tissue culture plates. Cells were fed with Dulbecco's minimum essential medium (MEM) supplemented with Fetal Bovine Serum and incubated at 37 °C in an atmosphere of 5% CO_2. Samples were prepared using Alg-Ccm conjugate maintained in MEM and supplemented with fetal bovine serum to attain a concentration of 10 mg/mL and then two other samples of the conjugate with concentration of 5 mg/mL and 2.5 mg/mL were obtained *via* dilution. 100 μL of each of different concentration of conjugate samples, negative control and positive control were placed in triplicate on subconfluent monolayer of L-929 mouse fibroblast cells and the cells were incubated at 37 ± 2° C for 24 ± 2 h. Then cell culture was examined microscopically (Leica inverted fluorescence microscope, DMI 6000; Leica Microsystems, Wetzlr, Germany) for testing the cellular response.

Quantitative assessment of the cytotoxic potential of Alg-Ccm was carried out by MTT assay which evaluates the metabolic reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple colored formazan crystals in viable cells. Conjugate samples were prepared as mentioned above. Equal volume (100 μL) of various dilutions of test samples (2.5, 5 and 10 mg/mL, *i.e.*; equivalent to 27.25 μg, 54.5 μg and 109 μg/mL curcumin respectively) and free curcumin were placed on subconfluent monolayer of L-929 cells. After incubation of the cells for 24 ± 2 h at 37 ± 2° C, free curcumin and conjugate sample medium were replaced with 50 μL MTT (1 mg/mL in serum free MEM) and cells were again incubated at 37 ± 2° C for 2 h. After discarding the MTT medium, 100 μL of isopropanol was added to each well and kept in an orbital shaker (Labline Instruments, Melrose Park, Illinois, USA) at 50 revolution per minute (rpm) for 20 min. Absorbance of the resulting solutions were read at 570 nm immediately using an automated microplate reader (Bio-Tek Instruments, Winsooski, Vermont, USA). After blank (only medium) subtraction, the results were expressed as optical density. The mean value of three replicates (for each sample) is reported here.

3. Results and discussion

3.1. Synthesis and structural characterization of Alg-Ccm conjugate

As a natural copolymer, alginate is highly useful for biomedical applications due to its striking characteristics like biodegradability, non-toxicity, non-immunogenicity, chelating ability *etc.* Hydrophobically modified alginate derivatives have also been studied extensively. In order to obtain a potential curcumin conjugate for therapeutic applications with improved aqueous solubility and stability of curcumin, the Alg-Ccm conjugate was synthesized. In order to synthesize Alg-Ccm, extremely hydrophilic polymer alginate was first finely dispersed in water/DMSO (1:1 v/v) mixture and then phenolic —OH group of curcumin was conjugated to the activated C-6 carboxylate functionality of alginate *via* esterification using DCC/DMAP as shown in Scheme 1. Dialysis and lyophilization resulted in solid, yellow colored Alg-Ccm conjugate.

The formation of Alg-Ccm conjugate was verified by FTIR spectra (Fig. 1A). Fig. 1A (i) shows the FTIR spectrum of sodium alginate in which a broad band around 3421 cm^{-1} was assigned to the O—H stretching, peak around 2925 cm^{-1} was due to aliphatic C—H stretching and the sharp peak at 1608 cm^{-1} was assigned to the carboxylate C—O stretching. Fig. 1A (ii) depicts the FTIR spectrum of the conjugate in which the O—H stretching appears at 3441 cm^{-1} and the peak is relatively sharper as the conjugate contains phenolic O—H moieties from curcumin. The peak at 1617 cm^{-1} was assigned to the stretching vibration of C=O (enol) functionality of curcumin

