ENGINEERING NANO-CURCUMIN WITH ENHANCED SOLUBILITY AND IN-VITRO ANTI-CANCER BIOACTIVITY

by

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ABSTRACT OF THE THESIS

Engineering nano-curcumin with

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A number of food components with various health-promoting benefits have been found in many natural plants. Although these phytochemicals show great potential in the functional food market, their bioavailability is a still critical factor that hinders their health benefits. Processing may also induce changes in the properties of food ingredients.

In this Master research project, the problem of poor bioavailability of one polyphenol, curcumin, was addressed by developing a novel delivery system. In order to improve its water solubility and oral bioavailability, researchers have tried numerous encapsulation methods. However, they always went through complicated preparing and processing steps. In this research, a novel nano-formulation of curcumin by wet-milling technology has been developed.

A suspension of curcumin and modified starch was wet-milled to create a stable nanodispersion. Some of the suspension was spray dried. The other was freeze dried.

Powdered samples were stored at 4°C in dark for storage. The average particle size of curcumin nanodispersion reduced from 2.4 μm to 150 nm after one-hour wet-milling process and increased to around 170nm after one-month storage. The solubility of the nano-sized curcumin was 8.8±0.3 mg/ml increased by $8x10^5$-fold compared to the unformulated curcumin. DSC and XRD results revealed that wet-milling process with modified starch not only reduced the particle size to nano-range, but also transferred some degree of crystal curcumin into the amorphous state. Moreover, the formulated curcumin showed enhanced in-vitro anti-cancer activity in Human hepatocellular carcinoma cell line in the range of 0.5-10 ug/ml.

Moreover, the difference of freeze-drying and spray-drying processes on the properties of nano-curcumin formulation was studied. Both DSC and XRD showed that the freeze-dried nano-curcumin powder had more degree of amorphous state than that of the spray-dried. Using Caco-2 cell monolayer model, it revealed that the permeation of the freeze-dried nano-curcumin was faster than that of the spray-dried one, which indicated that the amorphous form could provide the absorption advantage for the poor water soluble compounds.

This new formulation provides a promising opportunity in the functional food market. Also wet milling technique can be applied for oral delivery systems of other poor-water-soluble compounds for functional food applications.

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I am so obliged to my advisor Dr. Qingrong Huang for his support and guidance through the course of my studies and life in Food Science Department of Rutgers University. His knowledge and experience in academia guided me a very clear direction of my research project and provided lots of helpful suggestions especially when I encountered difficulties during the research work. Although we had limited research funding these years, Dr. Huang always tried his best to support me financially and purchased the necessary experimental supplies and advanced laboratory instruments in order to ensure my research proceeding smoothly. Also Dr. Huang is very helpful and supportive with my career development. Even though I decided to shift my major from science to arts, he showed his understanding and encouraged me to chase my dream. I feel so fortunate to meet Dr. Huang at this stage of my life.

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helped me with my experiments when I was busy, but also offered me much entertainment during the boring routine lab work.

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1 Introduction

1.1 Curcumin

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-diene-3,5-dione, is a natural polyphenol extracted from the dried rhizomes of the dietary spice turmeric, i.e., Curcuma longa. The commercial grade curcumin usually contains 70-90% curcumin, 10-15% demethoxycurcumin and less than 5% of bisdemethoxycurcumin (Figure 1).

Curcumin has been widely used in traditional Chinese medicine and Ayurveda since time immemorial. Since curcumin is a yellowish compound, it is also primarily used as a food colorant. In US, curcumin belongs to food colorants exempt from certification, with E number E100 (Damodaran, CRC Press: 2007).

To date, more and more types of biological and pharmacological activities of curcumin have been demonstrated, such as its anti-oxidant, anti-inflammatory and anti-cancer properties. Multiple signal transduction pathways are proposed to be the potential molecular targets for curcumin. It inhibits several cell signalling pathways at multiple levels, such as transcription factors NF-κB and AP-1 (Huang et al., 1991) and enzymes like cyclooxygenase-2 (COX-2) which is responsible for inflammation(Zhang et al., 1999). In addition, curcumin can up-regulate caspase family proteins and down-regulate anti-apoptotic genes like Bcl-2 and Bcl-XL (Woo et al., 2003). Moreover, several cDNA microarray studies have demonstrated the gene expression induced by curcumin in many cancer types (Hong et al., 2010; Teiten et al., 2009). Some animal models (Shankar TN, 1980) and human studies (Lao et al., 2006a; Lao et al., 2006b) proved that curcumin is safe even at high doses. Phase I clinical trials indicated that curcumin is well tolerated when taken as high as 8g per day (Cheng AL, 2001). The pharmacological activities and

safety of curcumin makes it a promising compound for treatment and prevention of a wide variety of human diseases. However, it has not yet been approved as a therapeutic agent, and the extremely low water solubility (Tønnesen et al., 2002) and low oral bioavailability of curcumin (Yang et al., 2007) has been highlighted as a major problem for this.

1.2 Hydrophobically modified starch

Hydrophobically modified starch (HMS), which was synthesized from waxy maize and n-octenyl succinic anhydride (n-OSA). HMS is widely used as wall material to encapsulate flavors in spray drying process (Shaikh et al., 2006).

1.3 Functional foods

The term "functional foods" was first proposed in 1980s in Japan. Institute of Food Technologists (IFT) gave its own definition of functional food in one of its expert reports. It stated that functional foods are "foods and food components that provide a health benefit beyond basic nutrition"(2005). Nowadays, with the development of molecular biology and chemical biology, the bioactive compounds are isolated from natural products and their interactions with human body are discovered. Therefore, functional foods will be a new trend in both food science research and food industry. In food industry, the forecasted market value in the year of 2014 will be greater than eight billion US dollars (Cui, 2009). In the food science field, the anti-oxidant properties of the bioactive compounds are usually publicized. However, the bioavailability of many of these bioactive compounds is far lower than the effective anti-oxidant concentration (Halliwell, 2005).

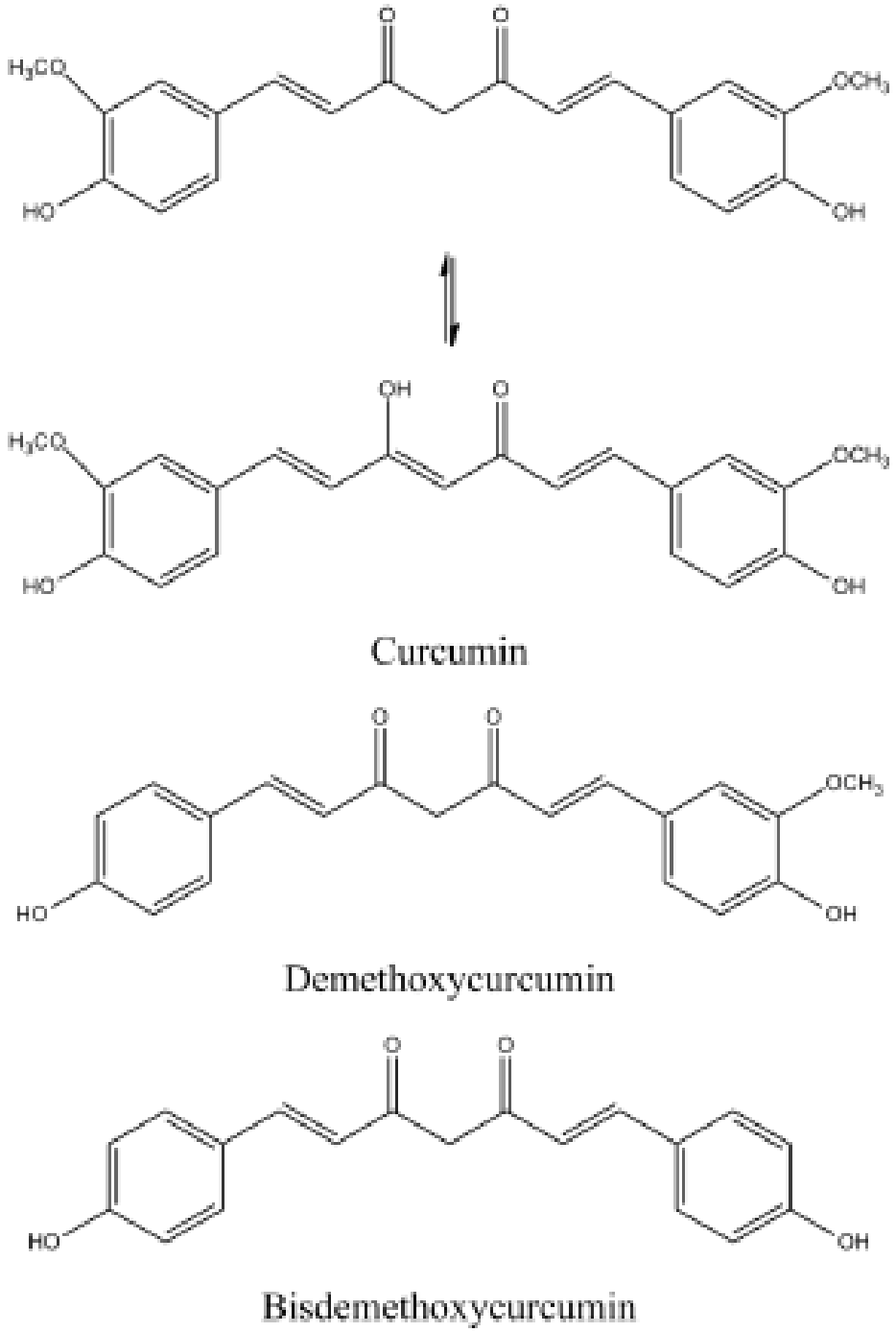


Figure 1. Chemical structures of curcumin in equilibrium of keto- and enol- forms, demethoxycurcumin and bisdemethoxycurcumin, in keto form.

1.4 Nanotechnology

Nanotechnology is an emerging technology that has shown great potential for delivering bioactive compounds in the functional foods to improve human health. The U.S. National Nanotechnology Initiative has defined "nanotechnology" as encompassing the science, engineering, and technology related to the understanding and control of matter at approximately 1–100 nm. Furthermore, it emphasizes nanotechnology research and development of materials, devices, and systems with novel properties and functions due to their nanoscale dimensions or components, and stresses the ability to measure/control/manipulate matter at this length scale to change those properties and functions and integrate along length scales upward for many fields of applications.

Nanotechnology deals with the capability to image, measure, model, control, and manipulate matter at dimensions of roughly 1–100 nanometers, where novel interfacial phenomena introduce new functionalities. This exceptional capability has led to a vast array of new technologies that have an impact on virtually every aspect of science and technology, industry, economy, the environment, and human lives.

Some nanoscale phenomena have been utilized in nutraceuticals and functional food formulation, manufacturing, and processes. New concepts based on nanotechnology are being explored to improve product functionality and delivery efficiency (Scott, 2003). Systems containing large interfacial areas such as emulsion, dispersion, and bicontinuous structured fluid are a rich source of new knowledge. Newly developed capabilities in nanoscale characterization offer a better visualization of these structures in nanometer resolution, and further a better understanding of their functionality (Tolles, 2003).

The applications of nanotechnology in food and agricultural systems are growing very fast. One important application of nanotechnology in food and nutrition is to design and development of novel functional food ingredients with improved water solubility, thermal stability, oral bioavailability, sensory attributes, and physiological performance (Huang et al., 2010). For example, phytochemicals, which are food supplements with health benefits, are commonly used as part of the daily diet. Because of their low solubility, many phytochemicals are poorly absorbed by human body, thus one of the most important and interesting applications of nanotechnology approaches for encapsulation of phytochemicals is to enhance the bioavailability of phytochemicals by changing the pharmacokinetics (PK) and biodistribution (BD).

