MICRO- AND NANO-ENCAPSULATION AND CONTROLLED-RELEASE OF PHENOLIC COMPOUNDS AND OTHER FOOD INGREDIENTS

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ABSTRACT OF THE DISSERTATION

Micro- and nano- encapsulation and controlled-release of phenolic compounds and other food ingredients

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The health promotive properties of phenolic compounds attracted a lot of attention in recent years because of their biological and pharmacological effects including antioxidative and cytoprotective functions. Green tea catechins and curcumin have been extensively studied and they both show strong anti-oxidant and anti-inflammatory properties, but low bioavailability is always a problem. Therefore, effective delivery systems could be a solution to enhance their oral bioavailability.

In this study tea catechins were encapsulated in two W/O/W double emulsion systems, protein-polysaccharide complex coacervates and emulsifiers/polysaccharide stabilized double emulsion. Physicochemical characteristics were determined for both systems. The coacervate encapsulation achieved 90.5% encapsulation efficiency, and the other double emulsion reached 94.5% efficiency. Coacervate-encapsulated catechins were stable in artificial gastric juice, and could target-release catechins in small intestinal juice triggered by pH.

Curcumin was dissolved in medium chain triglyceride (MCT) and further emulsified in water. Curcumin nano-emulsions had average particle sizes of 150.5nm and 148.4nm for 1% and 1.5% curcumin, respectively. The encapsulation efficiencies were 77.5% for 1% curcumin emulsion and 71.5% for 1.5% emulsion. Oral administration of nano-emulsified curcumin could inhibit TPA-induced edema on mouse ears by 100%, and significantly inhibited pro-inflammatory factors IL-1beta, IL-6, MMP-9, and cyclin D1 dose-responsively. The anti-inflammatory effects directly indicated enhanced bioavailability of curcumin.

Protein-polysaccharide coacervation was further applied to enzyme encapsulation. α-Amylase can form coacervate with κ-carrageenan under optimized conditions, and reach 99.3% encapsulation efficiency. Enzyme kinetics showed that encapsulation could strongly protect α-Amylase from acid denaturation, suggesting that the stoichiometric complexation of α-amylase did not alter the active binding sites of enzyme.

In summary, low cost, convenient and highly efficient encapsulation methods using food grade natural biopolymers have been developed to encapsulate nutraceuticals or enzyme. The encapsulation systems have protective, target-releasing, and bioavailability enhancing functions.

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Introduction

Polyphenols are a group of chemicals, and found in many plants with more than one phenol groups per molecule. Dietary polyphenolic compounds have attracted a lot of attention in recent years due to their biological significant functions, such as anti-oxidant, anti-inflammatory and anti-carcinogenic activities. Epigallocatechin-3-gallate (EGCG) from green tea, curcumin from turmeric, resveratrol from red grape skin, quercetin from onion, and hesperidin from citrus are the ones, which have been mostly studied. The bioavailability of the polyphenols varies widely, and also depends on the form of the compound in the dietary source. Manach et al. (2005) had reviewed the bioavailability of 97 polyphenol compounds and showed that proanthocyanidins, galloylated tea catechins, and the anthocyanins are the least absorbed pholyphenols.

Various approaches have been made to enhance the bioavailability of polyphenols, including green tea EGCG and curcumin. The absorption of EGCG can be improved by consuming another dietary supplement, piperine, together (Lambert et al., 2004). It reported that the enzymatic modification of hesperidin, a citrus flavonoid, could increase its bioavailability by changing the absorption site in the intestine (Nielsen et al., 2006). Using carriers to enhance the bioavailability was an effective approach. Complexation of resveratrol and cyclodextrin could increase the stability and bioavailability of resveratrol (Lucas-Abellan et al., 2007). Research also showed injection of liposome encapsulated EGCG into basal cell carcinomas could increase the uptake of EGCG by 20 fold (Fang et al., 2006).

As dietary supplements, it would be better if the polyphenols could be carried by food grade encapsulation material and enhance the bioavailability through oral

administration. Therefore, in this study the polyphenolic compounds and other active food ingredients are encapsulated in coacervate or fatty acids and further emulsified into water. Particle sizes reduction, stability, and encapsulation efficacy would be discussed, followed by *in vitro*/*in vivo* tests.

Chapter 1

Background and Literature Review

1.1. Polyphenols

1.1.1. Tea Catechins

Tea is the most popular beverage next to water, consumed by over two-thirds of the world's population. Tea as a beverage has been consumed for thousands of years. It is rich in natural antioxidants, which are believed to have health promotive functions. The distinguished contents of tealeaves are methylxanthines and polyphenols especially flavonols of the catechin type. The major green tea polyphenols are epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epicatechin (EC), galocatechin (GC), and catechin. Among the catechines, EGCG has shown the most protective activities (see Figure 1). EGCG accounts for about 60-70% of total tea catechins (Katiyar and Mukhtar, 1996). Whereas, black tea is undergone further fermentation and contains more oxidative products of catechins, such as bisflavonols, theaflavins (TF) and thearubigins (TR) (Ahmad, et al., 1998). Experimental studies have showed significant antimutagenic and anticlastogenic effects of both green and black tea and their polyphenols in multiple mutational assays. Green tea is believed to have greater beneficial potency than black tea due to higher content of unpolymerized catechins in green tea, especially EGCG (Katiyar and Mukhtar, 1996). The cancer inhibitory activity of EGCG has been demonstrated. The inhibition of EGCG against skin, stomach, colon, and lung carcinogenesis as well as the growth of human prostate and breast tumor in athymic mice have been reported. In vitro study has shown the modulation of gene expression of EGCG (Gupta, et al., 2002).

