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Enhanced in vitro anti-cancer activity of curcumin encapsulated in hydrophobically modified starch

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ABSTRACT

Curcumin is a natural polyphenolic compound with anti-oxidation, anti-inflammation, and anti-cancer properties. However, these benefits of curcumin suffer from its extremely low water solubility and bioavailability. In this study, we demonstrated that hydrophobically modified starch (HMS), a food-grade biopolymer, is able to form micelles and to encapsulate curcumin. Upon encapsulation, curcumin showed increased solubility by about 1670-folds. This may be due to the hydrophobic interaction and hydrogen bonding between curcumin and HMS, as suggested by results from infrared and fluorescence spectroscopy. The synchrotron small-angle X-ray scattering results indicated that the addition of curcumin did not alter the structure of HMS, whose radius of gyration remained at 14.1 ± 0.1 nm. Moreover, encapsulated curcumin revealed enhanced in vitro anti-cancer activity compared to free curcumin. This study provides a novel food-grade encapsulation formulation to increase the bioaccessibility of curcumin.

1. Introduction

One of the newest trends in food science and technology is functional food. According to the International Food Information Council (IFIC), functional food is defined as "foods that provide health benefits beyond basic nutrition" (Shibamoto, Kanazawa, Shahidi, & Ho, 2008; Vaclavik & Christian, 2008). In recent years, extensive research has been carried out to study the health promotion properties of different phytochemicals and to devise novel encapsulation materials and methods, trying to incorporate functional ingredients into foods (Pegg & Shahidi, 2007).

Among various functional food ingredients, polyphenols have attracted many researchers' attention because of their anti-oxidant, anti-inflammatory, and anti-cancer properties (Chan, Huang, Fenton, & Fong, 1998; Huang et al., 1994; Sharma, Gescher, & Steward, 2005; Sreejayan & Rao, 1996). Together with some other plant-derived polyphenols, curcumin [bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], which is extracted from plant turmeric (*Curcuma longa*), is among the best characterised polyphenols (Khanna, 1999). Since it has a strong yellowish colour, it is primarily used as a food colourant. Equally important, curcumin has a long history to treat inflammation and cancers in India and China (Joe, Vijaykumar, & Lokesh, 2004).

Curcumin functions as an anti-cancer agent by activating apoptosis signaling and inhibiting cell proliferation (Duvoix et al., 2005). On one hand, curcumin inhibits Bcl2 and activates Caspase 9 to induce apoptosis, and on the other hand, it blocks many cell proliferation signaling pathways, such as MAP kinase pathway, AKT pathway and mTOR pathways (Duvoix et al., 2005; Howitz & Sinclair, 2008; Joe et al., 2004; Syng-ai, Kumari, & Khar, 2004).

The application of curcumin as a health-promoting agent has been limited by its poor water solubility and bioavailability. While curcumin is dissolves in ethanol, acetone, chloroform, DMSO (dimethyl sulphoxide) and some other polar organic solvents, its solubility in pure water is estimated to be at most 11 ng/mL (Kaminaga et al., 2003). Even worse, once absorbed in human body, curcumin undergoes rapid degradation and excretion (Sharma et al., 2005).

Many approaches have been applied to increase the water solubility and/or bioavailability of food bioactives by methods such as emulsion, chemical modification and micelle encapsulation. The greatest loading capacity is achieved by using an oil in water (O/W) emulsion system. As reported, curcumin can be dissolved in hot soybean oil up to 1% (Sou, Inenaga, Takeoka, & Tsuchida, 2008). The curcumin O/W nanoemulsions using medium chain triacylglycerols as the oil phase, which contained 1% curcumin, showed significantly improved anti-inflammation activity (Wang et al., 2008).

In addition to emulsion system, cyclodextrin is also known to form inclusion complex with curcumin (Tang, Ma, Wang, & Zhang, 2002; Tomren, Masson, Loftsson, & Tonnesen, 2007; Tonnesen, Masson, & Loftsson, 2002). Among different cyclodextrin variants, hydroxypropyl-γ-cyclodextrin (HPγCD) has the highest encapsulation capacity, and the water solubility of curcumin in 10% HPγCD can reach about 2 mg/mL (Tomren et al., 2007).