1.5 Wet milling technique

In the past decade, solubilization technologies have been strongly desired to develop the pharmaceutical products for poorly water-soluble drugs, because more than 40% of the promising compound, so-called "candidate", in the development pipeline are reported to be categorized as poorly soluble (Schroter, 2006). To address this need, it was investigated that a solubilization methodology and process, so-called "solubilization tool" fitted for in vivo screening and profiling researches, would be established. To attain this object, the micronization of drug candidate to submicronsized particles has been proposed in the current research. Among this approach, the high-shear beads milling technique in aqueous medium was adopted since its preparation process is simple and the suspension to be orally administered to animals could be directly prepared (Niwa et al., 2011).

Technology has been pioneered by the company NanoSystems and it has lead to successful commercialization of drug nanoparticles. The media mill consists of milling chamber, milling shaft and recirculation chamber as depicted in Fig.2 (E. Merisko-Liversidge, 2003). High energy and shear forces generated as a result of impaction of the milling media with the drug provide the necessary energy input to disintegrate microparticulate drug into nanosized particles. The milling media is composed of glass, zirconium oxide or highly cross-linked polystyrene resin. In the media milling process, the milling chamber is charged with the milling media, water or suitable buffer, drug and stabilizer and the milling media or pearls are then rotated at a very high shear rate. The milling process is carried out under controlled temperature (crucial for thermolabile drugs) and can be performed in either a batch or recirculation mode. In batch mode, the time required to obtain dispersions with unimodal distribution profiles and mean diameters <200 nm is 30 – 60 min. The media milling process can successfully process micronized and nonmicronized drug crystals. Once the formulation and the process are optimized, very little batch-to-batch variation is observed in the quality of the dispersion (Date and Patravale, 2004).

There are many advantages to wet media milling. The primary reason to select media milling is that the process can produce uniform particle size distribution in the micron and submicron (or nanometer) range. Also, dry-milled materials that have the tendency to agglomerate when added to liquid may avoid agglomeration by being mixed with a liquid and processed in a wet media mill. Wet milling will encapsulate the dry particle, surrounding it with liquid and preventing re-agglomeration. Further stabilization

of the suspension using either electrostatic charge control or long-chain molecules can be achieved.

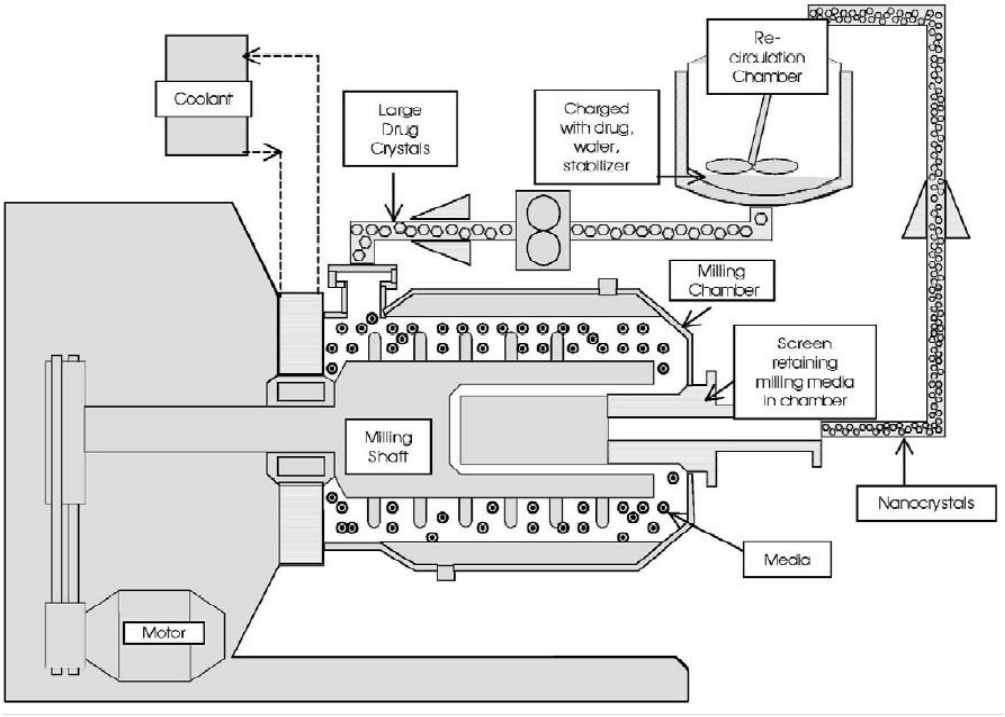


Figure 2. A schematic representation of wet milling process.

1.6 Flow-through cell dissolution test

The last decade has witnessed a rapid development in the area of novel drug delivery systems such as microspheres, liposomes, nanosuspensions, and microemulsions(Kostarelos, 2003). In order to assure the performance and safety of these novel delivery systems, as well as to assist in the product development process, in vitro testing methods must be developed. In vitro release plays an important role in characterizing the quality of the product and indicating the in vivo product performance(Kostarelos, 2003; Upkar Bhardwaj, 2010).

The flow-through cell method for the study of dissolution first appeared in 1957 as a flowing medium dissolution apparatus developed by FDA (Langenbucher, 1989). The method was adapted by USP, the European Pharmacopoeia (Ph. Eur.), and the Japanese Pharmacopoiea (JP), and the flow through cell became an official apparatus.

The system of USP4 consists of a reservoir containing the dissolution/release medium, a pump that forces the medium upwards through the vertically positioned flow-cell where the sample is placed, and a water bath to control the temperature in the cell. A filter is positioned at the inner top of the cell to retain undissolved material. The collected samples can be analyzed directly by a UV-vis spectrophotometer or a fiber-optic probe, or they can be collected in fractions and analyzed by HPLC or other appropriate methods.

USP Apparatus 4 can be operated under different conditions such as open or closed system mode; different flow rates and temperatures. The diversity of available cell types allows application to a wide range of dosage forms. Since the reservoir volume is not fixed, media volume can be decreased to accommodate systems where the concentration of drug released would otherwise be below the detection limit (e.g. systems

with low drug loading) or increased to allow ease of maintenance of sink conditions for poorly soluble compounds. USP Apparatus 4 also offers flexibility in monitoring release via in situ UV analysis. Such analysis can be achieved without any correction for scattering by the dispersed system or particle accumulation on the probes since the microspheres or other delivery systems are isolated (Zolnik, 2005).

1.7 Caco-2 cell monolayer as an in-vitro tool to predict intestinal absorption

In the intestine, a single layer of epithelial cells covers the inner intestinal wall and forms the rate-limiting barrier to the absorption of dissolved drugs. As a consequence, the proper

reconstitution of a human differentiated epithelial cell monolayer in vitro allows the prediction of oral drug absorption in humans (Artursson, 2001). The human colon carcinoma cell line Caco-2 has been found to serve this purpose well. Permeability coefficients across monolayers of the human colon carcinoma cell line Caco-2, cultured on permeable supports, are commonly used to predict the absorption of orally administered drugs and other xenobiotics (Hubatsch et al., 2007).

In culture, Caco-2 cells grown on permeable filters and slowly differentiates into monolayers with many phenotypes with many functions of small intestine enterocytes: tight junctions between adjacent cells, microvilli on the apical surface, expression of multiple drug transport proteins and metabolic enzymes (Figure 4).

The apparent permeation rate (Papp) across Caco-2 cell monolayers is a very good parameter to predict the in vivo absorption. Although the Papp obtained from different labs are different, there is a general trend that high Papp implies high absorption.

General speaking, Papp > 1 × 10-6 cm/s means high permeation while Papp < 1 × 10-7 cm/s implies low permeation (Artursson, 2001).

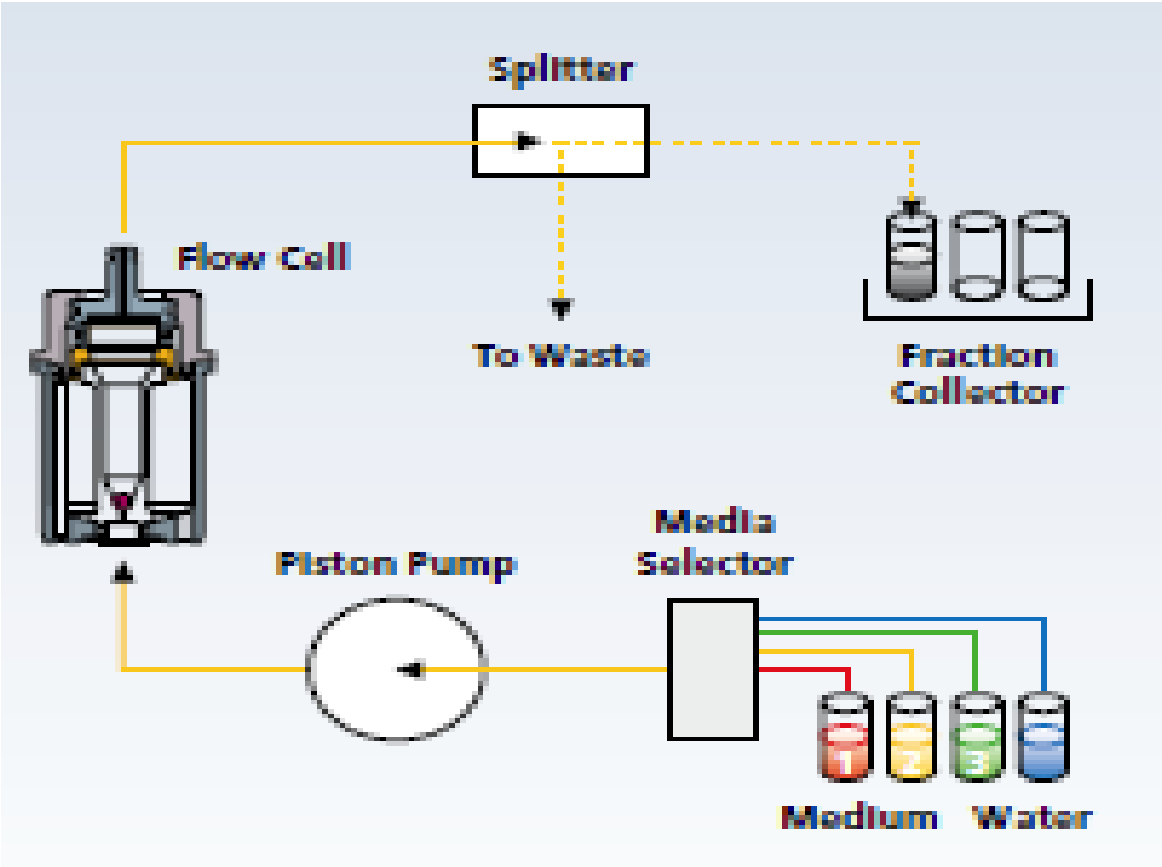


Figure 3. A schematic representation of USP4

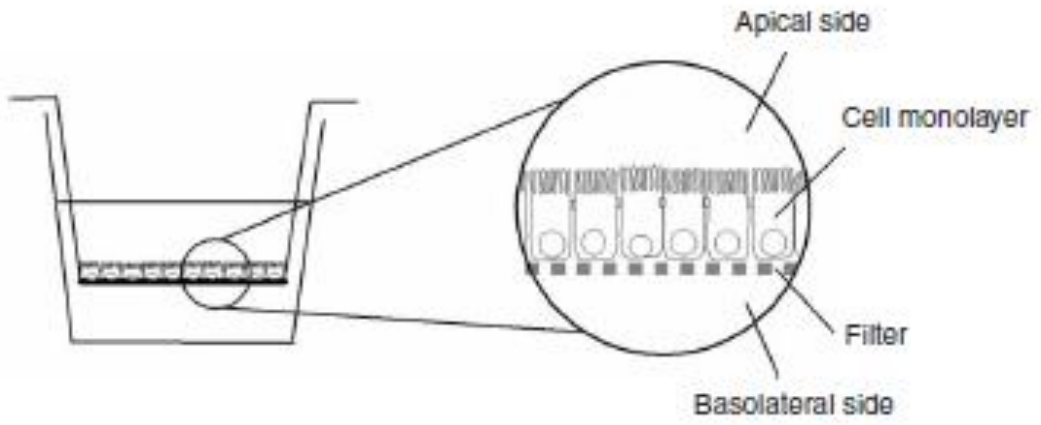


Figure 4. Diagram of a Caco-2 monolayer grown on a permeable filter support.(Hubatsch et al., 2007)