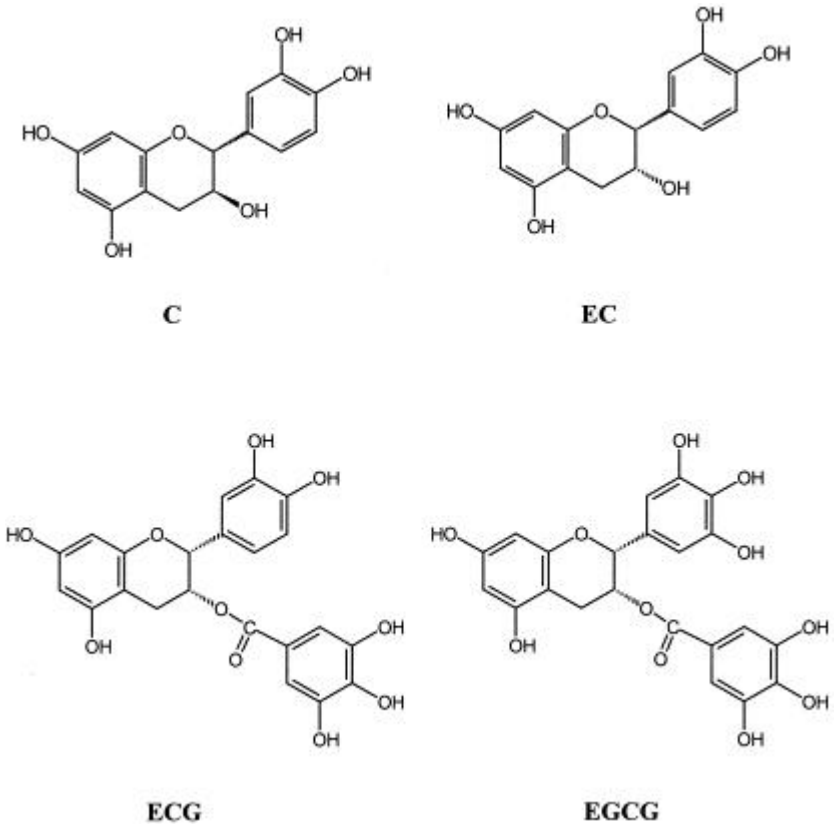


Figure 1. Chemical structures of green tea catechins: (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (Caturla, et al., 2003).

The cancer chemopreventive effects of tea polyphenols were found to be effective on decreasing cell transformation and proliferation or increasing apoptosis. Many cancer preventive effects of EGCG and other tea polyphenols have been reported (Lambert and Yang, 2003). Catechins also have great antioxidant potency. Tea can relieve oxidative stress induced by carcinogens and tumor promoters (Singh and Agarwal, 2002); EGCG can induce cell cycle in the G0/G1 phase to inhibit the proliferation of the tumor cells (Peng et al., 2006); EGCG affects the signal transduction and results in growth inhibition, cell cycle arrest, and apoptosis of a cell (Chen et al., 2004).

On the other hand, the effective tissue concentration of EGCG is very low. The EGCG levels in the tissues and blood corresponded to 0.0003-0.45% of the ingested dose (Natagawa and Miyazawa, 1997). Another study using [H3]-EGCG showed that following a single initial dose of [H3]-EGCG, only 10% of the initial dose was presented in the blood after 24h, and approximately 1% in the brain, lung, heart, liver, kidney and other tissue (Mukhtar and Ahmed, 2000).

In the experiments to study the mechanisms of cancer chemopreventive activity of EGCG and other tea polyphenols, the concentrations of 10-1000 μM were used to inhibit the growth in tumor cell lines. But EGCG has very poor bioavailability. Therefore, it is almost impossible to achieve such high concentrations in most of parts of a body except skin and gastrointestinal tract (Lambert and Yang, 2003).

The poor bioavailability maybe in part explained by Lipinski's Rule of 5. That is, if a compound has a molecular weight of >500, and contains 5 or more hydrogen-bonding donors and/or 10 or more hydrogen-bonding acceptors, it is very difficult to pass through transient pores formed in the plasma membrane. Apparently, EGCG has a large

molecular size and many hydrogen bond donors and acceptors, resulting in a molecule with a large hydration shell, which is hard to pass through the plasma membrane (Lambert et al., 2006).

Metabolism of EGCG in a body is as follows. Glucuronidation, sulfation, and methylation are the major metabolic interactions of tea catechins. The metabolism pathway is summarized in Figure 2. EGCG is conjugated with methyl groups with COMT (catechol-O-methyltransferase). 4', 4"-DiMeEGCG (4', 4"-di-O-methyl-epigallocatechin gallate) is the major product at low EGCG concentrations (<1μM). When high dose of EGCG (100mg) was administrated orally, 4"-MeEGCG (4"-O-methyl-(-)-epigallocatechin gallate) was the major methylation product of EGCG excreted in the bile of rats. Except for the conjugation reactions, EGCG and EGC also form the ring fission products (See Figure 2). The possible sites of methylation and glucuronidation are shown in Figure 2 (Lu, et al., 2003). B-ring and D-ring are very important in the metabolic pathway. A-ring might be modified to increase its bioavailability.