Additionally, chemical modification methods were also reported to increase the water solubility of curcumin. Glucose

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molecules have been conjugated onto curcumin molecule by either chemical reaction or by plant cell culture (Kaminaga et al., 2003). These glucose–curcumin prodrugs have much better solubility in water, and the solubility of curcumin 4′,4″-O-β-D-digentiobioside in water could reach as high as 240 mg/mL (Kaminaga et al., 2003).

Micellar encapsulation is another approach to increase the water solubility of nutraceuticals. Micelles can be formed by small molecular-weight surfactants, such as cetyltrimethylammonium bromide (CTAB) (Iwunze, 2004; Leung, Colangelo, & Kee, 2008; Wang, Wu, Liu, Jia, & Yang, 2006). Synthetic amphiphilic polymers, for example, PEO-b-PCL [poly(ethylene glycol)-block-poly(caprolactone)] and methoxy poly(ethylene glycol)-palmitate, were also reported to form polymer micelles to encapsulate curcumin (Ma et al., 2008; Sahu, Bora, Kasoju, & Goswami, 2008). The solubilisation capacity and loading efficiency have been extensively investigated. However, no food-grade polymers have been reported to encapsulate curcumin, which greatly limits the application of curcumin in functional food products.

In searching food-grade amphiphilic materials to encapsulate curcumin, we focused on hydrophobically modified starch (HMS), an abundant and low cost food ingredient synthesised with waxy maize and *n*-octenyl succinic anhydride (*n*-OSA) (Shaikh, Bhosale, & Singhal, 2006). HMS is widely used to encapsulate flavours during spray drying process (Krishnan, Bhosale, & Singhal, 2005; Shaikh et al., 2006; Soottitantawat et al., 2005a, 2005b; Xie, Zhou, & Zhang, 2007). Since HMS is an amphiphilic polymer, we hypothesise that following the polymeric micellar encapsulation strategy, HMS is also able to form polymer micelles and to encapsulate curcumin. In this study, we demonstrated that HMS micelles greatly increased the water solubility of curcumin. Moreover, curcumin encapsulated in HMS micellar cores exhibited increased anti-cancer activity in vitro.

2. Materials and methods

2.1. Materials

Curcumin was a generous gift from Sabinsa Corporation (Piscataway, NJ), which contains 85% curcumin, with 11% of demethoxycurcumin and 4% of bisdemethoxycurcumin (Wang et al., 2008). It was used without further purification. Hydrophobically modified starch (HMS) was obtained from National Starch and Chemical Company (Bridgewater, NJ) with a brand name of Hi-Cap 100. Pyrene, acetone and chloroform were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Determination of the critical aggregation concentration of HMS

The critical aggregation concentration (CAC) of HMS was determined by measuring the fluorescence spectrum of 6×10^{-7} M pyrene in 1× phosphate buffered saline (1× PBS) containing different concentrations of HMS (0.01–5%). The excitation fluorescence spectrum from 300 to 350 nm was obtained using Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA). The emission wavelength was set at 390 nm and the slit openings were set at 5 nm for both excitation and emission. The ratio of the intensity at 337 nm (I_{337}) to that at 334 nm (I_{334}) was calculated and plotted against the common logarithm of HMS concentration. The CAC of HMS was determined as the corresponding concentration of HMS at the turning point in the plot.

2.3. Loading of curcumin in HMS solution

Excessive amount of curcumin was mixed with 1% HMS solution and homogenised at 24000 rpm for 10 min with High Speed Homogeniser (ULTRA–TURRAX T-25 basic, IKA Works, Wilmington, NC) and stirred on a magnetic stirrer overnight at room temperature. On the next day, free curcumin was removed by high-speed centrifugation and filtration through 0.45 μm filter.