2 Materials and Methods

2.1 Materials

Curcuminoids was generously provided by Sabinsa Corporation (Piscataway, NJ), which contains 76% curcumin, 19% demethoxycurcumin and 5% bisdemethoxycurcumin. It was used without further purification. Hydrophobically modified starch (HMS) with the brand name of Hi-Cap 100 was obtained from National Starch and Chemical Company (Bridgewater, NJ). Milli-Q water (18.3 MΩ) was used in all experiments. Eagles minimum essential medium with Earle's balanced salt solution (MEM/EBSS), trypan blue, lucifer yellow, dimethyl sulfoxide (DMSO) and Hank balanced salt solution (HBSS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). fetal bovine serum (FBS), 100X non-essential amino acids, 100X penicillin and Streptomycin, 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA), 1M 4-(2-hydroxyethyl)-1-piperazineethane- sulfonic acid (HEPES) and bovine serum albumin (BSA) were all purchased from Fisher Scientific. HPLC grade of acetonitrile was obtained from J.T. Baker. The human hepatocellular caricinoma (HepG2) cell line and Caco-2 cell line were gifts from Dr. Mou-Tuan Huang (Rutgers, The State University of New Jersey, US). 96-well cell culture plates, transwell permeable polycarbonate inserts (0.4μm) and 12-well cell culture plates were purchased from Corning (Corning, NY, USA).

2.2 Preparation of nanodispersion

Nanodispersed curcumin was produced by wet-milling method in a milling machine (MiniCer, Netzsch-Feinmahltechnik GmbH, Staufen, Germany). The milling process was performed using Yttrium-stabilized zirconium oxide grinding beads with a diameter of 0.8-1.0 mm. Fifty-five grams of curcumin powder and eighty grams of HMS

were suspended in 260mL deionized water. Mixed overnight and then fed into the milling chamber. The suspension was agitated at the speed of 3100 rpm and recycled by a pump with the speed of 100 rpm. The milling temperature was controlled at 30°C by a cooling system. At 5, 10, 15, 30, 45 and 60 minutes of milling, the samples were collected for particle size determination.

2.3 Freeze dry & spray dry

Half of the wet-milled nanodispersion of curcumin was placed in the model Pulvis GB22 fluid bed spray dryer (Yamato, Santa Clara, Ca). The drying air was set to 0.43-0.45 m3/min, air inlet was set to 110°C and the outlet temperature was set to 200°C. The powdered product was then collected from the filter chamber and was split into appropriate plastic jars for storage.

The remaining sample was placed in multiple plastic 50ml sterile centrifuge tubes and frozen overnight (-20°C). The following day the samples were removed from the freezer, which were completely solidified and placed in the freeze dryer (Freezone 4.5, Labconco, Kansas City, MO), open to the system and under vacuum until all moisture was removed and a dry solid mass remained. The samples were then placed in plastic jars for storage.

2.4 Particle size measurements

The average diameter and size distribution of the wet-milled samples were observed under dynamic light scattering particle size analyzer (Model 90 Plus, Brookhaven Instrument Corp., Holtsville, NY) at a fixed angle of 90° at 25°C. Powdered samples after spray drying and freeze-drying were re-hydrated with deionized water and also

measured for particle size under the above method. All samples were weighed at 0.5g and diluted with 10g of deionized water and measured in triplicate to ensure accuracy.

2.5 Morphology observation

The morphology of the particles in the nanodispersion of curcumin was examined with a Nikon TE-2000-U inverted microscope equipped with a CCD camera (Retiga EXi, QImaging). Samples were analyzed with polarized light at 100-fold magnification to obtain overview information of the whole system. All images were processed by SimplePCI C-Imaging software (Compix Inc., Sewickley, PA, USA).

2.6 Quantification of curcumin by high-performance liquid chromatography (HPLC)

Curcumin was quantified by UltiMate 3000 HPLC system with 25D UV-VIS absorption detector (Dionex). A Supelco Ascentis ®RP-Amide 4.6x150mm, 3um column was used with the mobile phase consisting of (A) acetonitrile and (B) 2% acetic acid in water. 50 μm samples were injected and then eluted under gradient conditions: 0-2 min, 65% B and 35% A; 2-15 min, linear gradient from 35 to 90% A; 15-20 min, held at 90% A; 20-21 min, A went back to 35% linearly; 21-25 min, held at 35% to balance the column. The flow rate was 1mL/min and the UV detector was set at 420nm.

2.7 Water dispersity and loading capacity

The free dried powder of wet-milled curcumin was prepared for the water dispersity test. 0.2g of such powder was added into 5ml distilled water. The sample was held on an orbital shaker agitating at 160rpm at room temperature in dark. After 48 hours, the aqueous phase was filtered through 0.22μm filters. The filtrate was diluted by 1000 times with acetonitrile: water (2:1, v/v) for HPLC quantification. The loading capacity was expressed as:

$$\text{Loading capacity (\%)} = \left(\frac{\text{Total amount of curcuminoids dispersed}}{\text{Total amount of the formulated powder added}}\right) \times 100\%$$

2.8 Dissolution rate test

Powder dissolution study was carried out using flow-through cell USP apparatus 4(The CE 7smart, SOTAX, US) in 400 ml of DI water or simulated intestinal buffer at a temperature of 37±0.5 °C at 3mL/min. A powdered sample (equivalent 5 mg curcumin) was introduced directly into the dissolution medium. At regular time intervals of 15, 30, 45 and 60 min, suitable amount

of sample (1 ml) was withdrawn and same amount replaced by fresh medium to maintain the sink condition. The withdrawn samples were suitable diluted and analyzed through HPLC at 420 nm. All studies were carried out in triplicates.

2.9 Fourier-transform infrared spectroscopy (FT-IR)

To compare the structures of freeze-dried curcumin nanodispersion powder and raw curcumin, FT-IR was performed using a Thermo Nicolet Nexus 670 FT-IR system with attenuated total reflectance (ATR) accessory (Thermo Fisher Scientific, Waltham, MA). HMS and physical mixture of HMS and raw curcumin were measured as controls. The FTIR spectra were obtained in the region 600-4000 cm-1 at a resolution of 4 cm-1. Each sample was subjected to 512 repeated scans. The spectra were averaged and smoothed, and their baselines were calibrated with the Spectra Manager software.

2.10 Differential scanning calorimetry (DSC)

Thermal properties of freeze-dried curcumin nanodispersion powder compared with the raw curcumin and HMS, were investigated by DSC (model 823, Mettler-Toledo Instruments, Columbus, OH). About 5mg of each sample were placed in perforated

aluminum sealed pans. Samples were heated from 60°C to 250°C at the rate of 10°C/min. The melting point and heat of fusion were calculated using the TA DSC software.

2.11 X-ray diffraction (XRD)

Powder X-ray diffraction experiments were conducted using a D/M-2200T automated system (Ultima+, Rigaku) with Cu Kα radiation (λ=1.5406 A). The PXRD patterns were collected between 2θ angles of 3° to 50° at a scan rate of 3 deg·min-1. Graphite monochromator was used and the generator power settings were at 40 kV and 40 mA.

2.12 HepG2 cell culture and *in vitro* anti-cancer activity assay (MTT assay)

Human hepatocellular carcinoma cell line HepG2 was cultured in eagles minimum essential medium and supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in incubators at 37°C under 95% relative humidity and 5% CO2.

To examine the *in vitro* anti-cancer activity of the formulated curcumin complex, methyl thiazol tetrazolium bromide (MTT) assay was performed. Briefly, HepG2 cells (10,000 cells/well in 100 µL media) were seeded in 96-well microtiter plates and allowed to attach for 24 h. Then media was replaced with different concentrations of DMSO-dissolved or the formulated curcumin diluted by fresh media. Other cells were untreated as negative control, or treated only with DMSO or HMS at the maximum concentration used to dissolve and formulate 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 nm was recorded with Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). Relative cell viability was expressed as A560 normalised to that of the untreated wells. Data were presented as mean ± standard deviation with eight-well repeats.

2.13 Caco-2 cell culture and permeation experiments across Caco-2 monolayer

Cells were maintained in DMEM with 10% FBS, 1X non-essential amino acids and 1X penicillin and streptomycin, at 37°C with 5% CO2. Cells of passage 35-45 to keep relatively constant cellular phenotypes were used in this study.

To generate Caco-2 cell monolayers in the insert filters of 12-well plates, 0.5 mL Caco-2 cells were plated onto the insert (in the apical compartment) at the density of 6 × 105 cell/mL. 1.5 mL culture media were subsequently added in the lower (basolateral) compartment of each well. Media were changed every two days. Permeation experiments were performed after 21- 29 days of plating.

In the permeation experiments, 20 μg/mL curcumin in the donor media were obtained by diluting curcumin DMSO solution or BSA/mixed micelle solubilized curcumin into donor media. The donor and receiving media were used throughout the study as noted : HBSS + 25 mM HEPES, pH 7.4; HBSS + 25mM HEPES + 4% BSA, pH7.4. BSA was added to solubilize permeated curcumin, while the addition of BSA to the receiving media could mimic the in vivo condition. In the permeation direction of apical to basolateral (A-B) compartment, 0.4 mL donor media with 20 μg/mL curcumin was added to the apical compartment and 1.2 mL receiving media were added to the basolateral compartment. Plates were then put in a shaker at 100 rpm and 37 °C. After 15,

30, 45, 60 minutes of permeation, half volumes of the receiving media were removed and the same volumes of fresh media were replenished.

The removed receiving media were mixed with two volumes of acetonitrile by vortexing briefly and centrifuged at 16,000g in a bench-top microcentrifuge for 15 minutes. The supernatants were filtered through 0.45 μm filter and analyzed with HPLC for curcumin quantification.

Cumulative quantity of curcumin permeated at each time interval was calculated and plotted against time. The initial slope was then used to calculate the apparent permeation rate (Papp) using the following equation:

$$P_{app} = \left(\frac{dQ}{dt}\right)\left(\frac{1}{AC_0}\right)$$

where dQ/dt is the rate of curcumin permeation. A is the surface area of the insert (1.1cm2), C0 is the initial curcumin concentration.

To ensure the integrity of Caco-2 monolayers, transepithelial electrical resistance (TEER) value and the apparent permeation rate of lucifer yellow were determined. TEER value was measured before each experiment using Evohm2 epithelial voltmeter (World Precision Instruments), and calculated as

$$\text{TEER}(\Omega \cdot \text{cm}^2) = \left(\text{TEER}(\Omega) - \text{TEER}_{\text{background}}(\Omega)\right) \times \text{Area}(\text{cm}^2)$$

where TEER(Ω) is the electrical resistance across Caco-2 monolayers directly read from the Evohm2 epithelial voltmeter, TEERbackground(Ω) is that across the insert only (without cells). Area(cm2) is the area of the insert, 1.1cm2.

3 Results and Discussion

3.1 Development of HPLC method to quantify curcumin

Curcumin, demethoxycurcumin (D-Cur), and bisdemethoxycurumin (BD-Cur) are three major curcuminoids that coexist in most curcumin products (Figure 1). The amount of D-Cur and

BD-Cur could account for about 15% or more. Therefore, a sensitive HPLC method to separate and quantify the three curcuminoids was established first before.