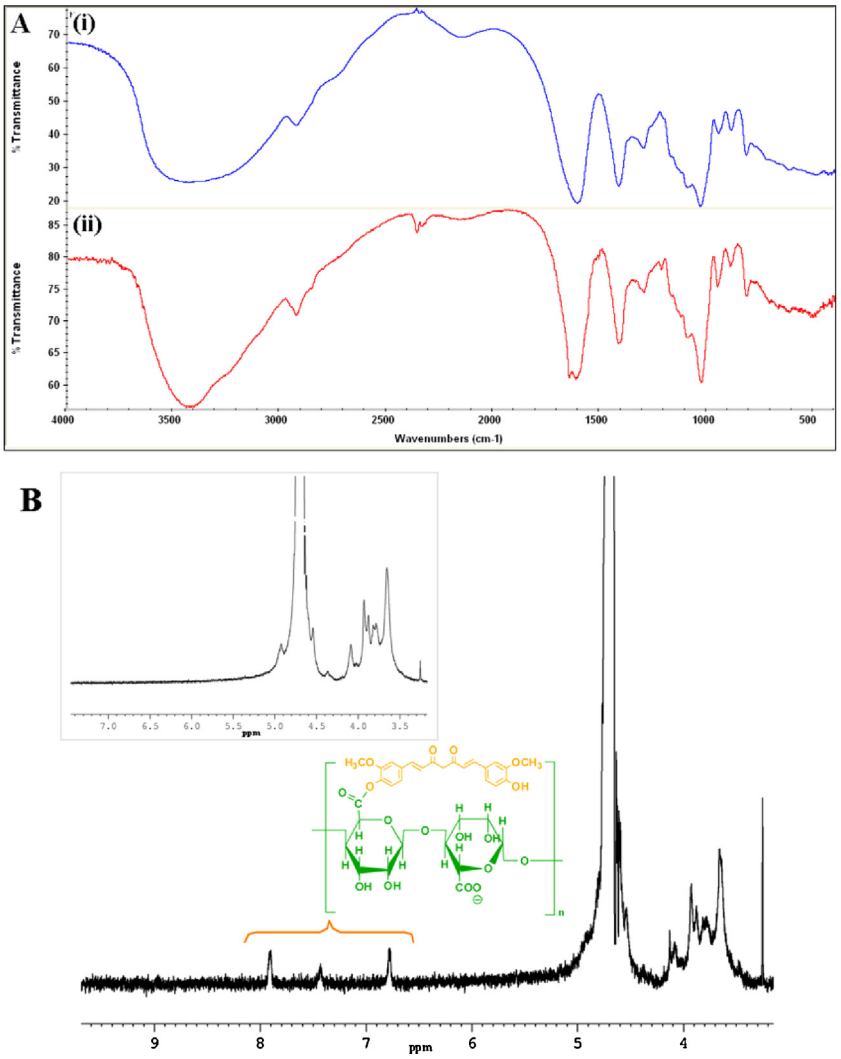


Fig. 1. (A) FTIR spectra of (i) Sodium alginate and (ii) Alg-Ccm conjugate, (B) ^{1}H NMR spectrum of Alg-Ccm conjugate (*inset*: ^{1}H NMR spectrum of alginate).

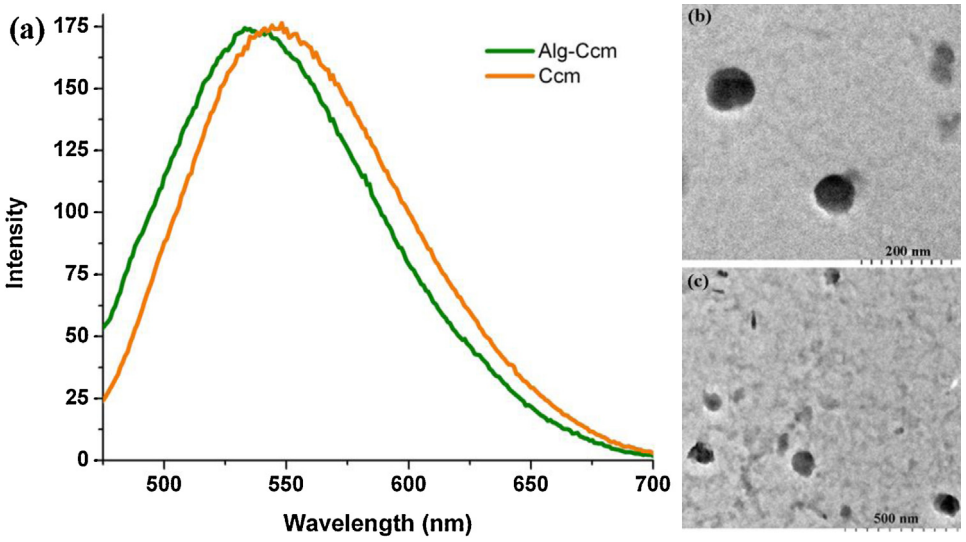


Fig. 2. (a) Fluorescence spectra of Alg-Ccm conjugate and free curcumin; (b) and (c) TEM images of the conjugate micelles at different magnifications.

in Alg-Ccm. The peaks seen around 1658 cm^{-1} and 1217 cm^{-1} were attributed to the C=O and C—O stretching frequencies of ester linkage, respectively. The aliphatic C—H stretches in Alg-Ccm were also nicely manifested through the peaks at 2928 cm^{-1} and 2847 cm^{-1}.

In order to confirm the formation of Alg-Ccm, ^{1}H NMR spectra of the conjugate was recorded. The ^{1}H NMR spectrum of alginate is illustrated as the inset picture in Fig. 1B in which the signals between 3.5 to 5 ppm are due to the methine protons of hexuronic acid residues (M and G) of alginate (Li, Liu, Huang, & Xue, 2011; Yang, Zhang, Wen, Liang, & Zhang, 2007). Fig. 1B depicts the ^{1}H NMR spectrum of the conjugate in which the additional peaks from 6.7 to 7.9 ppm clearly indicated the conjugation of curcumin to alginate backbone. However, the characteristic signal at 3.81 ppm assigned for methoxy proton of curcumin might have merged with the peak of C-5 methine proton of M unit of alginate in the conjugate.

Fig. 2(a) shows the fluorescence spectra of Alg-Ccm in water/EtOH (1:1, v/v) medium in comparison with that of pure curcumin. The hypsochromic shift by ~11 nm in the emission spectrum of Alg-Ccm compared to pure curcumin indicated the successful conjugation of curcumin to alginate. The characteristic absorption of Alg-Ccm in aqueous solution has been shown in Fig. S1 (Supporting information).