2.4. Quantification of curcumin extracted from HMS micelles

Equal volume of chloroform was added to curcumin HMS solution and subsequently vortexed for 10 min and then stirred on a magnetic stirrer overnight. After complete phase separation, the chloroform phase was diluted 10 times, and the UV–Vis absorbance at 419 nm was measured with a Cary UV–Vis spectrophotometer (Varian Instruments, Walnut Creek, CA). The quantity of curcumin was determined according to the calibration curve of curcumin in chloroform in the concentration range of 1–5 μg/mL.

2.5. Lyophilisation and reconstitution

Curcumin HMS solution was frozen at −20 °C overnight and then lyophilised using a Freezone 4.5 freeze-dry system (Labconco, Kansas City, MO). Deionised H_2O was used to reconstitute the curcumin HMS solution.

2.6. Infrared spectrum and fluorescence spectrum of HMS-encapsulated curcumin

The infrared spectrum of lyophilised curcumin HMS was measured by using a Thermo Nicolet Nexus 670 FT-IR system with attenuated total reflectance (ATR) accessory (Thermo Fisher Scientific, Waltham, MA). The fluorescence emission spectra of curcumin water solution and curcumin HMS solution were determined using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA). The excitation wavelength was set at 319 nm, and the emission spectra were ranged from 450 to 650 nm. The slit openings were set at 10 nm for both excitation and emission.

2.7. Synchrotron small-angle X-ray scattering (SAXS)

SAXS datasets were collected from solutions of 10 mg/mL HMS with and without the addition of curcumin at the BIOCAT undulator beamline 18-ID of APS, Argonne National Laboratory (Fischetti et al., 2004). To minimise radiation damage during data collection, samples were continuously pumped through a 1.5 mm-wide quartz capillary at 12.5 μL/s for an average exposure time of 0.6 s. The scattering intensity profiles were obtained by subtracting the average of 15 water-only profiles from the average of 15 starch–water or curcumin–starch–water profiles, which were performed with the program IGOR Pro (WaveMatrics), and macros written by the BIOCAT staff.

2.8. Cell culture and in vitro anti-cancer activity assay

Human hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (HB-8065, Manassas, VA) and were cultured in minimum essential medium (Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/mL penicillin (Invitrogen, Carlsbad, CA) and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained in incubators at 37 °C under 95% relative humidity and 5% CO_2.

Anti-cancer activity of curcumin was examined by methyl thiazol tetrazolium bromide (MTT) assay. Briefly, HepG2 cells were seeded in 96-well microtiter plates at a density of 10,000 cells per well in a final volume of 100 μL medium. After 24 h, the cells were treated with a medium containing DMSO-dissolved or

HMS-encapsulated curcumin of different concentrations. Other cells were untreated as negative control, or treated only with DMSO or HMS at the maximum concentration used to dissolve and encapsulate curcumin, respectively. After 24 h, cell culture media were aspirated and cells were incubated with 100 μL MTT solution (0.5 mg/mL in RPMI 1640 medium) for 2 h at 37 °C. Subsequently, MTT solution was carefully aspirated and the formazan crystals formed were dissolved in 100 μL DMSO per well. Light absorbance at 560 and 670 nm was recorded with Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). Relative cell viability was expressed as A560–A670 normalised to that of the untreated wells. Data were presented as mean ± standard deviation with eight-well repeats.

2.9. Statistical analysis

Anti-cancer effect of DMSO-dissolved and HMS-encapsulated curcumin was compared with *t*-test using SigmaPlot 10.0 software (Systat Software, San Jose, CA).