Figure 5 shows a typical HPLC chromatogram separating the three compounds in the curcumin samples used in this study. The retention times for BD-Cur, D-Cur, and curcumin were

approximately 9.80, 10.32, and 10.95 min, respectively. Meanwhile, because the three compounds had similar mass absorption coefficients(Peret-Almeida, 2005), the mass percentages of the three compounds were calculated directly from the peak areas of the three peaks:

76.0%± 1.0% for curcumin, 19.4%± 0.5% for D-Cur, and 4.6%±0.5% for BD-Cur.

Different standard concentrations (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 μg/mL) of total curcuminoids were analyzed by HPLC to generate calibration curves for the three curcuminoid compounds. Four calibration curves of different concentration ranges were generated for each of the three compounds and the total curcuminoids (Table 1& Figure 6,7).

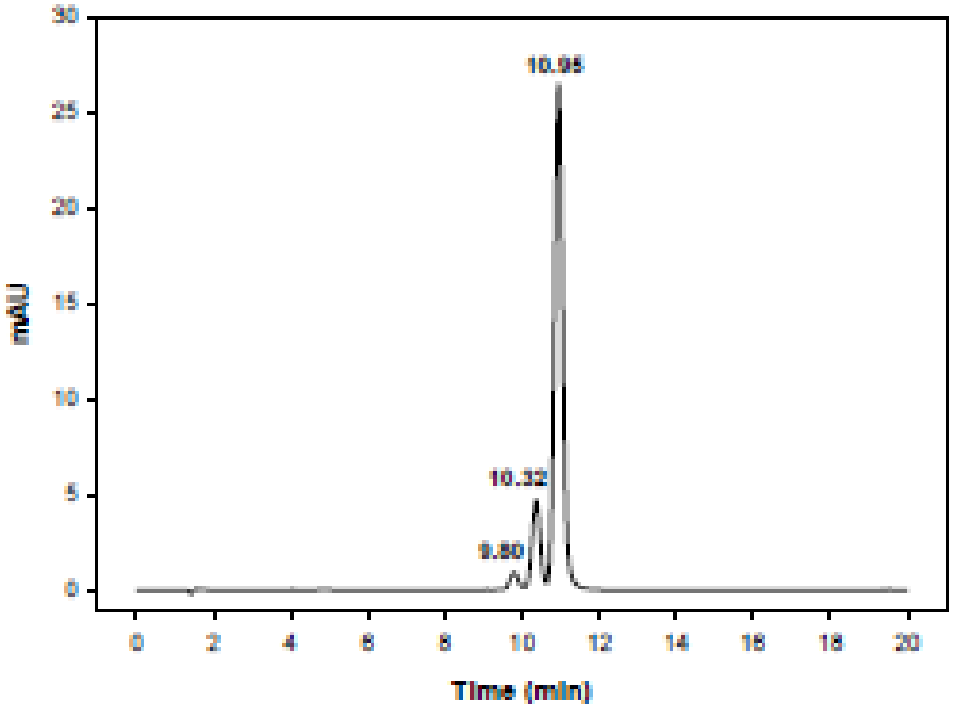


Figure 5. A typical chromatograph of three curcuminoids analyzed by HPLC. 50µL injection of 0.2 µg/ml total curcuminoids were shown here.

Table 1. Calibration Curves for Curcumin, D-Cur, BD-Cur, and Total Curcuminoids from HPLC

	Curcuminoids Concentration (ug/mL)										
	20	10	5	2	1	0.5	0.2	0.1	0.05	0.02	0.01
Curcumin	15.20603	7.60302	3.80151	1.520604	0.760302	0.380151	0.15206	0.07603	0.038015	0.015206	0.007603
	114.1378	57.4236	29.2412	10.9697	5.5064	2.9705	0.9024	0.498	0.2221	0.097	0.0443
	116.5575	57.3531	29.1655	10.9045	5.4895	2.9543	0.8961	0.4966	0.2192	0.0847	0.049
		58.748	28.8	10.964	4.9493	2.9647	0.9011	0.4932	0.2827	0.1253	0.0582
	115.3168	57.4297	29.255	10.9078	4.9018	2.9535	0.8903	0.4946	0.2789	0.1201	0.0567
avergae	115.3374	57.7386	29.11543	10.9365	5.21175	2.96075	0.897475	0.4956	0.250725	0.106775	0.05205
percentage	0.773972	0.775382	0.775688	0.77397	0.767263	0.771999	0.752284	0.761349	0.742834	0.736252	0.732325
Average	0.760302										
D-Cur	3.8827	1.94135	0.970675	0.38827	0.194135	0.097068	0.038827	0.019414	0.009707	0.003883	0.001941
	27.2099	13.6165	6.9294	2.6157	1.3302	0.7143	0.2413	0.1276	0.0641	0.0279	0.0127
	28.1869	13.6035	6.9265	2.6095	1.3287	0.7162	0.2393	0.1275	0.0673	0.0272	0.0136
		14.0773	6.835	2.6165	1.2538	0.7198	0.2388	0.1269	0.0725	0.0354	0.0164
	27.6297	13.6263	6.957	2.6131	1.2502	0.7203	0.2381	0.1263	0.0745	0.0339	0.0149
avergae	27.6755	13.7309	6.911975	2.6137	1.290725	0.71765	0.239375	0.127075	0.0696	0.0311	0.0144
percentage	0.185717	0.184395	0.184148	0.18497	0.190018	0.187123	0.20065	0.195215	0.206207	0.214446	0.202603
Average	0.194135										
BD-Cur	0.911259	0.45563	0.227815	0.091126	0.045563	0.022782	0.009113	0.004556	0.002278	0.000911	0.000456
	5.9601	2.9945	1.5007	0.5817	0.2911	0.1564	0.0573	0.0284	0.017	0.0065	0.003
	5.9774	3.0012	1.5081	0.5727	0.2976	0.1556	0.0563	0.0271	0.0167	0.006	0.0049
		2.9865	1.4997	0.582	0.2878	0.1569	0.0563	0.0308	0.0184	0.0082	0.0058
	6.0843	2.9986	1.5217	0.5844	0.2842	0.1582	0.0547	0.0268	0.0167	0.0079	0.0048
avergae	6.007267	2.9952	1.50755	0.5802	0.290175	0.156775	0.05615	0.028275	0.0172	0.00715	0.004625
percentage	0.040312	0.040223	0.040164	0.04106	0.042719	0.040878	0.047066	0.043437	0.050959	0.049302	0.065072
Average	0.045563										
Total	149.0201	74.4647	37.53495	14.1304	6.79265	3.835175	1.193	0.65095	0.337525	0.145025	0.071075

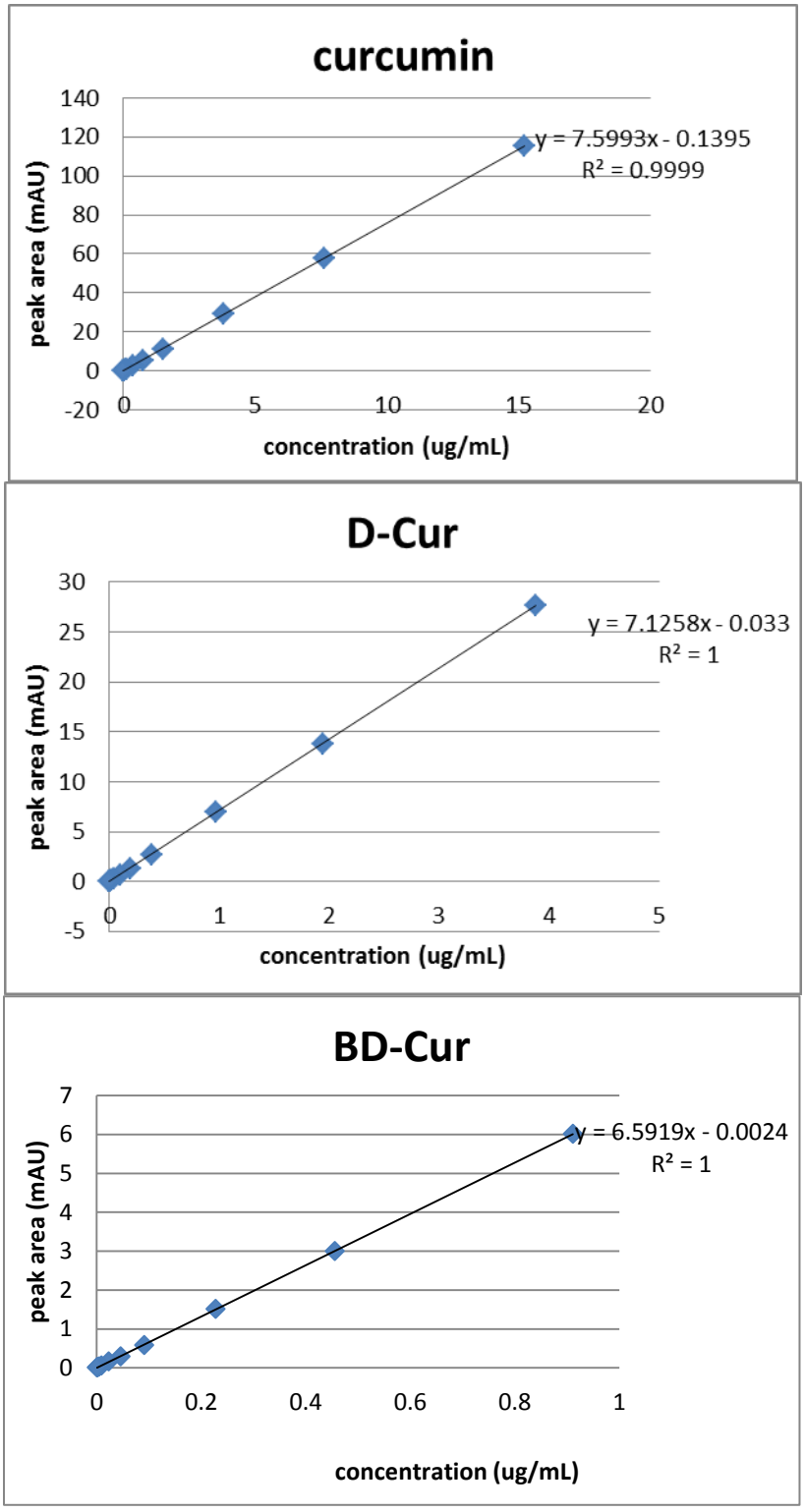


Figure 6. Separate calibration curves for curcumin, D-cur, BD-cur.

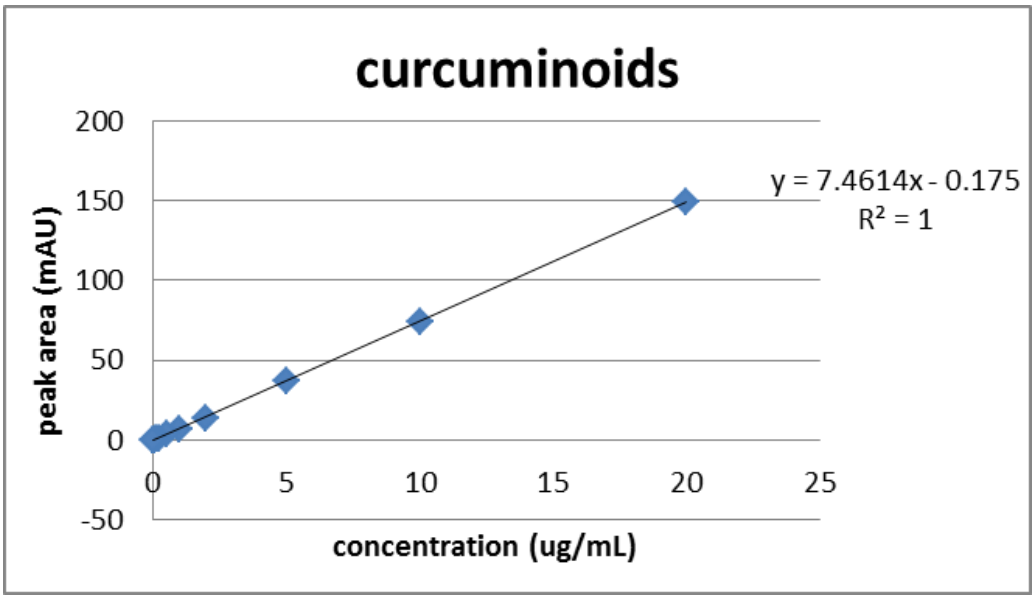


Figure 7. Calibration curve for total curcuminoids.