3.2. Self-aggregation of Alg-Ccm conjugate in aqueous medium

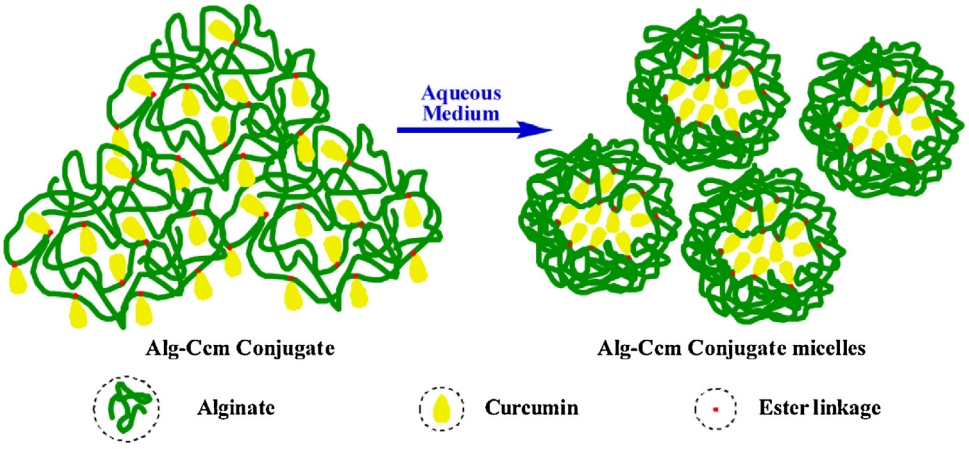
In order to look into the possibility of self-assembly of Alg-Ccm conjugate, the aqueous solution of the conjugate was analyzed by DLS which indicated the formation of nanosized micelle like structures with an average hydrodynamic diameter of 459 ± 0.32 nm as shown in Fig. S2 (Supplementary information). It was further confirmed by the TEM images of the conjugate shown in Fig. 2(b) and (c). The TEM images depict spherical particles with an average diameter of 62.5 ± 11 nm. The size of Alg-Ccm micelles determined by DLS was found to be much higher than the value obtained by TEM analysis and this observation is quiet justified. Because, in DLS the size determined is the hydrodynamic diameter of the nanoparticles in solution phase, whereas in TEM the entities are visualized in completely dry state (Manju & Sreenivasan, 2011a,b). Thus in this case, the Alg-Ccm micelles were in swelled state due to the extensive hydrophilicity of alginate and appeared much larger than when they were in complete dry condition (*i.e.* on the TEM grid).

Another important characteristic of polymeric nanoparticles is the zeta potential which represents an index for particle stability in nanoparticle dispersion. The zeta potential of Alg-Ccm micelles was found to be – 45.43 ± 0.2 mV (at 25 °C and pH 7.4). This indicated that the micelles were stable enough in aqueous medium (Hans & Lowman, 2002). A polymer-drug conjugate can promote passive tumor targeting, hence it should have a longer circulation time to achieve maximum EPR effect and it should not generate any unwanted immune response. It has also been reported that positively charged particles or particles with hydrophobic surfaces can facilitate plasma protein adsorption on their surface and are susceptible to reticuloendothelial system (RES) clearance (Anitha et al., 2011; Gessner et al., 2000). In Alg-Ccm conjugate micelles, the outer shell is made of strongly hydrophilic, non-immunogenic polymer alginate and the micelles possess negative (–ve) surface charge. Hence Alg-Ccm micelles might evade protein adsorption to a large extent giving rise to enhanced circulation time with elevated EPR effect.

3.3. Determination of critical micelle concentration of Alg-Ccm conjugate

Amphiphilic derivatives of alginate containing long alkyl chains or cholesteryl groups; as well as amphiphilic block copolymers *etc.* are well studied examples to form micelles in aqueous solution due to hydrophobic interactions (Choi, Lee, Choi, & Park, 2006; Galant, Kjøniksen, Nguyen, Knudsen, & Nyström, 2006). In the present case, Alg-Ccm can also form micelle type core-shell structure where the highly hydrophilic polymer alginate will acquire the shell layer and curcumin will constitute the hydrophobic inner core as illustrated in Scheme 2.

In order to characterize the self-assembly behavior of Alg-Ccm, the critical micelle concentration (CMC) of the conjugate was determined using 1-PyCHO as the probe molecule. 1-PyCHO, an exocyclic carbonyl compound possesses its $n \rightarrow \pi^*$ singlet and triplet states close in energy to the $\pi \rightarrow \pi^*$ states and hence it is an extensively used photoinduced probe for characterization of CMC (Oton & Acuna, 1980; Singh, Srivastava, & Kumar, 2012). Fig. 3(a) shows the absorption spectra of 1-PyCHO in water with increasing concentration of Alg-Ccm conjugate. The absorption spectrum of 1-PyCHO is highly sensitive to the polarity of the solvent used and in aqueous solution the spectrum consists of intense bands in 300–450 nm regions due to the ^1B_b and ^1L_a states of the main pyrene chromophore (Dutta, Misra, & Pal, 1996). Absorbance of the probe increases with increase in the concentration of the conjugate without any spectral shift (Fig. 3a). Hence, it is assumed that there is a complex formation between the probe molecule and the amphiphilic conjugate resulting in the enhancement of absorbance. CMC value of Alg-Ccm was determined by plotting the absorbance ratio ($O.D./O.D._0$) (at 368 nm) against – log [conjugate] as shown in Fig. 3(b). O.D. and $O.D._0$ represent the absorbances of 1-PyCHO



Scheme 2. Schematic presentation of micellization of Alg-Ccm conjugates in aqueous medium.