3. Results

3.1. Capability of HMS to form polymer micelles

To demonstrate the ability of hydrophobically modified starch (HMS) to form polymer micelles, the pyrene fluorescent method was first used to determine the possible critical aggregation concentration (CAC) of HMS. The fluorescent spectrum of pyrene is sensitively affected by its microenvironment. When pyrene is in hydrophilic environment, one of its excitation peaks is at 334 nm. Once pyrene migrates into hydrophobic environment, such as the core portion of polymer micelles, this peak shifts from 334 to about 337 nm. By plotting the ratio of the fluorescence intensity at 337 nm to that at 334 nm (I_{337}/I_{334}) versus polymer concentrations, the CAC of amphiphilic polymers can be determined (Zhao, Winnik, Riess, & Croucher, 1990). As shown in Fig. 1, I_{337}/I_{334} increased with the increase of HMS concentration. At 0.36%, a turning point appeared, indicating the onset of the self-assembly of HMS and the CAC of HMS.

Compared with other surfactants and synthetic amphiphilic polymer micelles, HMS has a relatively high CAC. This may be due to its big portion of hydrophilic polysaccharide group and low degree of substitution (typically less than 3%) limited by the food industry regulation.

3.2. Encapsulation of curcumin in HMS solution

HSM solution 1% was prepared to encapsulate curcumin in HSM micelles. Curcumin powder was loaded under high-speed homogenisation and stirred on a magnetic stirrer overnight. After removal of free curcumin, curcumin HMS solution was clear and yellowish (Fig. 2A), compared with curcumin water solution as a control (Fig. 2B). To quantify the amount of curcumin in the HMS micelles, curcumin was extracted with chloroform. The concentration of curcumin was calculated using the calibration curve of curcumin chloroform solution. It is determined that the absolute concentration of curcumin in filtered HMS solution is 18.4 μg/mL. Compared with the estimated water solubility of 11 ng/mL (Kaminaga et al., 2003), HMS can at least increase the solubility by 1670-folds.

Curcumin HMS solution was subsequently lyophilised and reconstituted with water. Lyophilised curcumin HMS solution afforded a yellowish powder with no noticeable free curcumin powder. After being reconstituted with water, this powder dissolved back to clear solution very quickly and easily, with no noticeable curcumin precipitates (Fig. 2C). Our results suggested

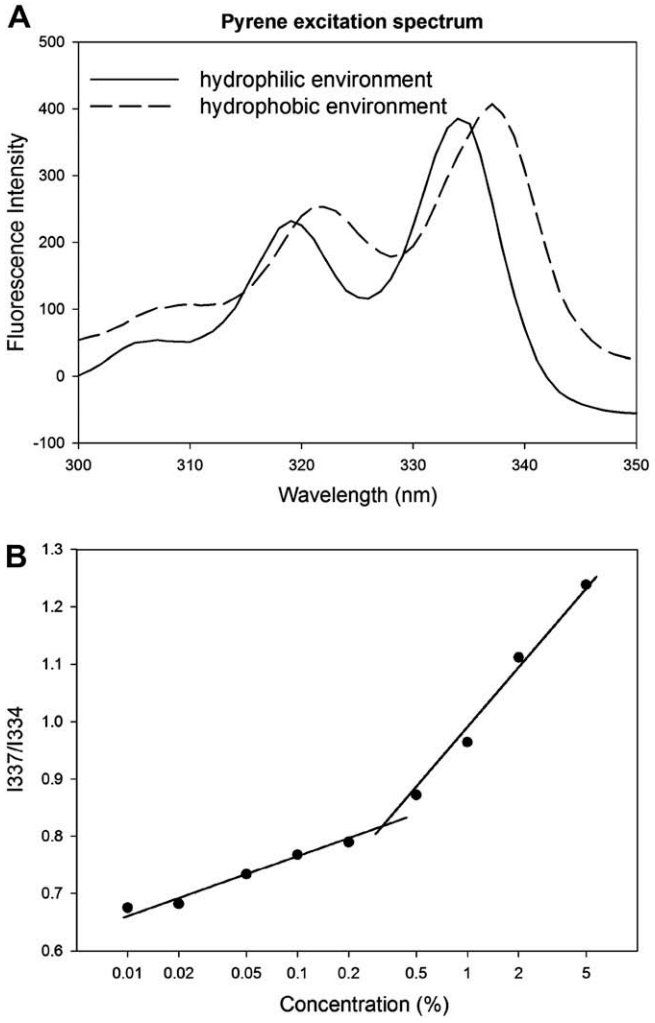

Fig. 1. (A) Excitation spectra of pyrene at hydrophilic (solid line) and hydrophobic (broken line) microenvironments; and (B) the semi-logarithmic plot of the ratio of pyrene fluorescence intensity at 337 nm to that at 334 nm versus hydrophobically modified starch (HMS) concentration. The critical aggregation concentration (CAC) of HMS was determined as the concentration of the crossing point where two lines meet.