3.2 Physiochemical properties of wet-milled nano-curcumin

3.2.1 Morphological properties of curcumin nanodispersion

To visualize the difference of curcumin nanodispersion by wet-milling process, photographic pictures of curcumin dispersion after wet-milled for 1 hr as well as the pure curcumin in water were taken and presented in Figure 8. The non-treated curcumin water solution was nearly colorless and most curcumin powder precipitated at the bottom of the vial (Figure 5a). In contrast, the wet-milled curcumin nanodispersion afforded a yellowish homogenous solution without free curcumin powder sediment. Moreover, the micrographs clearly showed that after wet-milling process the dispersion became more homogenous and the crystals turned into the nano/sub-micro size (Figure 9).

3.2.2 Effect of the wet-milling process on the particle sizes

The wet-milling process was performed to make the curcumin nano/submicro-dispersion. Figure 10 obtained from DLS measurements showed that the particle size was reduced sharply from approximately 2.4 μm to 150 nm in the first 5 minutes and without much change during the rest milling time.

During the wet milling process, the modified starch acted as the stabilizer, which could adsorb around the curcumin particles, as steric barriers, effectively prevented the agglomeration of the primary particles prepared by ground mixture (A. Pongpeerapat, 2008). According to this theory, it was assumed that the adsorption tendency of such hydrophilic part of the polymers on the surface of curcumin particles might contribute to the milling efficiency. The hydrophobic side chains might have strong interaction with the hydrophobic surface of the particles, resulting in effective size reduction.

Therefore, the significant particle size reduction could be achieved not only by intense shear force and high frequency compression provided by the milling media but also by molecular interaction with dispersing agents.

Moreover, the particle sizes were measured as a function of storage time shown in Figure 11. The particle sizes gradually increased from about 130nm to 170nm during the first month and finally it reached 290nm at end of 70 days' storage. But the particle size was still in the submicron range, which meant this formulation showed good stability in manner of particle size.

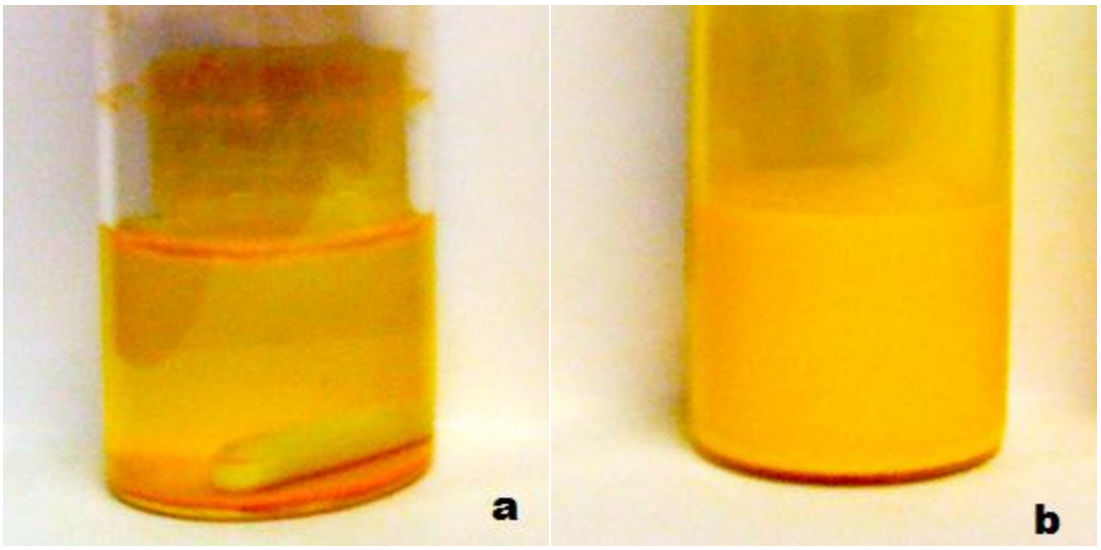


Figure 8. Photographic pictures of (a) pure curcumin in water and (b) wet-milled curcumin nanodispersion.

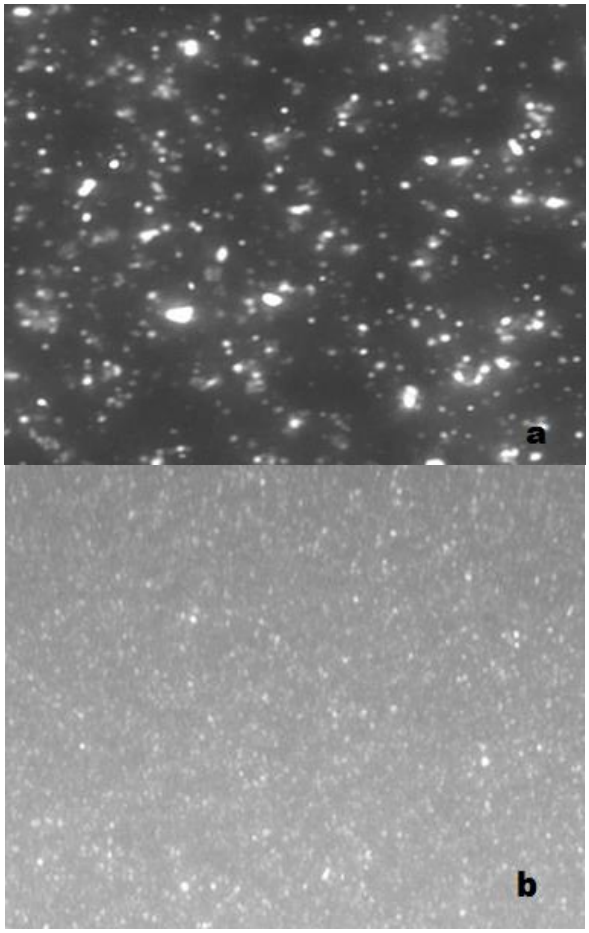


Figure 9. Micrographs of curcumin dispersion before (a) and after (b) one-hour wet-milling process.

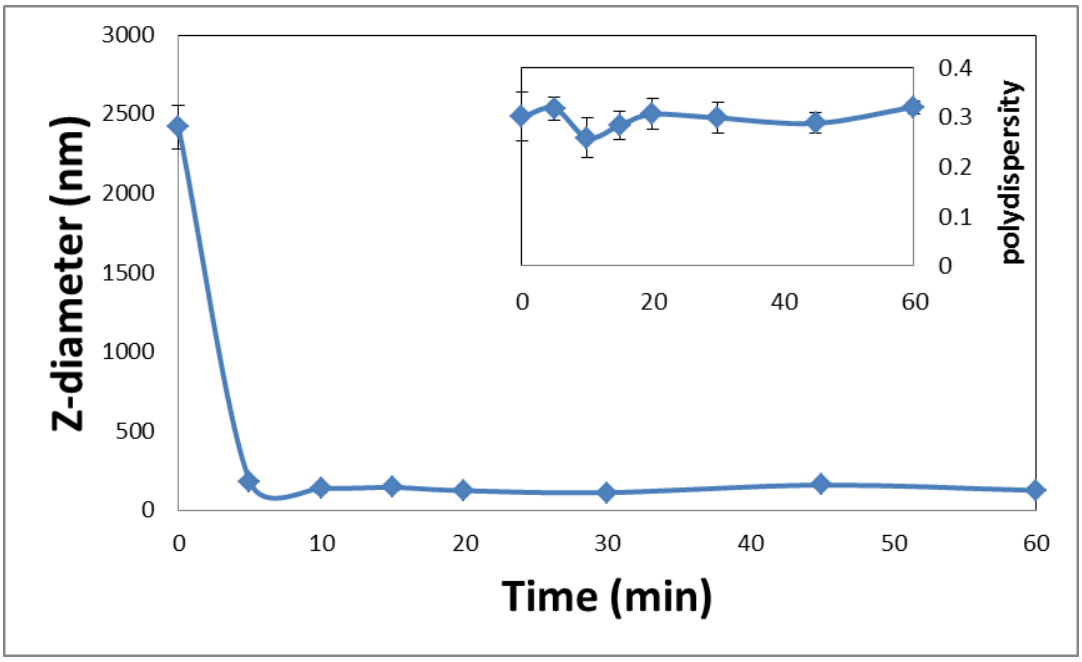


Figure 10. (Inset of a) Polydispersity index and z average diameter as a function of time during wet-milling process.

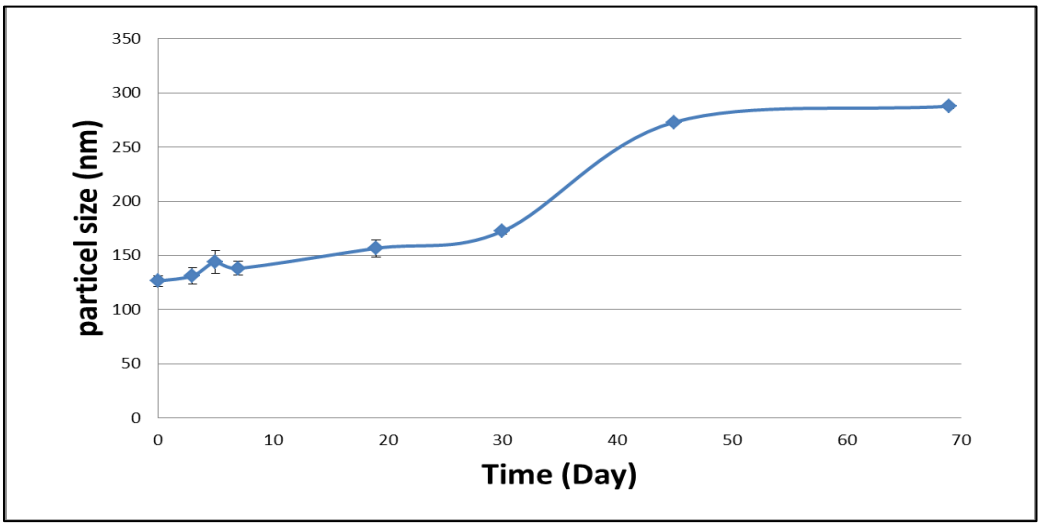


Figure 11. z average diameter as a function of storage time.

3.2.3 Determination of the water dispersity and the dissolution rate

The excess amount of the freeze-dried wet-milled curcumin powder was dispersed into distilled water and gently agitated for 2 days to reach fully saturation. According to the HPLC analysis, the water dispersity of the formulated curcumin was 8.8±0.3 mg/ml. After calculation, the loading capacity was 22%. Compared with the pure curcumin's water solubility of 11 ng/mL (Kaminaga et al., 2003), this formulation can enhance the water solubility by $8x10^5$ times.

For the dissolution process in USP4, the wet milled curcumin powder was almost fully dissolved in the first 20 minutes, while only about 5% of the unformulated one was dissolved after one hour. Moreover, the dissolution rate of the formulated curcumin powder was 9×10^{-4} mg/(mL·min).