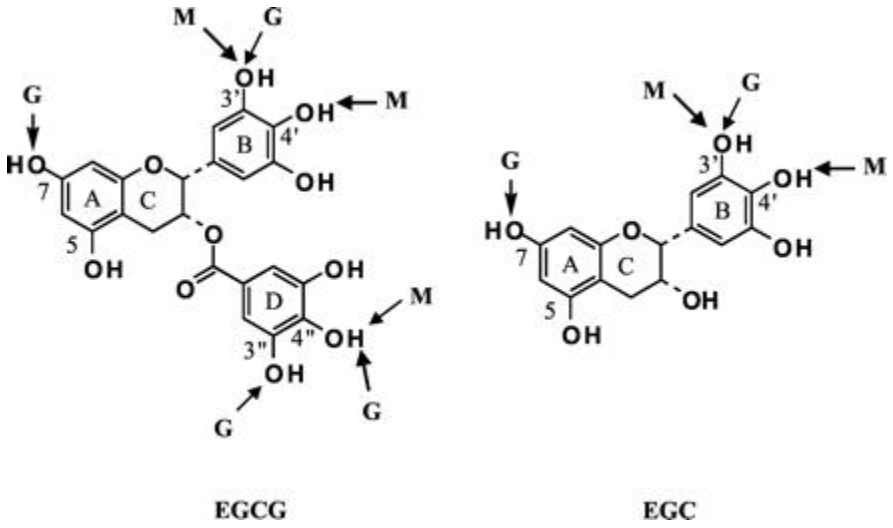
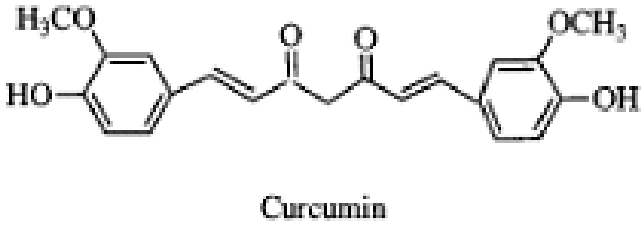


Figure 2. A summary of possible sites of methylation (M) and glucuronidation (G) of EGCG and EGC (Lu, et al., 2003).

1.1.2. Curcumin

Curcumin, chemically named 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a polyphenol extracted from the rhizomes of turmeric (*Curcuma longa*). The chemical structure of curcumin is shown in Figure 3 (Khanna, 1999). Curcumin has very strong yellow color and can be used as a natural food color. Curcumin has exhibited antioxidant (Sharma, 1976), anti-inflammatory (Srimal and Dhawan, 1973), antimicrobial (Kim et al., 2003) and anticarcinogenic (Miller et al., 2008) activities. Various cell/animal models and human studies have demonstrated the preventive or therapeutic functions of curcumin (Miller et al., 2008; Kuttan et al., 1985; Hsu and Cheng, 2007). It inhibits breast, bladder, prostate, or leukemia cancer in cell culture. Curcumin affects arachidonic acid metabolism, inhibits COX and LOX, and induces apoptosis (Hong et al., 2004; Wallace, 2002). However, low bioavailability is a general problem for oral administration of curcumin. Oral intake of curcumin inhibited chemically induced esophagus, fore-stomach and colon cancer, but had negligible effect on lung or breast cancer in mice due to the low circulation concentration in blood (Huang et al., 1998). Hsu and Chen (2007) showed in phase I clinical trial that curcumin only had efficacy on the tissues (colorectum, oral mucosa and skin) exposed to drug directly, but had no clear effect on other chronic inflammation or cancer.



Curcumin

Figure 3. The chemical structure of curcumin (Khanna, 1999).

Curcumin is almost insoluble in water at neutral or acidic pH (Maiti, 2007). After oral administration, curcumin is poorly absorbed, which results in extremely low serum concentration (Ravindranath and Chandrasekhara, 1980). Free form of polyphenols could not pass through cell membranes, and were rapidly excreted from the general circulation (Wenzel et al., 2005; McGhie et al., 2003). Once absorbed, curcumin undergoes conjugation like sulfation and glucuronidation, and extensive reduction into less active forms at various tissue sites (Wahlstrom and Blennow, 1978; Pan et al., 1999). Curcumin also has very limited tissue distribution and a rapid clearance rate. The major route of elimination is through feces, and the curcumin level is negligible in urine (Holder et. al, 1978).

Several delivery systems have been studied to increase the bioavailability of curcumin, including nanoparticles, liposomes, and micelles (Anand et al., 2007). Nanoparticle formulations have been developed and showed increased solubility and promising results *in vitro*, but no *in vivo* oral administration result is available yet (Bisht et al., 2007; Tiyaboonchai et al., 2007). Liposome encapsulated curcumin has shown enhanced bioavailability and inhibition to pancreatic and colorectal cancer *in vitro* and *in vivo* (intravenous) (Li et al., 2007a; Li et al., 2007b). Micelle formulations have shown increased intestinal absorption *in vitro* (Suresh and Srinivasan, 2007). Curcumin-phospholipid complex demonstrated about 2-fold increase in plasma concentration in rat after oral intake (Liu et al., 2006). Yet, no therapeutic activity has been reported with oral administrated curcumin. Organic solvent and other non-food grade ingredients are involved in the previous studies. Therefore an effective food grade delivery system is

needed to increase the oral bioavailability and efficacy of curcumin as a natural nutraceutical.

An earlier preliminary study in our group reported that nanoemulsion delivery systems of curcumin showed some initial anti-inflammation activities on mice (Wang et al., 2008). In addition, the anti-inflammation activity of curcumin emulsions was determined by emulsion sizes, and the inhibition effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced edema of mouse ear changed from 43% to 85% when the emulsion sizes decreased from 618.6 nm to 79.5 nm. In this dissertation, we focus on a more systematic study to investigate the efficacy of curcumin nanoemulsions using either *in vitro* skin absorption model or *in vivo* mouse model to test their anti-inflammation activity using different biomarkers. In addition to study the TPA-induced edema of mouse ear using curcumin nanoemulsions with formulations different from our previous paper (Wang et al., 2008), we also investigate the dose-responsive effects and significant inhibition of pro-inflammatory factors IL-1beta, IL-6, MMP-9 and cyclin D1 by curcumin nanoemulsions.

1.2. Coacervation

One or more biopolymers in the water attract each other, form a concentrated phase, and separate from water. Single polymer phase separation is called simple coacervation (Burgess, 1994). Phase separation involved two or more biopolymers refers as complex coacervation or associative phase separation (Tolstoguzov, 1991; Piculell and Lindman, 1992). The attractions between two polymers are weak and non-specific. Normally they are electrostatic, Van der Waals, hydrophobic interactions, and hydrogen bonding (Imeson et al., 1977; Imeson et al., 1978; Piculell et al., 1995).