that curcumin was indeed trapped in the HMS micelles and the complex of HMS and curcumin could resist against freeze-drying.

The structure of HMS with or without the addition of curcumin was studied by synchrotron small-angle X-ray scattering. The small-angle scattering cross section per unit volume, $I(q)$, for a starch solution can be written as:

$$I(q) = \phi V_p (\Delta\rho)^2 P(q) S(q) \quad (1)$$

where φ and V_p are the volume fraction and the molecular volume of HMS molecules, respectively; $\Delta\rho$ is the X-ray electron density contrast between HMS and water; $P(q)$ is the form factor; and $S(q)$ is the structure factor. At current HMS concentration, $S(q) = 1$. HMS is a flexible polymer chain, thus the form factor can be fitted by Debye function (Higgins & Benoit, 1994), which is written as:

$$P(q) = \frac{2}{(qR_g)^4}\left[\exp(-q^2R_g^2) + q^2R_g^2 - 1\right] \quad (2)$$

where R_g is the radius of gyration fitted from the Debye function. Fig. 3 shows the X-ray scattering intensity profiles of 10 mg/mL HMS water solutions with or without the addition of curcumin. Our results showed that 10 mg/mL HMS solution has a slightly higher intensity at low q than 10 mg/mL HMS–curcumin solution.

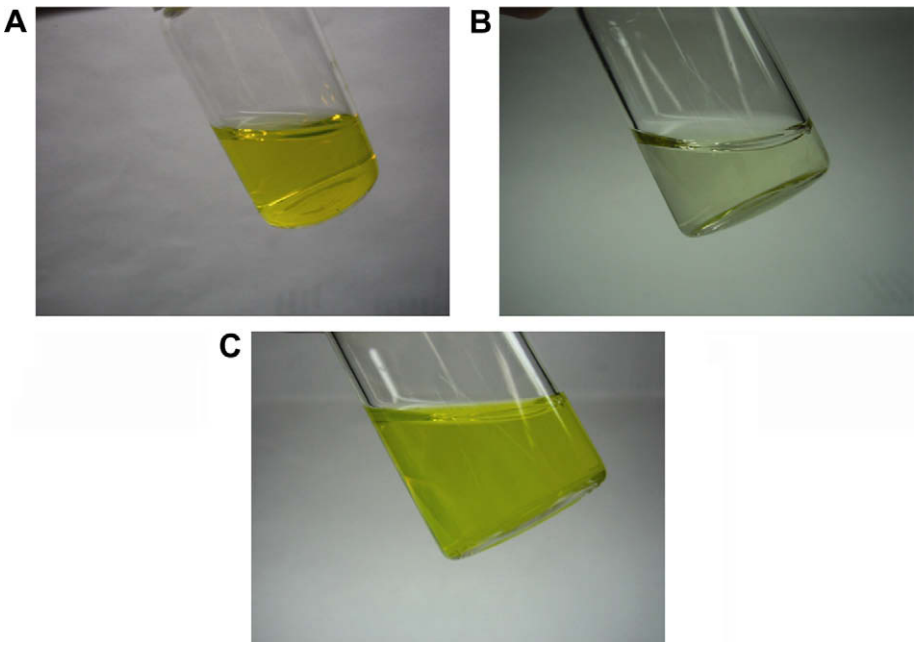

Fig. 2. Photographic images of curcumin in HMS solution (A) and water loaded with curcumin (B), as well as reconstituted curcumin HMS solution (C). Curcumin was loaded in 1% HMS solution and water, respectively.