The significant increase of the formulated curcumin powder in solubility and dissolution rate might be attributed to formation of soluble complex of curcumin and modified starch. Also, for poorly-water-soluble compounds, surface area of the compound particles drives dissolution. As described by the Nernst–Brunner and Levich modification of the Noyes–Whitney model of dissolution:

$$\frac{dX}{dt} = \left(A \times \frac{D}{\delta}\right) \times \left(C - \frac{X}{V}\right)$$

where X is the amount of compound in solution, t is time, A is the effective surface area, D is the diffusion coefficient of the compound, d is the effective diffusion boundary layer, C is the saturation solubility of the drug, and V is the volume of the dissolution medium. This equation proves that the nano-sized curcumin can have a dramatic effect on the solubility. Also HMS functioned as a stabilizer to prevent the aggregation of curcumin particles.

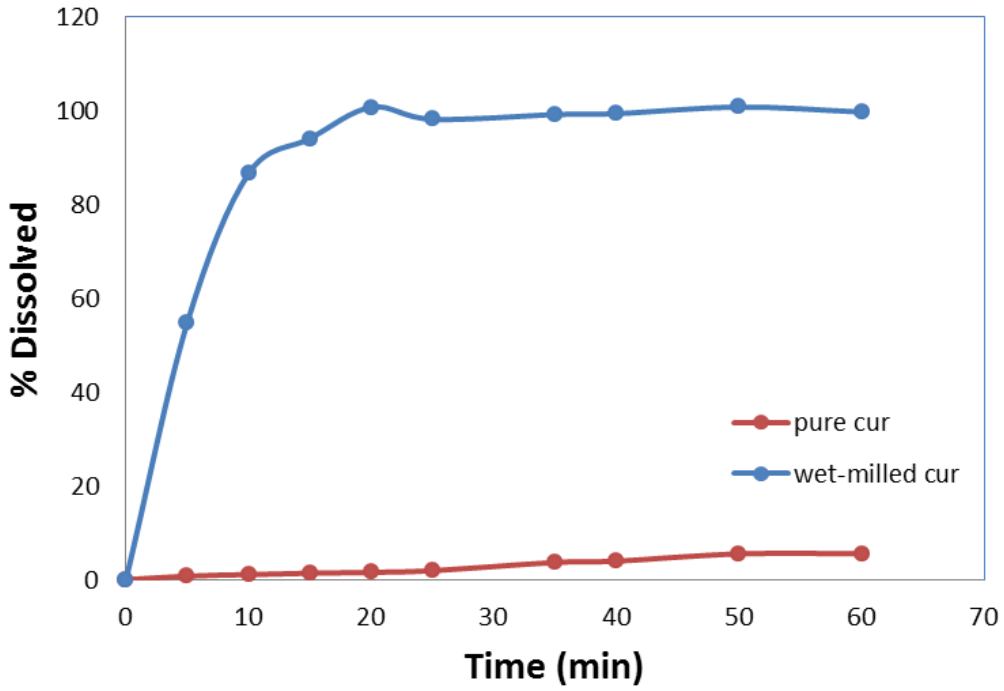


Figure 12. Dissolution profiles of pure curcumin and wet milled curcumin powder.

3.2.4 FTIR spectroscopy

The FTIR spectra of HMS, curcumin and formulated curcumin powder are shown in Figure 13. Also the FTIR spectrum of the physical mixture was measured for comparative analysis. In the fingerprint region from 500 to 2000 cm-1, the formulated curcumin powder retained majority of the characteristic peaks of both HMS and pure curcumin though with less intensity. The intensity of the physical mixture was even less, which might be due to not evenly distribution as that of the formulated one. As there was no band shift or new peaks, it was speculated that there was no any covalent interactions between the curcumin and HMS. On the other hand, the region from 3100 to 3600 cm-1 represents the presence of hydrogen bonds. The intermolecular hydrogen bonds between curcumin and HMS were not strong as the intramolecular ones of HMS itself. These results indicated that the wet-milling process with HMS did not influence the chemical properties of curcumin significantly.

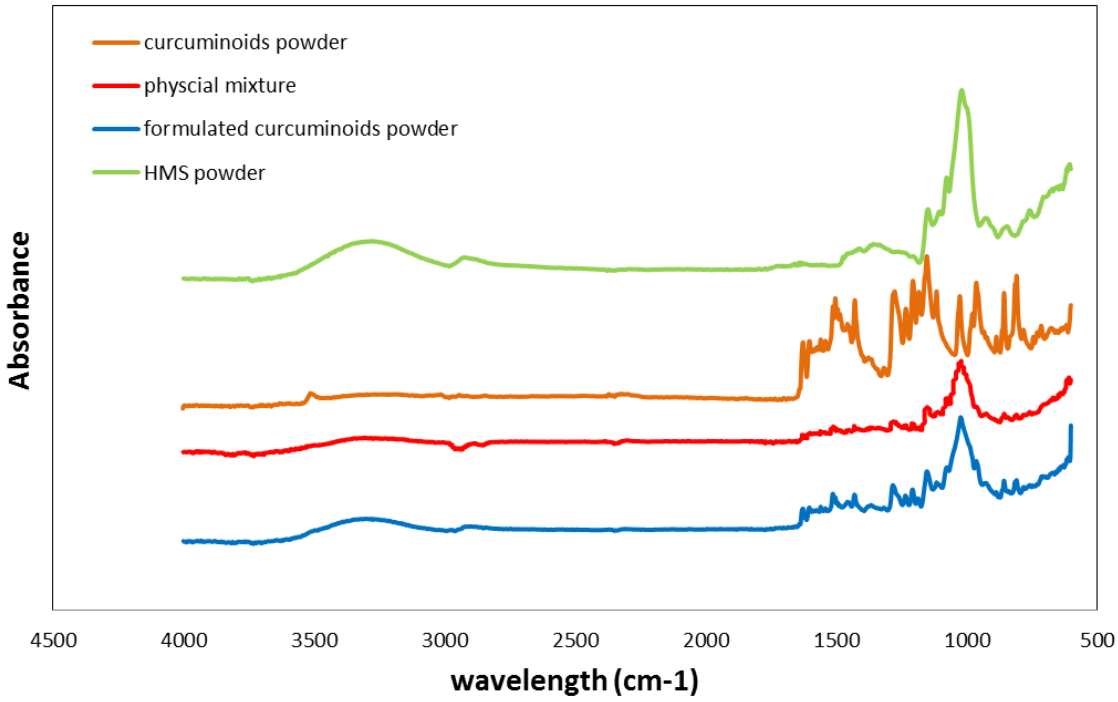


Figure 13. FTIR spectra of HMS, curcumin powder, physical mixture and formulated curcumin powder.

3.2.5 Thermal properties by DSC and XRD analysis

To understand the effect of the wet-milled process on the melting temperature and the melting enthalpy of curcumin, DSC was conducted. Figure 14 exhibits the thermograms of pure curcumin powder, HMS powder and the wet-milled curcumin powder. The pure curcumin powder used in this study showed a sharp melting endothermic peak at 173.18 °C and a melting enthalpy of 76.18 J/g. It can also be seen that the melting point of the wet-milled curcumin powder was 169.85 °C, similar to the original curcumin powder. However the enthalpy of fusion of the formulated one was 12.89 J/g, which was much lower than that of the original one. The DSC scan of HMS powder did not show any endothermic peak around 170°C in addition to no obvious interaction between curcumin and HMS from FTIR results, which indicated that HMS would not influence the thermal properties of curcumin significantly. The melting enthalpy value is proportionate to the degree of crystallinity in the samples. These results suggested a certain loss of crystallinity of curcumin during the wet milling process. This may contribute to the higher bioavailability of curcumin, because the amorphous form can yield a high apparent solubility and dissolution rate which provide the absorption advantage for the poorly water soluble compounds (Kaushal et al., 2004).

The XRD analysis of pure curcumin and the formulated one were performed and illustrated in Figure 6. The characteristic peaks of pure curcumin appeared at a series of scattering angles 2θ at 7.94°, 9.24°, 12.24°, 14.56°, 16.7°, 18.2°, 19.48°, 21.18°, 23.46°, 24.64°, 25.74°, 27.44° and 29.12°, which is similar to the one of curcumin found in the Cambridge Structure Database. This result meant that the pure curcumin powder before processing was in a relatively high crystallinity form. In comparison to the pure curcumin,

the XRD patterns showed that after formulating the majority of Bragg peaks disappeared, which indicated that the wet-milling process could partly break down curcumin crystals and turn them into the amorphous state. However, there were some peaks with less intensity located at 8.92° and 17.32°, in the XRD curve of the formulated one, which suggested that a small amount of curcumin crystals still exist. These results were in agreement with those from DSC analysis.

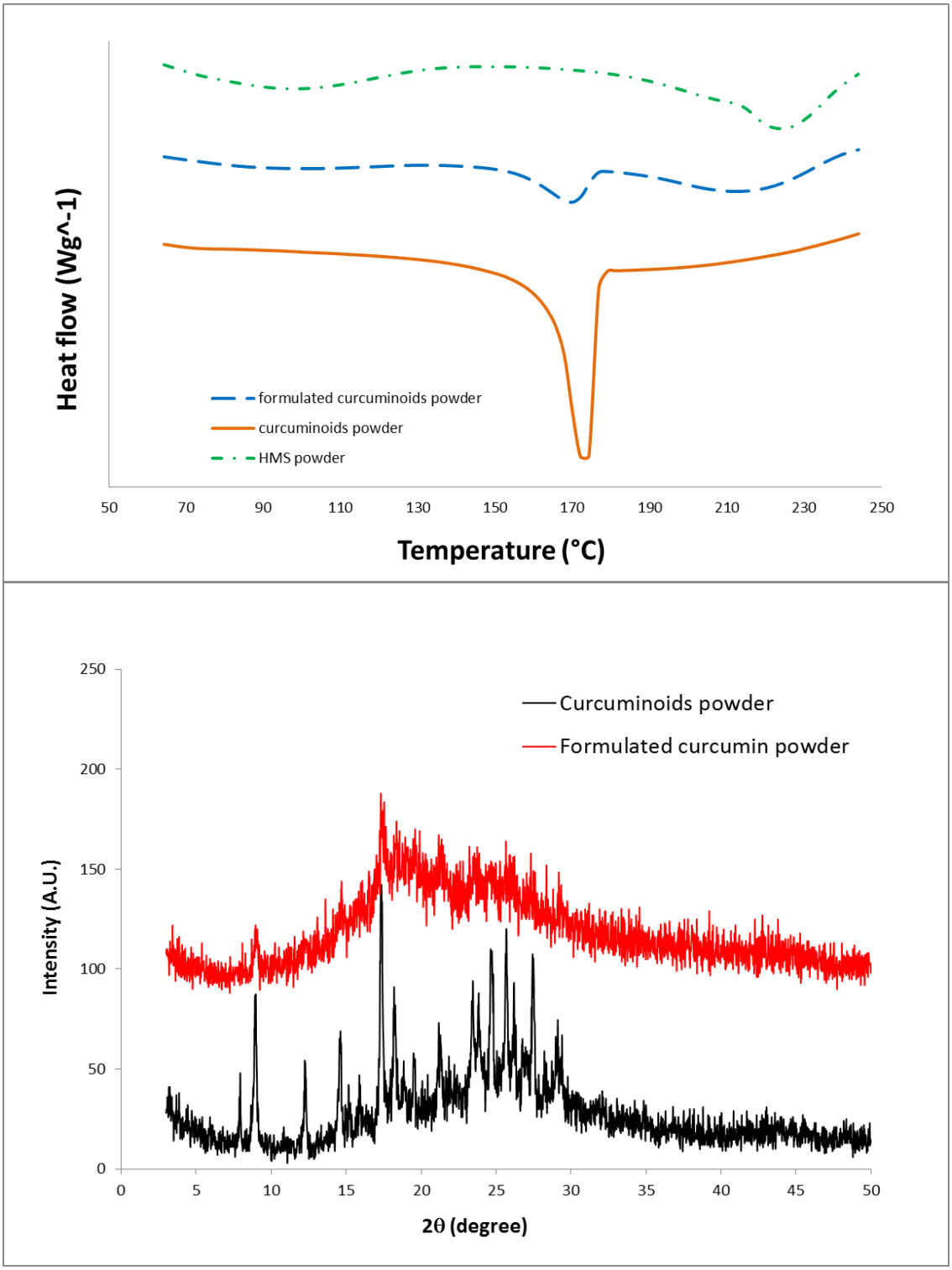


Figure 14. (a) DSC thermograms of curcumin powder, HMS powder and formulated curcumin powder and (b) XRD results of curcumin powder and formulated curcumin powder.