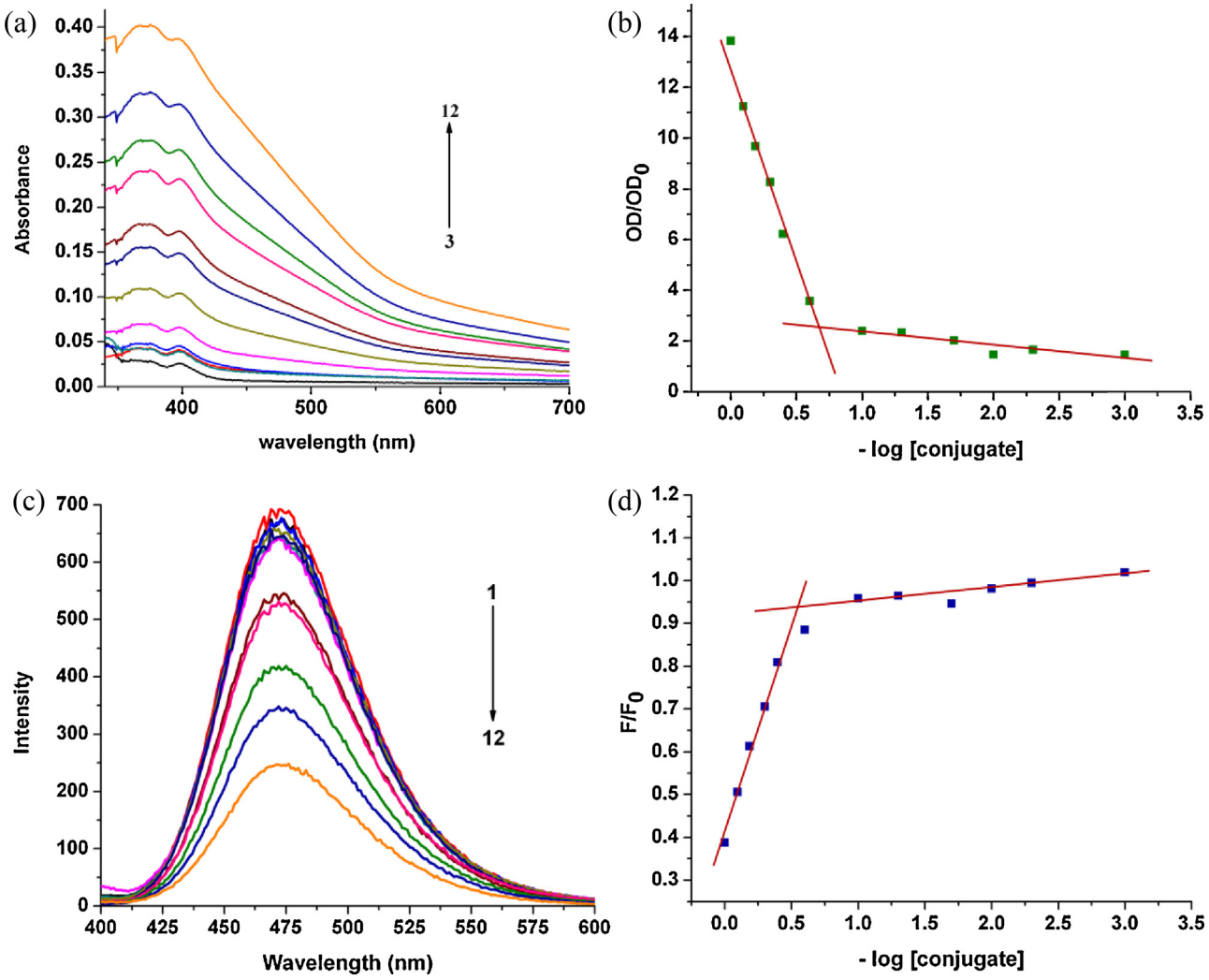


Fig. 3. Spectral data for the CMC determination of Alg-Ccm conjugate: (a) absorbance spectra of 1-PyCHO in water with increasing concentration of Alg-Ccm conjugate (b) plot of O.D./O.D.$_0$ Vs – log [conjugate]; (c) emission spectra of 1-PyCHO in water with increasing concentration of Alg-Ccm conjugate (d) plot of F/F_0 Vs – log [conjugate].

in presence and absence of Alg-Ccm. The break point in the curve indicates the CMC value of Alg-Ccm (Fig. 3b) and the CMC values are tabulated in Table S1 (Supplementary information).

Fluorescent probes are competent enough to provide microscopic information regarding the nature of micellar aggregates in aqueous solution. For 1-PyCHO, a relatively polar probe, the sensitive parameter for evaluating the polarity of probe's environment is its fluorescence maxima (λ_{max}) (Ananthapadmanabhan, Goddard, Turro, & Kuo, 1985). On exciting the probe at 368 nm, it shows an emission spectrum with λ_{max} at 474 nm due to the monomeric excited singlet state and also shows strong solvent dependence (Banerjee, Chatterjee, Pramanik, & Bhattacharya, 2007). Fig. 3(c) illustrates the relative lowering of fluorescence intensity of 1-PyCHO at 474 nm with increasing concentration of Alg-Ccm conjugate. The decrease of fluorescence intensity of 1-PyCHO at its λ_{max} indicates the solubilization of probe molecule in a more hydrophobic environment than water and in this case it is the hydrophobic core of the micelles formed by Alg-Ccm. Fluorescence intensities in presence of conjugate (F) at 474 nm relative to the intensities in absence of conjugate (F_0) were plotted against – log [conjugate] as depicted in Fig. 3(d) and the break point indicated the CMC value of Alg-Ccm (see Table S1). The CMCs obtained by absorption and emission studies (Table S1) were in good agreement with each other.