The coacervation process reduces the free energy of the system leading by electrostatic interaction. The complex entropy decreases during complex formation, but enthalpy arises from increasing intermolecular interactions (Piculell and Lindman, 1992).

1.2.1. Complex Coacervation and Control Factors

Complex coacervation is widely used in foods, cosmetics, and pharmaceutics. We focus on discussion in the formation of protein-polysacchride complex coacervation. Tiebackx (1911) conducted the first study of protein-polysaccharide coacervation through electrostatic interaction. The formation of coacervation starts from mixing of two oppositely charged biopolymers in water solution. They interact through electrostatic force and concentrate together. the system between gelatin and acacia gum was firstly studied by Bungenberg (1936 and 1949). The most significant factor affecting the complex formation is the charge densities of molecules. The electrostatic force depends mostly on pH and ionic strength of the solution (Xia and Dubin, 1994). Low charge density suppresses formation of coacervate. At certian pH two kinds of molecules carry equal amount of opposite charges, resulting in maximum coacervate yield (Burgess and Carless, 1985). This pH refers as electrical equivalence pH (EEP). In a high concentration salt solution, complex coacervation is weakened. The ions form a dense layer arround the biopolymers and suppress the electrostatic interaction (Bungenberg, 1936; Tsushida and Abe, 1982).

Electrostatic interaction is the main driving force for complex coacervate formation. The electrostatic interaction-related physiochemical parameters, such as pH, ionic strength, polysaccharide charge density, protein/polysaccharide concentration and ratio, strongly influence the formation, (Schmitt et al., 1998).

1.2.1.1. pH Effects

pH is the most important factor to determine the degree of functional groups ionization of polymers. Tiebackx (1911) studied gelatin (0.5 wt%) and acacia gum (2 wt%). His result showed that the amount of complex formation depended on the acidity. An anionic polysaccharide is mixed with protein in a neutral pH solution, and then acid was added. Maximum coacervation forms at EEP, which is a pH below isoelectric point (pI). Protein displays an overall positive or negative charge, depending on the pH of the solution and the pI of protein. At a pH higher than the pI, proteins will carry a negative charge. If the pH is lower than the pI, the overall charge will be positive. At EEP proteins are positively charged, which is opposite to anionic charges of polysaccharide (Xia and Dubin, 1994). Coacervation method has been used to purify whey protein. Whey protein was mixed with CMC in solution, and the pH was adjusted to 3.2 to form the coacervate. Protein could be recovered by simply adjusting pH back to basic pH. The protein recovery efficiency was 90% in this method (Hansen et al., 1971).

1.2.1.2. Ionic Strength Effects

Ionic strength is another important factor. It affects coacervate formation by interaction with charged polymers and reducing the electrostatic attraction between polymers. The charges of the polymers are neutralized by microions. Low ionic strength affects weakly on the coacervate formation but high salt concentration has strong hindering effect (Xia and Dubin 1994). High salt concentration decreases the coacervation yield sharply (burgess and carless, 1986).

1.2.1.3. Effects of Other Factors

Other factors influencing complex formation include charge density, molecular weight of polymer, polymer ratio, polymer concentration, and other environmental factors.

Charge density is the number of charges per unit length of polymer. Coacervation can only occur when the charge density is over a critical value (Schmitt, 1998). Highly charged polymers have strong electrostatic interaction (Park, et al., 1992).

Studies showed that increasing molecular weight of polymers resulted in increased coacervation yield (Overbeek and Voorn, 1957). It was explained that the solution compatibility decreased as the molecular weight of the polymers increased by lowering the combinational entropy of mixing.

For each specific coacervation system the maximum coacervate is obtained at a specific ratio of protein to polysaccharide. At this ratio, the complex is nearly zero charged and no polymers remain soluble in the solution (Tolstoguzov, 1986 and 1997).

Some external factors also affect the formation of coacervate. Coacervation has a higher yield at a lower temperature because it lows the mobility of molecules, but favor hydrogen bonding and hydrophobic interactions (Kelly et al., 1994). Shearing rate and time influence the coacervate particle sizes (Tirkkonen et al., 1994).

1.2.2. Typical Biopolymers used in Coacervation

1.2.2.1. Polysaccharides

Carrageenan is extracted from red seaweed varieties. Carrageenan exists in several forms that are mainly classified as iota-, kappa- and lambda-carrageenan. Carrageenan and furcelleran contain sulfated galactose unites. Kappa- (contains 19% sulfate) and iota (contains 25-27% sulfate) -carrageenan solubilize only in hot water and

form thermally reversible gels. Lambda-carrageenan (contains 32% sulfate) is cold-water soluble and, is primarily a thickener.

Furcelleran, also known as Danish agar, is an extract of red algae. Furcelleran has similar structure as kappa-carrageenan, but furcelleran has a smaller degree of sulphatation (Lahaye, 2001). It swells in cold water and solubilizes after heating to between 75 and 80°C. Upon cooling, furcelleran forms thermally reversible gels.

Guar gum is from the seed of *Cyamopsis tetragonoloba*. It is a galactomannan (Petkowicz et al., 1998), and disperses easily in cold water.

Alginates are extracted from brown seaweed and are linear unbranched polymers. They are soluble in both hot and cold water, and can thicken and bind to other oppositely charged polymers. It can form thermal stable gel when calcium ions are present (Donati et al., 2005).

Pectin is prepared from apple pomace and citrus peels. Pectin is an acidic structural polysaccharide, and has complex structure. Its exact functional properties vary, depending on the methyl ester content of the molecule. High-methoxyl (HM) pectins have a degree of esterification greater than 50%, and form thermal irreversible gels in presence of sufficient sugar at low pH. Low-methoxyl (LM) pectins have a degree of esterification less than 50%, and can form thermal reversible gel in the presence of calcium ions at low pH (Tsoga et al., 2004).