3.2.6 The *in vitro* anti-cancer efficacy of the formulated curcumin in HepG2 cells

To evaluate the *in vitro* anti-cancer efficacy of the formulated curcumin, cell proliferation assay was performed in HepG2 cell line. These cells were treated with DMSO-dissolved pure curcumin and the formulated curcumin of the same concentrations for 24h and then examined by MTT assay. Cells treated with DMSO or HMS alone were used as controls. The viable cell numbers were normalized against untreated controls and were taken as a measure of treatment cytotoxicity.

In Fig. 15, either DMSO or HMS did not show significant effect on cell viability which indicated no cytotoxicity of DMSO or HMS alone in these cells at indicated concentrations. Whereas, both DMSO-dissolved pure curcumin and the formulated ones have shown dose-dependent anti-proliferative effect in HepG2 cell lines. Furthermore, the formulated curcumin were more effective in anti-carcinogenesis compared to the pure ones. Especially at the concentrations of 0.5, 1 and 5ug/ml, the anti-cancer activity of the formulated curcumin was significantly higher than that of the pure ones ($P =< 0.001$), suggesting that the formulated ones was more effective to kill cancer cells.

Curcumin described to efficiently induce apoptosis in various cell lines (Duvoix, 2005). Ghosh et al. reported that curcumin exhibited notable anti-proliferative activity towards lymphoblast leukemic cells by induce DNA damage in cancer cells, finally leading to the apoptosis of cancer cells (Ghosh, 2009). It has been suggested that curcumin induces apoptosis in tumor cells by mitochondria-dependent mechanisms (Aggarwal, 2003; Reuter, 2008) suggested that curcumin activate cytochrome c caspase-3.

Although this MTT assay cannot reveal how this formulated curcumin functioned in the HepG2 cells, it can suggest several possible mechanisms for its in vitro anti-cancer

activity. One is modified starch could assist nano-sized curcumin particles to be delivered into the cells. Another is modified starch and curcumin might have synergic effect of activating cytochrome c caspase-3 to induce apoptosis. There is also possibility that dispersed curcumin particles were not able to enter the HepG2 cells and deposit on the membrane that would inhibit the normal cellular metabolism.

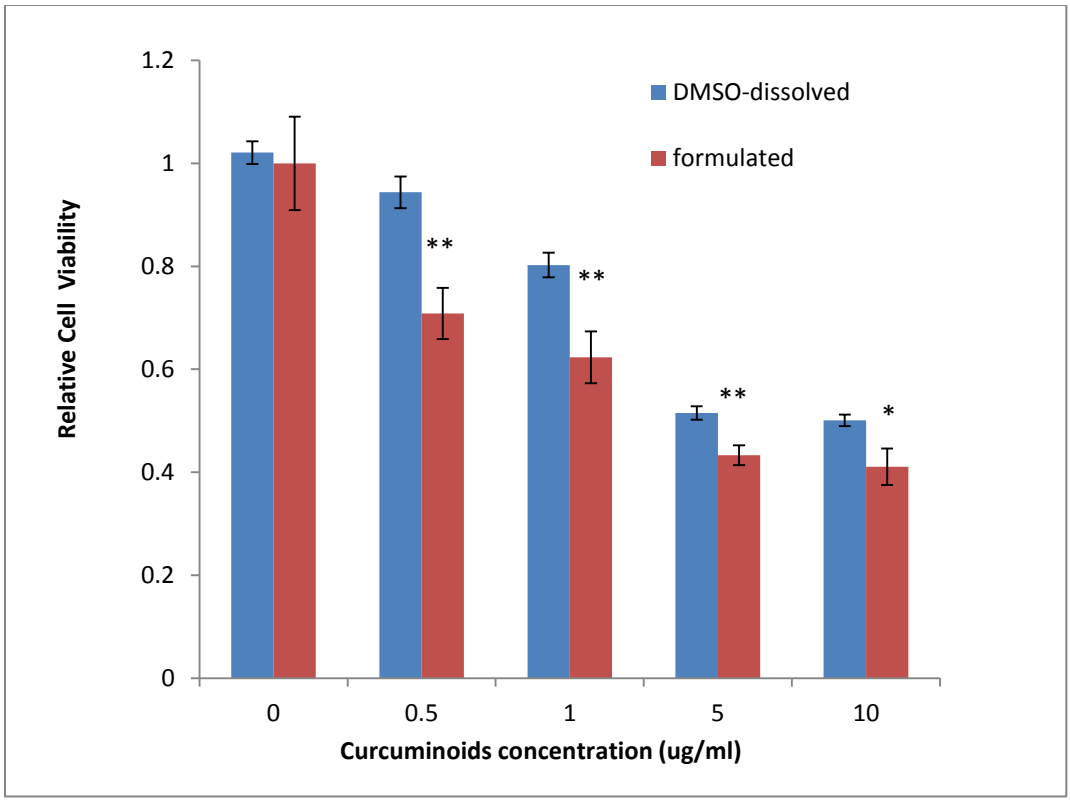


Figure 15. The plot of relative cell viability versus curcumin concentration for pure curcumin (dissolved in DMSO) and formulated curcumin. Conditions of DMSO alone and HMS alone were expressed as curcumin at zero concentration. **P=<0.001; *P=<0.01.

3.3 Comparison of freeze drying and spray drying processes on the properties of wet-milled curcumin powder

The processes of spray-drying and freeze-drying are commonly used to produce particulate products in various industries, notably food, pharmaceuticals, and chemicals. There are advantages and disadvantages to each technique. Spray-drying is relatively cheap, but can incur quality losses from the higher temperatures used - such as loss of volatile compounds and thermal degradation of heat sensitive materials. The lower temperatures used in freeze-drying minimize these losses, and also lead to a porous structure. However, freeze-drying involves higher capital and operating costs, due to the low temperatures, high vacuum, and long residence times required (Stapley, 22-25 August 2004).

3.3.1 Comparison of freeze drying and spray drying processes on the thermal properties of wet-milled curcumin powder

To compare the effects of the freeze dried and spray dried processes on the melting temperature and the melting enthalpy of curcumin, DSC was conducted. Figure 16 exhibits the thermograms of both freeze dried and spray dried formulated curcumin powder. Both of them showed a sharp melting endothermic peak around 173.18 °C, which was similar with the one of unformulated curcumin powder. However, the enthalpy of fusion of the freeze dried one was 12.89 J/g, which was a little lower than that of the spray dried one 15.93 J/g. As we know, the melting enthalpy value is proportionate to the degree of crystallinity in the samples. These results suggested a certain more loss of crystallinity of curcumin during the freeze drying process than the spray drying process.

The conclusion from DSC results was in agreement with that from XRD. The XRD analysis of freeze dried and spray dried ones were performed and illustrated in Figure 13. Some of the characteristic peaks of freeze dried formulated curcumin powder disappeared, while the spray dried one remained the majority of the Bragg peaks and with higher intensity. This result meant that the freeze drying process could turn more curcumin crystals into the amorphous state than the spray drying process.

3.3.2 Comparison of freeze drying and spray drying processes on permeation of wet-milled curcumin powder

Two quality control procedures were performed to confirm that the Caco-2 cell monolayers were confluent and suitable for the permeation study: A. Only the wells with the TEER values greater than 300 $\Omega \cdot cm^2$ were used (Artursson, 2001); B. the permeation rate of lucifer yellow, a paracellular transport marker, is expected to be less than 1×10^{-6} cm/s (Mou-Tuan, 1997), which was determined as $0.10 \pm 0.01 \times 10^{-6}$ cm/s in this study. Therefore, the Caco-2 cell monolayers were appropriate to be used in the permeation study.

Subsequently, the permeation rates of freeze dried and spray dried formulated curcumin powder dispersed in water were determined in the direction of A-B, with donor media pH set at 6.5, to mimic the acidic microenvironment in the small intestine (Huang, 1995). As shown in Figure 14, over 3% of freeze dried formulated curcumin was transported through the Caco-2 cell monolayers in 120 minutes. The corresponding Papp was determined as $3.64 \pm 0.6 \times 10^{-6}$ cm/s. In comparison, only about 1.5% of spray dried formulated curcumin was transported through the Caco-2 cell monolayers in 120 minutes. The corresponding Papp was determined as $1.8 \pm 0.6 \times 10^{-6}$ cm/s. Both of their permeation rates were larger than 1×10^{-6} cm/s, which was thought as a fast permeation rate and

implied a fast absorption rate in vivo (Artursson, 2001; Hubatsch et al., 2007). However, the permeation rate of the freeze dried one was significantly higher than that of the spray dried one, which might was due to the higher percentage of amorphous state of curcumin in the freeze dried one.

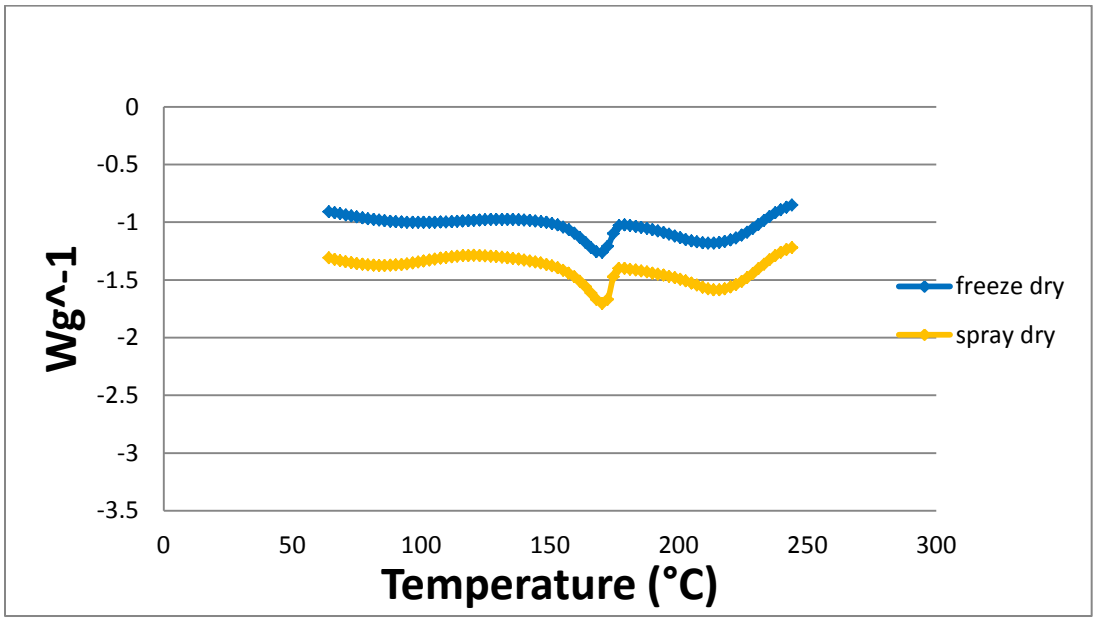


Figure 16. DSC thermograms of freeze dried and spray dried curcumin powder.