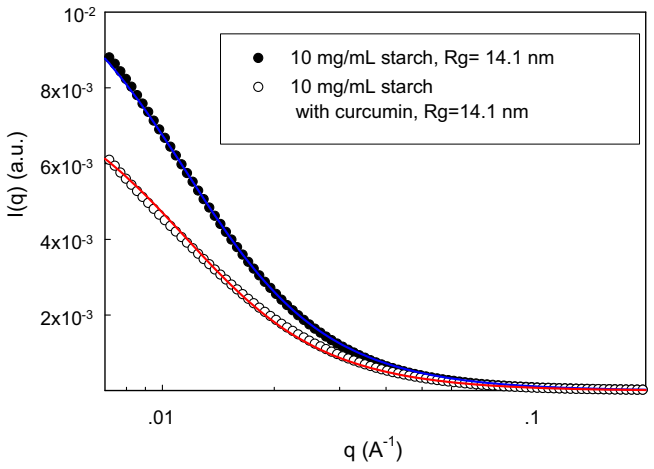

Fig. 3. Small-angle X-ray scattering intensity profiles of 10 mg/mL HMS water solution with (empty circles) or without (solid circles) the addition of curcumin. The solid lines are fits to Eq. (2).

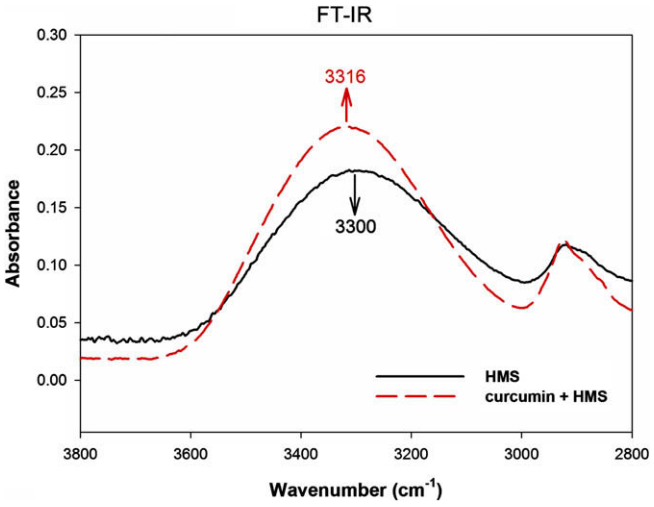

Fig. 4. FT-IR spectra of HMS (solid line) and HMS loaded with curcumin (dashed line) in the wavenumber range from 3800 to 2800 cm^{-1}.

From the fits of scattering intensity profiles to Eq. (2) we conclude that no matter whether there is curcumin in the 10 mg/mL HMS solutions, the radius of gyration of HMS remained at 14.1 ± 0.1 nm, further suggesting that the addition of curcumin did not perturb the micellar structure of HMS.

3.3. Interaction between curcumin and HMS

To investigate the interaction between HMS and curcumin, the infrared (IR) spectra of lyophilised curcumin HMS powder and HMS powder alone were measured. Compared with that of pure HMS, the IR spectrum of curcumin HMS powder showed a band shift from 3300 to 3316 cm^{-1} as evidenced in Fig. 4, which was assigned to the vibrational band of the hydroxyl (–OH) group of HMS. The band shift in the IR is mostly likely due to the formation of intermolecular hydrogen bonding between HMS and curcumin.

Considering that curcumin itself is a fluorescent compound, and the fact that the fluorescence spectrum of a compound is usually affected by its microenvironment, we compared the emission spectrum of curcumin in HSM solution with that of curcumin in water. As shown in Fig. 5, the emission peak of curcumin in water was at 542 nm, while the peak shifted to 531 nm when curcumin was encapsulated in HMS solution. This result further confirmed that the microenvironment of curcumin was changed upon HMS-encapsulation.