1.2.2.2. Proteins

Gelatin is the only food protein hydrocolloid extracted from an animal source. Gelatin is available in two types. Type A is from pork skin treated with acid, while Type B is from beef bones and calf skin treated with alkali. Gelatin swells in cold water, is

soluble in hot water and forms gels that set at 20°C and melt at 30°C. Type A gelatin has a pI of 6.5 to 8.5 for bone-derived and 7.5 to 9.5 for skin-derived. Type B has a pI of 4.8 to 5.1.

Globular proteins interact strongly with polysaccharides and form coacervates. BSA and whey proteins are often used as food grade protein. BSA is separated from bovine blood and has high stability. The isoelectric point (pI) of BSA is 4.6. Whey protein is from the watery byproduct of cheese making. It contains alpha-lactalbumin, beta-lactoglobulin, bovin serum albumin, immunoglobulins, and proteose-peptones. Beta-lactoglobulin accounts for ~65% of the total protein and alpha-lactalbumin is ~25%. Alpha-lactalbumin (pI 5.1-5.3) can act as emulsifier, and beta-lactoglobulin (pI 4.5) has gel forming property (Kinsella and Whitehead, 1989).

1.2.3. Soluble Complexes

Soluble complex and insoluble complex can form through electrostatic interaction. When opposite charges on two polymers are not equal, they interact with each other, on the other hand the net charges of the complex also interact with solvent molecules. The repulsion force in the complex is strong enough to solublize the polymers. However, when the opposite charges of two polymers are equal, the whole complex is fully neutralized and precipitates as insoluble complex (Tolstoguzov, 1997; Xia and Dubin, 1994; Mattison et al., 1995). After the insoluble complex forms, non-electrostatic intractions involve to stabilize the coacervate. The interactions include hydrophobic interaction, Van der Waals interaction, and hydrogen bonding (Stainsby, 1980). Therefore, the complexes are less sensitive to pH or ionic strength change to recover to soluble molecules (Antonov, 1996).

Coacervate forms at pH above the pI of a protein. In albumin and anionic polysaccharides, soluble complex forms at pH above the pI of albumin (4.7). It was explained as charged patches on protein present local positive charges. The patches form coacervates with negatively charged polysaccharides. The interaction is strong enough to overcome the repulsion between polymers (Noguchi, 1956 and 1959).

1.2.4. Applications of Coacervation

Complex coacervation has been studied for a century and utilized in lots of industry. The major applications include macromolecule purification, micro-encapsulation, and using coacervate as new food/bio-materials.

Using coacervation method to purify macromolecules is simple and low cost. The basic of the method is adjusting pH and other conditions to get the maximum coacervation, the dense precipitate, and tuning pH back to release the macropolymers (Daniels and Mittermaier, 1995). Serov et al. (1985) used apple pectin to recover whey protein with reclaim efficiency of 90%. Hidalgo and Hansen (1971) reported a method of using carboxymethyl cellulose to specifically form complex with whey protein, thus purify and fractionate protein.

Using complex coacervation in microencapsulation is another important industrial application. Coacervate forms a solid film around the emulsion droplet, hence to encapsulate the sensitive ingredients (Thies, 1982). To produce the encapsulation system, protein was first used as an emulsifier or a stabilizer to emulsify the oil, which contains oil soluble ingredients. Then polysaccharide was added to form coacervate with protein around emulsified droplet. The coacervate is subsequently freeze dried (Jizomoto et al., 1993; Dong and Rogers, 1993; Chilvers and Morris, 1987). The release mechanism of the

material is the same as that of coacervation. pH and salt concentration are the triggers. Gao et al. (1984) used gelatin and acacia gum or peach gum to encapsulate pesticide and fenthion. The method extended the drug activity with very high encapsulation efficiency and controlled-release properties. Jizotomo (1993) encapsulated oil soluble drugs with coacervate of gelatin and acacia gum. Coacervates formed at pH 4.0 with shelf life of more than one year. They also showed increased absorption in GI track of the micro-encapsulated drug in animal compared to free drug and drug in oil.

1.3. Emulsions, Emulsifiers and Stabilizers

1.3.1. Emulsions

Emulsion is a complex dispersed system containing oil, water, and emulsifiers. According to IUPAC 1972, “an emulsion is a dispersion of droplets of one liquid in antoher one with whch it is incompletely miscible. In emulsions the droplets often exceed the usual limits for colloids in size” (Israelachvili, 1994). Simple emulsion includes O/W emulsion when oil droplets disperse in continuous water phase, and W/O emulsion when water disperses in continuous oil phase. When the term oil or water is used, it refers as a less polar or a more polar phase, respectively.

1.3.2. Emulsifiers

Emulsifiers or surfactants are suface active molecules consisting of a hydrophilic “head” group and a lipophilic “tail” group (Faergemand and Krog, 2003; Krog and Sparso, 2004). At oil-water interface, the hydrophilic group interact with water and tail groups have high affinity to oil. During homogenization, emulsifiers quickly absorb to the oil-water interface to reduce the interfacial tension. By reducing surface energy of oil-water interface, emulsifiers prevent droplet aggregation. In this way emulsifiers improve

emulsion formation and stability (Charalambous and Doxastakis, 1989; Stauffer, 1996, 1999).

A wide range of emulsifiers can be used in food. They can be divided into ionic, such as lecithin and fatty acid salts, and non-ionic, such as Twin and Span. The hydrophilic-lipophilic balance (HLB) of a emulsifier is described as a number showing the relavire affinity to the oil and water phases (Becher, 1983, 1985; Davis, 1994). High HLB value means a high ratio of hydrophilic groups to lipophilic groups, and vice versa. The most common emulsifiers used in food are non-ionic and their HLB values are listed in Table 1 (summarized from hydrophile-lipophile balance numbers chart of Sigma-Aldrich catalog). Each emulsifier has its unique molecular structure and shows different phisicochemical property during emulsion formation (Krog, 1997; Faergemand and Krog, 2003). So it is important to select the most suitable emulsifier for each particular system.