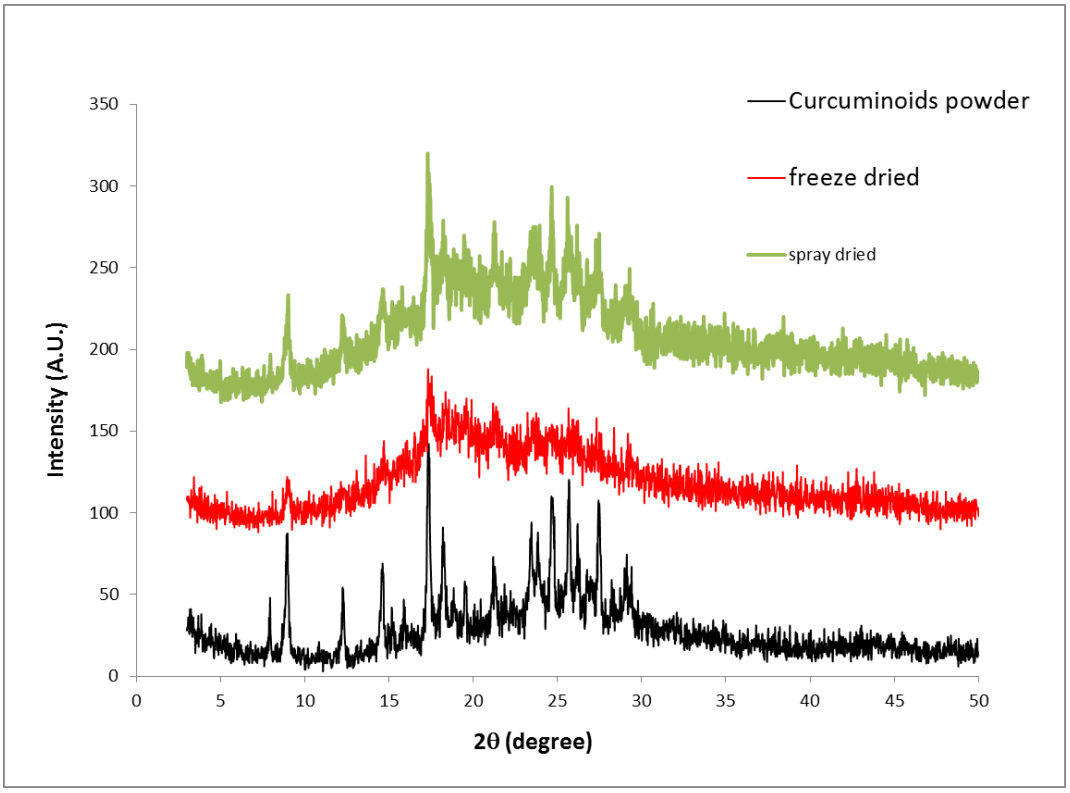
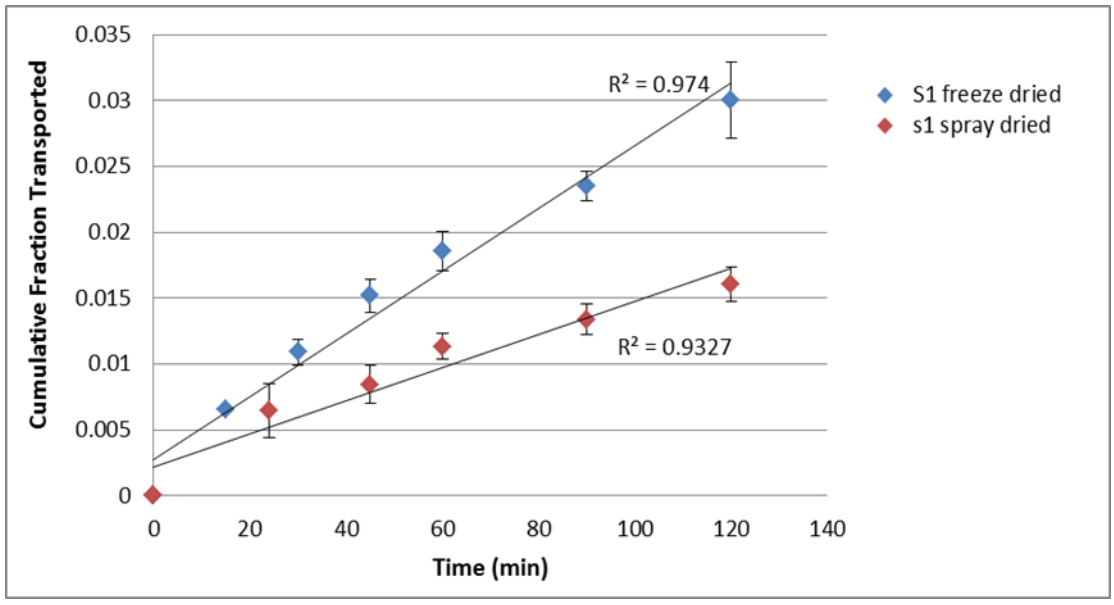


Figure 17. XRD results of curcumin powder, freeze dried and spray dried wet milled curcumin powder.



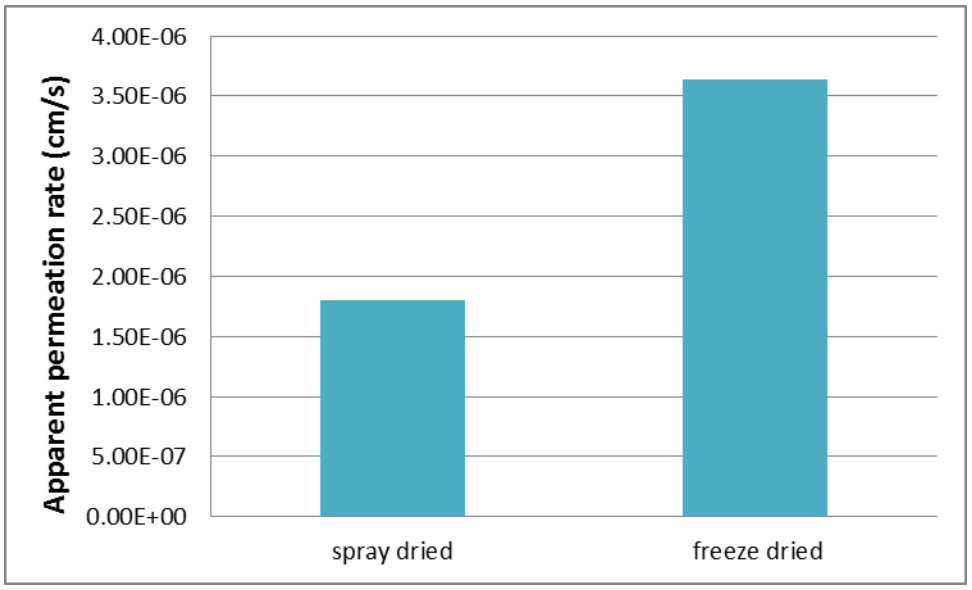


Figure 18. Transport of freeze dried and spray dried formulated curcumin powder.

4 Conclusions

In this study, a novel delivery system for curcumin was prepared by wet milling technique. The physiochemical characteristics and the in vitro anti-cancer property have proven that it is a promising formulation for the further product development. Wet milling is a recent technique widely used to form nanodispersion in pharmaceutical industry (Gowthamarajan, 2010). The curcumin nanodispersion consists of water, curcumin and HMS as stabilizer. During the wet milling process, high shear forces generated during impaction of the milling media with curcumin provided the energy input to fracture curcumin crystals into nano-sized particles. HMS was capable of wetting the surface of curcumin crystals and providing a steric barrier, which promoted the particle size reduction process and generated physically stable formulations. The nanodispersion was freeze-dried as a dry powder for storage or solid dosage development. The dried powder could re-disperse into nano-sized particles when placed in water, which is critical to the development of a solid dosage form that maintains the benefits of this novel delivery system. The nanodispersion prepared by wet milling technique is advantageous as opposed to the traditional formulation approaches. One of its major advantages is its safety at higher dosing, while in traditional formulations, hash solvents or co-solvents often present for the poor-water-soluble compounds, which limits the dosing as a result of toxicity of the excipients(E. Merisko-Liversidge, 2003). Another advantage is this method could handle large quantities of substances and provide a universal process suitable for formulation development and commercialization in industries.

In the toxicity experiments, DMSO solution of pure curcumin was used as control groups, because attempts to dissolve a workable concentration of curcumin in distilled

water were not successful. However, the formulated curcumin powder could disperse in water relatively stable without involving organic solubilizers. It is expected that the improvement of cytotoxicity on cancer cells was due to the higher water solubility and cell uptake. The nano-sized particles stabilized by HMS could disperse in media homogeneously, which provided the cells with longer "effective exposure time" (EET) to curcumin. In contrast, the cells treated with pure curcumin might experience a short EET due to premature precipitate (Safavy et al., 2007).Since HMS is a biomolecule synthesized with waxy maize and n-octenyl succinic anhydride (n-OSA) (Shaikh et al., 2009), the interaction between HMS and the cancer cells could be the reason of enhanced cellular absorption and anti-cancer activity.

In summary, curcumin nanodispersion was prepared by wet milling method, with the particle size of 150nm. Upon this new formulation, the loading capacity of curcumin was 22% and the water dispersity was 8.8±0.3 mg/ml which was increased about 105-folds. FTIR results showed that the chemical properties of curcumin remained the same with HMS as stabilizer. Both DSC and XRD analysis proved that wet milling process could turn curcumin crystals into amorphous state at a certain level. Furthermore, the anti-cancer activity of the formulated curcumin was greatly increased compared to the pure one. All of these results show that nanodispersion by wet milling method is a promising deliver system for increasing the bioavailability of water-insoluble polyphenols. Its high water solubility and efficacy in the cell-line models highlight the potential in vivo models. Human trails will be conducted to evaluate its feasibility in clinical applications as an improved chemotherapeutic agent.

5 Future studies

As an integral component of Asian food for thousands of years, native curcumin has been proved to be safe in various studies (Dhillon et al., 2008; Hatcher et al., 2008; Javvadi et al., 2008). It is intriguing to test whether nano-formulation of curcumin will cause any undesirable toxicity. To test this hypothesis, we will treat several primary cell cultures (liver, kidney, and cortical neuron) with various doses of formulated curcumin, native curcumin or saline control for 24hrs and then evaluate and compare the cell viability among different groups using MTT assay. We anticipate to see no statistically significant differences among 3 groups, thereby confirming our hypothesis that nano-formulation of curcumin will not cause any cytotoxicity.

Although widely reported as a bioactive chemical with many beneficial physiological effects, curcumin still has limited clinical application due to its poor bioavailability. We hypothesize that nano-formulation of curcumin may significantly enhance its absorption and half-life in the body, as suggested by our Caco-2 in vitro experiment. We will test our hypothesis by orally giving 30mg/kg of formulated curcumin, native curcumin or saline control to 200g male Wistar rats and then collecting blood samples at various time points (0, 2, 4, 8, 12, 16, 24hrs). The concentration of curcumin in the serum will be determined by HPLC and we expect to see better bioavailability and longer half-life of formulated curcumin compared with that of its native form.

If our nano-formulated curcumin proves to be safe and reveals better bioavailability than its native form in the aforementioned studies, we may test its therapeutic efficacy in an animal model of liver cancer, because plenty of studies have suggested the anti-cancer property of native curcumin in hepatocellular carcinoma

research (Anand et al., 2008; Darvesh AS, 2012). Briefly, 200g male Wistar rats will receive plain drinking water or 200 ppm nitrosodiethylamine (NDEA) in drinking water 5d/week for 4 weeks to induce hepatocarcinogenesis (Thapliyal et al., 2003). Starting 2 weeks before NDEA treatment, the NDEA group of rats will receive 0, 0.2, 1, 5% formulated curcumin diet or native curcumin diet until 10 weeks after NDEA treatment. Body and liver weight will be determined at the end of the experiment as an assessment of hepatocellular carcinoma progression and we expect to see significant increase of both absolute and relative liver weights in rats treated with NDEA, which may be fully rescued by formulated curcumin but not native curcumin. Animal mortality rate, carcinoma incidence and focal liver lesions among different groups will also be determined, and formulated curcumin is expected to have better protective efficacy than native curcumin in NDEA-treated rats.

6 References

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