3.4. Enhanced in vitro anti-cancer activity of curcumin encapsulated in HMS

By inducing apoptosis, curcumin is demonstrated as a potent anti-cancer agent (Cao, Jia, Zhou, Liu, & Zhong, 2006). To investi-

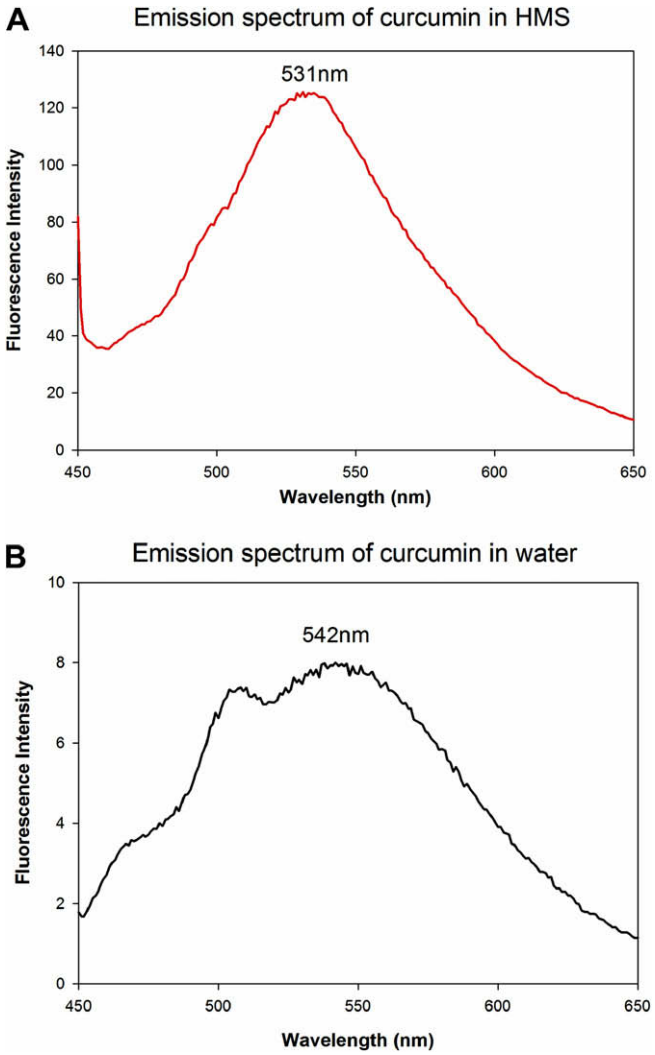

Fig. 5. Comparison of fluorescence emission spectra of curcumin in HMS solution (A) and in water (B).

gate the in vitro anti-cancer activity of curcumin encapsulated in HMS, HepG2 cells were treated, respectively, with DMSO-dissolved curcumin and HMS-encapsulated curcumin of the same concentrations. After treatment for 24 h, the cell viability was determined with MTT assay. As shown in Fig. 6, HepG2 cells treated with DMSO or HMS alone reveals comparable cell viability to untreated cells, suggesting that at the concentration used, DMSO and HMS have no cytotoxicity. Furthermore, HMS-encapsulated curcumin was more effective than DMSO-dissolved curcumin in anti-carcinogenesis. At the concentrations of 0.4, 2, and 10 μg/mL, the anti-cancer activity of HMS-encapsulated curcumin was significantly higher than that of DMSO-dissolved curcumin ($P < 0.001$), suggesting that HMS-encapsulation is more effective to deliver curcumin to cancer cells.

4. Discussion and conclusion

In this study, curcumin was encapsulated into polymer micelles formed by hydrophobically modified starch. Upon encapsulation, curcumin showed increased water solubility. As the peak position shifted in either infrared or fluorescence spectra, it was suggested that the microenvironment of curcumin was changed upon encapsulation, and there may be a hydrogen bonding interaction between curcumin and HMS.