3.4. Curcumin content and aqueous solubility of the conjuagte

The average numbers of curcumin ester linked to hexuronic acid residues (G/M) in alginate is defined as the degree of substitution (DS) of Alginate-Curcumin (Alg-Ccm) conjugate. Alg-Ccm conjuagte exhibited an intense yellow color with an average curcumin content of 1.09 ± 0.53 mg in 100 mg of the conjugate. Thus the degree of substitution (DS, in %) of Alg-Ccm was found to be 0.5. Hence, on an average, 1 unit of curcumin was conjugated to every 200 hexuronic acid residues. Alg-Ccm conjugate dissolved readily in distilled water and aqueous buffer solution (pH 7.4). The aqueous solubility of Alg-Ccm conjugate was found to be more than 10 mg/mL, which corresponds to 109 μg/mL curcumin. Curcumin is practically having an extremely low aqueous solubility but a significant enhancement in its aqueous solubility was observed after conjugation with highly hydrophilic polymer alginate and the images shown in Fig. 4(a) support the fact.

3.5. Stabilization of curcumin inside the core of Alg-Ccm micelles

The rapid hydrolytic degradation of curcumin is one of the major reasons behind its poor bioavailability. Several studies have proved that this hydrolytic degradation occurs much faster above the neutral pH (Wang et al., 1997) and these

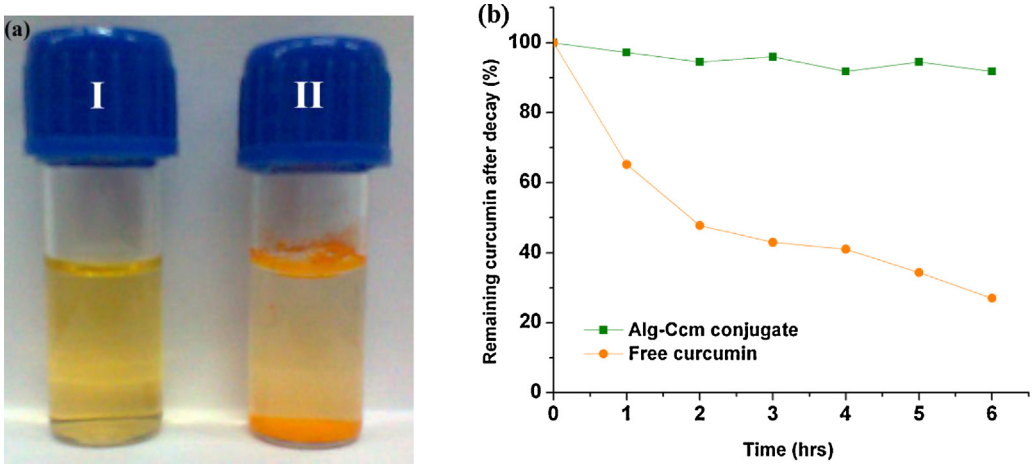


Fig. 4. (a) Images of solutions of Alg-Ccm conjugate (I) and free curcumin (II) in PBS at pH 7.4, the conjugate gives a clear yellow solution but free curcumin forms flakes indicating poor solubility; and (b) Plot showing degradation of free curcumin and Alg-Ccm conjugate in PBS at pH 7.4.

studies have also revealed that partially deprotonated curcumin is first fragmented into *trans*-6-(40-hydroxy-30-methoxyphenyl)-2,4-dioxo-5-hexenal which further degrades into other smaller fragments like feruloyl methane, vanillin and ferulic acid. These smaller fragments contribute negligibly toward the absorption of light (Agarwal & Shishodia, 2006). Hence a decrease in the absorbance (at 420 nm) can indicate the decrease in concentration of pure curcumin in a solution. Here to study the stability of curcumin in conjugated state, we incubated solutions of free curcumin and Alg-Ccm in PBS (pH 7.4) for 6 h at 37 °C and estimated absorbances of each solution at 1 h intervals. We reasoned that the absorbance of Alg-Ccm would also decrease drastically in case of hydrolytic degradation. It was observed that free curcumin decayed very fast where as the conjugate was highly stable (as shown in Fig. 4b). In aqueous solution the conjugate remained in self-assembled condition in which hydrophobic curcumin was inside the core of conjugate micelle protected by the hydrophilic alginate shell. Thus micelle structure shielded curcumin from hydrolytic degradation to a large extent and made Alg-Ccm highly stable in aqueous medium.

3.6. Evaluation of cytotoxic activity of Alg-Ccm conjugate

Curcumin has the ability to kill cells and it has also been reported that the —OH and —OMe groups in Ccm are responsible for its antioxidant and antiproliferative properties, respectively (Ravindran et al., 2009). In order to confirm whether conjugation of curcumin to a hydrophilic polymer can exert any effect on the intrinsic potential of curcumin to kill cells, the cytototoxicity of Alg-Ccm was evaluated with L-929 mouse fibroblast cells. For this study, 10 mg of the conjugate was dissolved in 1 mL of culture medium (10 mg/mL of Alg-Ccm solution, *i.e.*; equivalent to 109 μg curcumin) and this solution was then diluted to get other two concentrations of conjugate samples (5 mg/mL and 2.5 mg/mL corresponding to 54.5 μg and 27.25 μg curcumin, respectively). Then cells were incubated with 100 μL of each of the conjugate solutions to study the cellular response. The images shown in Fig. 5 clearly indicated severe toxicity of the conjugate toward L-929 cells. Fig. 5(a) and (b) showed almost complete destruction of the original spindle shape morphology of the cell layers. Fig. 5(c) indicated moderate cytotoxic reactivity (due to reduced concentration of curcumin) toward the cells. These images also pointed out that the Alg-Ccm micelles were internalized into cells. Destruction of cells by the conjugate micelles proved that the ability of curcumin to kill cells was retained in the conjugate.