Normally a low HLB value (3.5-8) emulsifier is mostly soluble in oil and stabilize W/O emulsion; a high HLB value (8-16) emulsifier is mostly soluble in water and stabilize O/W emulsion; an intermediate HLB value (7-9) emulsifier doesn't have particular preference to water or oil, and normally act as wetting or spreading agent (McClements, 2005).

Two or more emulsifiers can be mixed and get a new HLB number. The method to calculate HLB value of mixture of A, B, C, X% of emulsifier A (HLB = A), Y% of emulsifier B (HLB = B), and Z% of emulsifier C (HLB = C), is by the following formula.

$$HLB(A,B,C) = (AX + BY + CZ) / (X+Y+Z)$$

Table 1. HLB values of some food grade emulsifiers (Sigma-Aldrich, Inc)

Emulsifier	HLB
Sorbitan trioleate (Span 85)	1.8
Sorbitan tristearate (Span 65)	2.1
Glyceryl monostearate, NF	3.8
Sorbitan monooleate, NF, (Span 80)	4.3
Sorbitan monostearate, NF, (Span 60)	4.7
Sorbitan monopalmitate, NF, (Span 40)	6.7
Sorbitan monolaurate, NF, (Span 20)	8.6
Polyoxyethylene sorbitan tristearate, (Tween 65)	10.5
Polyoxyethylene sorbitan trioleate, (Tween 85)	11.0
Polyethylene glycol 400 monostearate	11.6
Polysorbate 60, NF, (Tween 60)	14.9
Polyoxyethylene monostearate (Myrj 49)	15.0
Polysorbate 80, NF, (Tween 80)	15.0
Polysorbate 40, NF, (Tween 40)	15.6
Polysorbate 20, NF, (Tween 20)	16.7

1.3.3. Emulsion stabilizers

Weighting agents are commonly used in beverage to increase the specific gravity of flavor oil. They are oil soluble and have high specific gravity (>1 g/cm3). However, they do not have undesirable flavor, odor or color, and must be approved to use in food. Ester gum, sucrose acetate isobutyrate (SAIB), Damar gum, and brominaed vegetable oils (BVO) are often used in soft drinks (Tan, 2004).

Some polysaccharides have high emulsifying property, such as gum Arabic, modified food starch, and gum Tragacanth. They can be used independently as emulsifiers or conjuncted with other surfactants as emulsifiers and stabilizer (Tan, 2004). Gum Arabic is reported to have HLB value of 8.0 (Chun, et. al., 1958) as emulsifier. Purity Gum 1773 and Purity Gum 2000 from National Starch and Chemical Company can also be used in beverage as emulsifier and stabilizer similar to gum Arabic (Gerlat, 2000).

Thickening agents were used in O/W emulsion to increase the viscosity of the aqueous phase (Mitchell and Ledward, 1986). Natural or food grade polymers, such as polysaccharides and proteins, are often used for this purpose (McClements, 2005).

1.3.4. Multiple Emulsions

The most seen multiple emulsion is double emulsion. Depending on how water and oil phases are located, double emulsions can be divided into two types, water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O). Here water is referred as the polar phase and oil is the less polar phase, and they are not immiscible (Matsumoto and Kang, 1989; Salager, 2004).

1.3.4.1. Multiple Emulsion Formation

Double emulsions have been reported to be prepared by several different methods, through phase inversion (Seifriz, 1925; Matsumoto et al., 1985), one-stage emulsification (Matsumoto et al., 1987), two-stage emulsification (Matsumono, 1985; Matsumoto, 1989), membrane emulsification (Okochi and Nakano, 1996; Mine et al., 1996), and microchannel emulsification (Sugiura et al., 2004).

Two steps emulsification is the most common method for preparing double emulsion. The method is relatively easy, highly efficient, and highly reproducible. The first step is to produce an ordinary O/W or W/O emulsion using a hydrophilic or lipophilic emulsifier, respectively. The first stage emulsion, O/W or W/O is considered as one inner phase, and can be re-emulsified into oil or water, respectively. The ratio of hydrophilic emulsifier to lipophilic one existing in the system influences the formation, yield, and physicochemical properties of the double emulsion system (Matsumoto and Kang, 1989; Khan, et al., 2006).

Phase inversion is another popular method to prepare W/O/W emulsion. This one-step method is firstly reported by phase inversion of concentrated W/O emulsion to W/O/W emulsion (Matsumoto, 1983; Matsumoto, et al., 1985). The procedures are described as follows: 1. Dissolve hydrophilic emulsifier into aqueous phase; 2. dissolve lipophilic emulsifier into oil phase; 3. the aqueous solution is then introduced into the defined volume of oil phase successively under mixing. Intimately a W/O emulsion is formed, and the oil is the continuous phase. When the volume fraction of hydrophilic water phase exceeds 0.7, the continuous phase is replaced by water. The oil vascular globules become stable when the volume fraction of water is 0.75 (Matsumoto and Kang, 1989). Emulsion systems prepared by this method always generate a wide distribution of

globule size and indefinite number of encapsulated compartments (Hou and Papadopoulos, 1997).

1.3.4.2. W/O/W Emulsions as Delivery System

1.3.4.2.a. Parenteral Administration

W/O/W double emulsion has been used in parenteral formulation in water soluble drug target delivery system (Florence and Whitehill, 1982) through multiple administration pathways. The advantage of W/O/W delivery system includes fast absorption in intramuscular delivery (Porter and Charman, 1997), and high bioavailability of drug in intravenous delivery (Kanke, et al., 1980; Vyas and Khar, 2002). The mechanism of metabilization of W/O/W emulsions is that macrophages of reticuloendothelial system (RES) engulf the emulsions rapidly and transport to lymphatics node and liver (Khopade and Jain, 2001; Vyas and Khar, 2002).