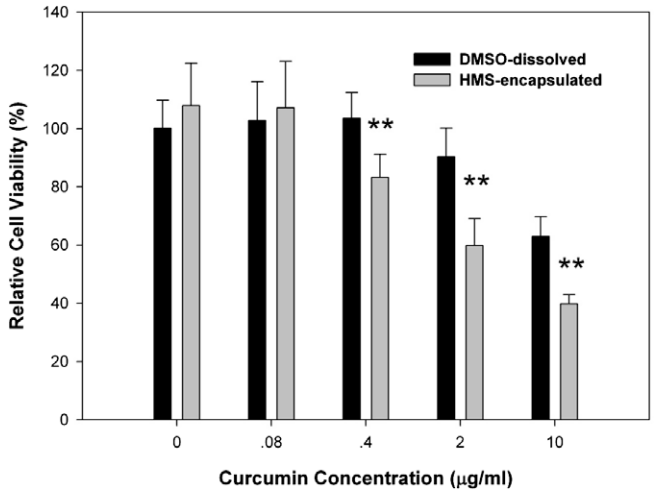

Fig. 6. The plot of relative cell viability versus curcumin concentration for free curcumin (dissolved in DMSO, black bars) and HMS-encapsulated curcumin (grey bars). Conditions of DMSO alone and HMS alone were expressed as curcumin at zero concentration. Data was presented as mean ± standard deviation, $n = 8$. **Denotes that the differences are statistically significant.

Compared with other polymeric micelles used to encapsulate curcumin, the critical aggregation concentration of HMS is relatively high and the encapsulation capacity of HMS is relatively low. This may be due to the low degree of substitution (DS) of HMS. The DS of HMS does not exceed 3%, which is quite different from amphiphilic block co-polymers, such as PEO-b-PCL (Bhosale & Singhal, 2006). On the other hand, we showed that freeze-dried HMS-encapsulated curcumin could be easily reconstituted. It suggests the structural stability of HMS–curcumin micelles, which is further evidenced by the constant HMS R_g with and without curcumin. To the best of our knowledge, this is the first report regarding this property.

From our results, an increase in curcumin solubility may be due to the combination of hydrophobic microenvironment of curcumin and hydrogen bonding between modified starch and curcumin. This hydrogen bond interaction has also been conceived between curcumin and some of other molecules studied, such as phosphatidylcholine (Began, Sudharshan, Sankar, & Rao, 1999). Using thermodynamic analysis, Began et al. (1999) found that the thermodynamic parameters for the binding of curcumin to phosphatidylcholine is driven by both entropy and enthalpy, suggesting that curcumin forms hydrogen bond with phosphatidylcholine in addition to hydrophobic interaction. On the other hand, the extensive research on the interaction between curcumin and human serum albumin only suggested the hydrophobic interaction but no hydrogen bonding (Barik, Mishra, Kunwar, & Priyadarsini, 2007; Reddy, Sudharshan, Rao, & Lokesh, 1999).

In the literature, different micelle encapsulation formulations of curcumin revealed variations in in vitro anti-cancer/cytotoxicity activity. Micelles formed by PEO-b-PCL showed less anti-cancer activity, probably due to the prolonged release profile and the lack of direct interaction between curcumin and cells (Ma et al., 2008). Curcumin encapsulated in mPEG-palmitate nanocarriers, on the other hand, had comparable activities to DMSO-dissolved curcumin (Sahu et al., 2008). In contrast, our results suggested the enhanced activity of encapsulated curcumin. Since HMS is a slightly-modified biomolecule, the interaction between HMS and HepG2 cells could be the reason of enhanced anti-cancer activity. As a matter of fact, curcumin–casein micelle complex also revealed a stronger activity than free curcumin (Sahu, Kasoju, & Bora, 2008).

In summary, we have demonstrated that hydrophobically modified starch is able to self assemble to form micelles and to encap-

sulate curcumin into its hydrophobic core. Upon encapsulation, the water solubility of curcumin increased about 1670-folds and the anti-cancer activity was also greatly increased. This study suggested that hydrophobically modified starch could be used as a self assembled biopolymer to encapsulate water-insoluble bioactives in functional food.

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