Besides, the cytotoxicity of the conjugate was quantitatively assessed using MTT assay. The percentage of cell viability quantified from MTT assay is shown in Fig. 6. The MTT assay of L-929 cells after contact with different dilutions of conjugate solutions corresponding to curcumin equivalent of 10.9 μg, 5.4 μg and 2.7 μg killed nearly 85%, 62% and 44% of the cells respectively. The result represents (Fig. 6a) a concentration dependent decrease in cell viability. The percentage of viable cells increased (for diluted conjugate solutions) as there was a decrease in the concentration of equivalent curcumin with dilution. The comparison of cytotoxic effect of conjugated curcumin with free curcumin (maximum amount of free curcumin *i.e.*10.9 μg is shown here) also reflected the improvement in cytotoxic potential of the conjugate (Fig. 6b). The conjugate exhibited significant cytotoxic effect and a low quantity of Alg-Ccm conjugate was able to produce considerable cytotoxicity. But

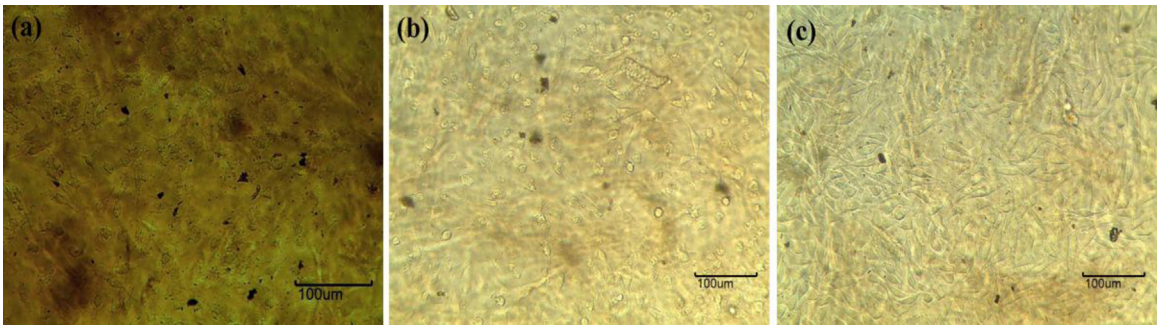


Fig. 5. Microscopic images showing the morphology of L-929 cells after contact with different concentrations of Alg-Ccm conjugate solution corresponding to curcumin equivalent of (a) 10.9 μg, (b) 5.4 μg and (c) 2.7 μg.

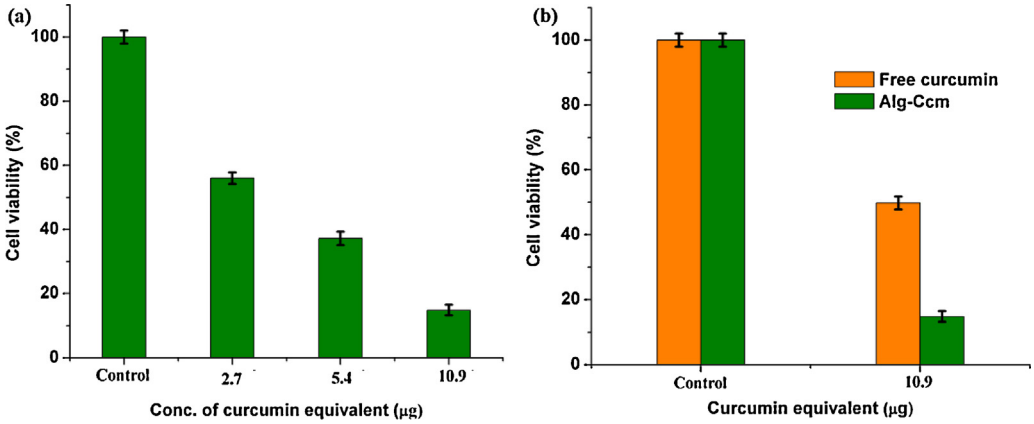


Fig. 6. (a) Cytotoxic activity of Alg-Ccm conjugate against L-929 cells, and (b) a comparison of cytotoxic activity of Alg-Ccm conjugate and free curcumin against L-929 cells. Cell viability (%) of the extract prepared using the negative control (ultrahigh density polyethylene) is taken as 100%. The error bars indicate mean value ± standard deviation ($n = 3$).

free curcumin showed less cytotoxicity. Here, we opine that free curcumin could get less effective exposure time due to its poor aqueous solubility, instability and consequent precipitation resulting in low cytotoxic potential. Whereas Alg-Ccm could get more exposure time due to the enhanced aqueous solubility, better cellular internalization of conjugate micelles and improved stability at physiological condition giving rise to a better cytotoxic activity.