1.3.4.2.b. Oral Administration

It has been reported that W/O/W emulsion increased the bioavailability of griseofulxin in oral delivery (Onyeji et al., 1991). The double emulsion system can also reduce the adverse effect of drug by avoiding direct contact of drug to gastrointestinal wall (Onyeji and Omotosho, 1993). After oral administration, the W/O/W emulsions are recognized as lipids in stomach and small intestine. The lipids are hydrolyzed to fatty acids and absorbed into enterocyte or directly to intestinal macrophage system. Then they are transported to systemic circulation (Porter and Charman, 1997). The delivery systems carry the instable water soluble drugs through the harsh pH, ionic and enzymatic environments in the gastrointestinal tract (Charman, 1992). The mechanism follows more of lipid digestion and transportation (Tso, 1994).

Multiple emulsion increases bioavailability of drug by reducing the first-pass metabolism in liver to the drug. The investigated drugs include insulin (Suzuki, et al., 1998; Cournarie, et al., 2004), griseofulvin (Onyeji, et al., 1991), isoniazid (Khopade and Jain 1998a; 1998b), vancomycin (Shively and Thompson, 1995), nitrofurantoin (Onyeji and Omotosho, 1993; Onyeji and Adesegun, 1995) and Pyrenetetrasulonic acid tetrasodium salt (PTSA) (Shima, et al., 2005). Other advantages of using W/O/W double emulsion for oral delivery include the ability to modulate drug absorption rate and applicability to lymphatic targeting drugs (Khan, et al., 2006).

1.3.5. Advantages of Lipid Based Delivery System

O/W and W/O/W emulsions are considered as lipid-based delivery systems. The most widely used lipid-based delivery systems are emulsion and liposome. They have been reported to enhance intestinal drug solubilization. They can accommodate not only water-soluble but also lipid soluble materials (Porter et al., 2008). It has been reported that emulsion increased the bioavailability of griseofulxin in oral delivery (Onyeji et al., 1991). After oral administration, the emulsions are recognized as lipids during digestion. The lipids can be hydrolyzed to fatty acid and absorbed into either enterocyte or directly to intestinal macrophage system, and then transported to systemic circulation (Porter and Charman, 1997). During lipid vesicular digestion, co-administration of drug may partition in gastrointestinal tract. The system also provides a reservoir of solubilized drug in intestinal melieu for absorption. Therefore, the systems potentially alter the drug uptake, and efflux disposition. They may also change the pathway and reduce the first-pass metabolism (Porter et al., 2007). Multiple emulsions have been utilized to increase

the bioavailability of drug by reducing the first-pass metabolism in liver to the drug (Cournarie, et al., 2004; Onyeji, et al., 1991).

1.4. Inflammation

Inflammation is the chain reaction of immune system responding to bacterial infection and/or tissue injury (O'byrne and Dalgleish, 2001). The harmful factors, including bacterial infection, injury, trauma, ultraviolet light irradiation, and non-digestive particles activate an innate immune response (Parham, 2000). Inflammation can be classified as acute and chronic. The acute inflammatory responding to infection or trauma is a defense mechanism of the body. The blood flow increases and moves plasma and leukocyte to the site to kill bacteria and virus, or facilitate wound repairing. The prolonged chronic inflammation often results in tissue destruction and fibrosis. Prolonged inflammation also increases the risk of developing certain degenerative diseases (O'Byrne, et al., 2000), such as lung cancer (Shukla et al., 2003), stomach cancer (Farinati et al., 2003), and skin cancer (Sluyter and Halliday, 2001).

The infection or injury activates immune cells and increases the expression of pro-inflammatory cytokines, tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). The cytokines thus up-regulate the expression of cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and other enzymes to produce reactive oxygen species (ROS). Persistent and prolonged inflammation may lead to chronic disorder such as cardiovascular disease, arthritis, degenerative diseases, and cancer (Dalgleish and O'byrne, 2002). Natural phenolic compounds inhibit inflammation by blocking nuclear factor kappa B (NFκB), down regulation of the over expression of pro-inflammatory factors and enzymes, and acting as ROS scavenger (Huang et al., 2004).

1.5. Enzyme Encapsulation

Enzymes are catalytic biomolecules, which have the advantages of great specificity and reactivity under physiological conditions. Like most of the proteins, they are highly susceptible to physiological parameters such as pH, heat, and chemical denaturation agents. There is always a lot of interest in developing techniques for utilizing and recovering enzymes in food, chemical, and biotech industries. Encapsulation, a process involving coating or entrapment of a pure material or a mixture into another material, can enhance the catalysis capability, chemical and thermal stability of enzymes (Kayes, 1977).

Polysaccharides and proteins are the two key components in both natural and processed foods. In solutions, charged polysaccharides and proteins may undergo two types of phase separation at above or below the isoelectric points of proteins: the solid-liquid phase separation called precipitation (Nguyen, 1986; Kokufuta, et al., 1981), and the liquid-liquid phase separation called coacervation (Lenk and Thies, 1987; Burgess and Carless, 1984). Coacervates formed by polysaccharides and oppositely charged proteins have been widely used in cosmetic formulations (Goddard, 1990), and microencapsulation of food ingredients, cells (Gibbs, et al., 1999), and pharmaceuticals (Kayes, 1977; Deasy, 1984). The studies of enzymatic activity of enzymes in protein/polyelectrolyte complexes have been carried out previously (Tibackx, 1911; Bungenber, 1949). For example, Larionova et al. have used protein-polyelectrolyte complexes to increase the thermal stability of proteins (Tibackx, 1911); Morawetz et al. and Kokufuta have studied the trypsin activity in hemoglobin/polyacrylic acid complexes (Bungenber, 1949) and trypsin/poly-(vinyl alcohol sulphate) complexes, respectively

(Sanchez and Paquin, 1997). Later, Xia and co-workers showed activity retention of alcohol dehydrogenase (ADH) when the ADH was in the coacervate form (Xia et al., 1993). Most of the researches so far focused on the studies of enzyme activity in the complex form, very few work has been done in understanding the activity of the enzyme after being released from the complexes. Enzymes are catalytic proteins that are capable of binding to oppositely charged polysaccharides and forming stoichiometric complexes. During the process of complex formation, enzymes are trapped on the polysaccharide charge surfaces. A fundamental question needs to be addressed is whether stoichiometric complexation has a strong influence on the active binding sites of the enzyme.