4. Conclusions

In the present study, we have developed a polymer-drug conjugate by direct covalent conjugation of hydrophobic drug curcumin with the C-6 carboxylate group of hydrophilic, biodegradable and biocompatible natural polymer sodium alginate *via* esterification reaction. Alg-Ccm conjugate was found to be extensively water soluble and thereby the aqueous solubility of curcumin (in conjugated form) was enhanced by several folds than that of free curcumin. The conjugate self-aggregated in aqueous solution forming nanosized micelles and curcumin occupied the core of the micelles enhancing the stability of curcumin in aqueous medium. Outer shell of the micelles is made of hydrophilic and non-immunogenic polymer alginate and the micelles possess negative surface charge. As a result, the conjugate may possibly improve the time of systemic circulation with enhanced EPR effect. Alg-Ccm conjugate also showed excellent cytotoxic activity compared to free curcumin. Hence, Alg-Ccm can effectively play the role of a potential curcumin conjugate. Besides, the conjugate micelles can encapsulate other hydrophobic drug molecules inside the hydrophobic core and can also be used as a nanosized delivery vehicle for other hydrophobic drugs.

Acknowledgements

The Council for Scientific and Industrial Research (CSIR), India, is gratefully acknowledged for financial assistance. The authors also thank the Director and the Head of Biomedical Technology Wing, SCTIMST for providing necessary facilities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.067.

References

Aggarwal, B. B., Kumar, A., & Bharti, A. C. (2003). Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer Research*, *23*, 363–398.
Agarwal, B. B., & Shishodia, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology*, *71*, 1397–1421.
Aggarwal, B. B., & Sung, B. (2009). Pharmacological basis for the role of curcumin in chronic diseases: An age-old spice with modern targets. *Trends in Pharmacological Sciences*, *30*, 85–94.
Alexis, F., Rhee, J. W., Richie, J. P., Moreno, A. F. R., Langer, R., & Farokhzad, O. C. (2008). New frontiers in nanotechnology for cancer treatment. *Urologic Oncology*, *26*, 74–85.
Anand, P., Kunnumakkara, A. B., Newman, R. A., & Aggarwal, B. B. (2007). Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics*, *4*, 807–818.
Ananthapadmanabhan, K. P., Goddard, E. D., Turro, N. J., & Kuo, P. L. (1985). Fluorescence probes for critical micelle concentration determination. *Langmuir*, *1*, 352–355.
Anitha, A., Deepagan, V. G., Divya Rani, V. V., Menon, D., Nair, S. V., & Jayakumar, R. (2011). Preparation, characterization, *in vitro* drug release and biological studies of curcumin loaded dextran sulphate–chitosan nanoparticles. *Carbohydrate Polymers*, *84*, 1158–1164.
Banerjee, P., Chatterjee, S., Pramanik, S., & Bhattacharya, S. C. (2007). Interaction of pyrene-1-Carboxaldehyde with micelles and mixed micelles of polyoxyethylene nonyl phenol (Igepal): A spectroscopic study. *Colloids and Surfaces A*, *302*, 44–50.
Bava, S. V., Puliappadamba, V. T., Deepti, A., Nair, A., Karunagaran, D., & Anto, R. J. (2005). Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappaB and the serine/threonine kinase Akt and is independent of tubulin polymerization. *Journal of Biological Chemistry*, *280*, 6301–6308.
Bisht, S., Feldmann, G., Soni, S., Ravi, R., Karikar, C., Maitra, A., & Maitra, A. (2007). Polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"): A novel strategy for human cancer therapy. *Journal of Nanobiotechnology*, *5*, 1–18.
Chattopadhyay, I., Biswas, K., Bandyopadhyay, U., & Banerjee, R. K. (2004). Turmeric and curcumin: Biological actions and medicinal applications. *Current Science*, *87*, 44–53.
Choi, S. H., Lee, J. H., Choi, S. M., & Park, T. G. (2006). Thermally reversible pluronic/heparin nanocapsules exhibiting 1000-fold volume transition. *Langmuir*, *22*, 1758–1762.
Dutta, A. K., Misra, T. N., & Pal, A. J. (1996). A Spectroscopic study of nonamphiphilic pyrene assembled in langmuir-blodgett films: Formation of aggregates. *Langmuir*, *12*, 459–465.
Galant, C., Kjøniksen, A. L., Nguyen, G. T. M., Knudsen, K. D., & Nyström, B. (2006). Altering associations in aqueous solutions of a hydrophobically modified alginate in the presence of β-cyclodextrin monomers. *Journal of Physical Chemistry B*, *110*, 190–195.
Gessner, A., Waicza, R., Lieske, A., Paulke, B. R., Mäder, K., & Müller, R. H. (2000). Nanoparticles with decreasing surface hydrophobicities: Influence on plasma protein adsorption. *International Journal of Phamaceutics*, *196*, 245–249.
Goel, A., Kunnumakkara, A. B., & Aggarwal, B. B. (2008). Curcumin as "Curecumin": From kitchen to clinic. *Biochemical Pharmacology*, *75*, 787–809.
Hans, M. L., & Lowman, A. M. (2002). Biodegradable nanoparticles for drug delivery and targeting. *Current Opinion in Solid State and Materials Science*, *6*, 319–327.
Lantz, R. C., Chen, G. J., Solyom, A. M., Jolad, S. D., & Timmermann, B. N. (2005). The effect of turmeric extracts on inflammatory mediator production. *Phytomedicine*, *12*, 445–452.
Li, S. D., & Huang, L. (2008). Pharmacokinetics and biodistribution of nanoparticles. *Molecular Pharmaceutics*, *5*, 496–504.
Li, Q., Liu, C. G., Huang, Z. H., & Xue, F. F. (2011). Preparation and characterization of nanoparticles based on hydrophobic alginate derivative as carriers for sustained release of vitamin D3. *Journal of Agricultural and Food Chemistry*, *59*, 1962–1967.

Maeda, H. (2010). Tumor-selective delivery of macromolecular drugs via the epr effect: Background and future prospects. *Bioconjugate Chemistry*, *21*, 797–802.
Manju, S., & Sreenivasan, K. (2011a). Conjugation of curcumin onto hyaluronic acid enhances its aqueous solubility and stability. *Journal of Pharmaceutical Sciences*, *359*, 318–325.
Manju, S., & Sreenivasan, K. (2011b). Synthesis and characterization of a cytotoxic cationic polyvinylpyrrolidone-curcumin conjugate. *Journal of Pharmaceutical Sciences*, *100*, 504–511.
Manju, S., & Sreenivasan, K. (2012). Gold nanoparticles generated and stabilized by water soluble curcumin-polymer conjugate: Blood compatibility evaluation and targeted drug delivery onto cancer cells. *Journal of Colloid and Interface Science*, *368*, 144–151.
Mythri, R. B., Harish, G., Dubey, S. K., Misra, K., & Bharath, M. M. S. (2011). Glutamoyl diester of the dietary polyphenol curcumin offers improved protection against peroxynitrite-mediated nitrosative stress and damage of brain mitochondria in vitro: Implications for Parkinson's disease. *Molecular and cellular biochemistry*, *347*, 135–143.
Oton, J. M., & Acuna, A. U. (1980). Fluorescence quantum yield of pyrene-1-carboxaldehyde in protic solvents. *Journal of Photochemistry*, *14*, 341–343.
Park, K., & Lee, J. H. (2007). Photosensitizer effect of curcumin on UVB-irradiated HaCaT cells through activation of caspase pathways. *Oncology Reports*, *17*, 537–540.
Ravindran, J., Prasad, S., & Aggarwal, B. B. (2009). Curcumin and cancer cells: How many ways can curry kill tumor cells selectively? *American Association of Pharmaceutical Scientists Journal*, *11*, 495–510.
Ringman, J. M., Frautschy, S. A., Cole, G. M., Masterman, D. L., & Cummings, J. L. (2005). A potential role of the curry spice curcumin in Alzheimer's disease. *Current Alzheimer Research*, *2*, 131–136.
Ringsdorf, H. (1975). Structure and properties of pharmacologically active polymers. *Journal of Polymer Science: Polymer Symposia*, *51*, 135–153.
Ruby, A. J., Kuttan, G., Babu, K. D., Rajasekharan, K. N., & Kuttan, R. (1995). Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Letters*, *94*, 79–83.
Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Murakami, A., Sethi, G., Limtrakul, P., Badmaev, V., & Aggarwal, B. B. (2007). Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis*, *28*, 1765–1773.
Shi, M. X., Cai, Q. F., Yao, L. M., Mao, Y. B., Ming, Y. L., & Ouyang, G. L. (2006). Antiproliferation and apoptosis induced by curcumin in human ovarian cancer cells. *Cell Biology International Reports*, *30*, 221–226.
Singh, P., Srivastava, A., & Kumar, R. (2012). Synthesis of amphiphilic poly(*N*-vinylcaprolactam) using ATRP protocol and antibacterial study of its silver nanocomposite. *Journal of Polymer Science Part A: Polymer Chemistry*, *50*, 1503–1514.
Thangapazham, R. L., Puri, A., Tele, S., Blumenthal, R., & Maheshwari, R. K. (2008). Evaluation of a nanotechnology-based carrier for delivery of curcumin in prostate cancer cells. *International Journal of Oncology*, *32*, 1119–1123.
Van, S., Das, S. K., Wang, X., Feng, Z., Jin, Y., Hou, Z., Chen, F., Pham, A., Jiang, N., Howell, S. B., & Yu, L. (2010). Synthesis, characterization and biological evaluation of poly(L-γ-glutamyl-glutamine)-paclitaxel nanoconjugate. *International Journal of Nanomedicine*, *5*, 825–837.
Wang, Y. J., Pan, M. H., Cheng, A. L., Lin, L. I., Ho, Y. S., Hsieh, C. Y., et al. (1997). Stability of curcumin in buffer solutions and characterization of its degradation products. *Journal of Pharmaceutical and Biomedical Analysis*, *15*, 1867–1876.
Yallapu, M. M., Jaggi, M., & Chauhan, S. C. (2010). Beta-Cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells. *Colloids and Surfaces, B: Biointerfaces*, *79*, 113–125.
Yang, L., Zhang, B., Wen, L., Liang, Q., & Zhang, L. M. (2007). Amphiphilic cholesteryl grafted sodium alginate derivative: Synthesis and self-assembly in aqueous solution. *Carbohydrate Polymers*, *68*, 218–225.
Zhou, P., Li, Z., & Chau, Y. (2010). Synthesis, characterization and *in vivo* evaluation of poly(ethyleneoxide-co-glycidol)-platinate conjugate. *European Journal of Pharmaceutical Science*, *41*, 464–472.