Chapter 2

Objectives and Hypotheses

2.1.1. Objective 1 – Enhance polyphenol bioavailability through single or double emulsion encapsulation

Create the multiple emulsion systems of green tea catechin and curcumin through various methods: using coacervate to encapsulate W1/O emulsion in water; double emulsion stabilized with polysaccharide; single emulsion to trap polyphenols. Study the physio-chemical properties of the systems. Reduce particle sizes, increase emulsion stability, and measure the encapsulation efficiency and *in vitro* release of polyphenols. Study anti-inflammatory activity of encapsulated polyphenols *in vivo*.

2.1.2. Hypothesis 1

Catechins and curcumin as anti-inflammation/anti-cancer polyphenols can be encapsulated in multiple emulsion systems. Emulsifier-stablized emulsion and coacervate-stabilized emulsions can increase the bioavailability of polyphenols through oral administration to animals. With a higher bioavailability, the encapsulated polyphenols should show enhanced anti-inflammation and anti-cancer activities. The pro-inflammatory factors should be inhibited. The biodistribution pathway and pharmacokinetics may be different from the free polyphenols.

2.2.1. Objective 2: Enhance enzyme activity through coacervate encapsulation

Use complex coacervates to encapsulate α-amylase. Study enzyme/polysaccharide coacervation characteristics. Compare the stability of encapsulated enzyme and free enzyme under acidic pH environment. Evaluate enzyme activity and kinetics after being released from capsules.

2.2.2. Hypothesis 2

α-Amylase as an enzyme can be encapsulated through the formation of coacervate with polysaccharides. The enzyme will be stabilized during coacervation. The encapsulation method will not destroy the enzyme activity, and can protect the enzyme from the harsh denaturing factors.

Chapter 3

Materials and Methods

3.1 Tea Catechins Encapsulated by Coacervates

3.1.1. Materials

Green tea extract, containing 98% catechins, 0.2% Caffeine was obtained from Indfrag Ltd. (India). Gelatin Type A was purchased from Fisher Scientific, (Fair Lawn, NJ). Kappa (κ), iota (ι), and lambda (λ) carrageenan, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and furcelleran was a gift from FMC biopolymer (Princeton, NJ, USA). Artificial Gastric Juice was made of 4g NaCl, 3.2g Pepsin A from porcine stomach mucosa, 7ml hydrochloric acid (1N) per liter to reach pH 1.5. Artificial Intestinal Juice was made of 15g sodium bicarbonate, 5.2g bile salt, 0.4g trypsin, 0.2g chymotrypsin, 0.006g elastease, 0.2g lipase per liter (pH 8.5). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water (18.3 MΩ) was used throughout the experiments.

3.1.2. Methods

3.1.2.1. Coacervation

Turbidimetric Titration Turbidity measurements were made on Brinkmann PC910 colorimeter equipped with a 2.0cm path-length fiber optic probe. 0.5g/L furcelleran or carrageenans, and gelatin were dissolved in saline solution separately in 0.01, 0.05, 0.1, 0.2 and 0.5mol/l NaCl salt solutions. Protein and polysaccharide were mixed at different ratios and solution pH values were adjusted to above 9.5. “Type I” titrations were carried out at room temperature by adding 0.05M HCl using a micrometer buret to a protein-polyelectrolyte solution, and recording T%. A protein blank correction was made under the same conditions in the absence of polyelectrolyte. Then particle sizes of each

individual polymer and the soluble complex were measured by using a dynamic light scattering (DLS)-based BIC 90 plus particle size analyzer (Brookhaven Instrument Corporation, New York, NY, USA). In addition, particle sizes during complex formation of BSA and kappa-carrageenan were measured.

3.1.2.2. Catechine Encapsulation

The suitable carrageenan and optimum conditions were chosen from turbidimetric titration phase diagrams of gelatin-A and four type of carrageenans. 0.25 g of tea catechins were dissolved in 1ml 100% ethanol, and further diluted 5 times into water, which generated 10% w/v catechins concentration. 5% sorbitan monostearate was mixed well in canola oil. Catechins solution (15% w/w) was added dropwise into oil under homogenization at 13,500rpm, and continued to homogenize for another 20 min to form stable W/O emulsion. 1.5 ml of emulsified catechins was mixed into 50 ml of 0.5g/l gelatin-A, and then 50 ml of 0.5g/l k-carrageenan was added. Under magnetic stirring, pH was adjusted to 3.3 to form coacervate and encapsulate catechins. The coacervate suspensions were then examined under inverted optical microscope (Nikon TE 2000, Nikon Corporation, Japan). Coacervate encapsulated catechins were carefully centrifuged at 2,000rpm for 5min to separate from water phase. Catechins in both phases were extracted by acetone. Encapsulation efficiency was determined by measuring catechin concentration using HPLC.

3.1.2.3. In vitro Release

The separated catechin coacervate phase was incubated in artificial stomach juice for 2h at 37°C with magnetic stirring. Then the coacervate was removed through centrifuging at 2,000rpm for 5min. The released catechins in the stomach juice

supernatant could be detected by HPLC. The catechin coacevates were then transferred into artificial small intestine juice and incubated for 4h at 37°C under magnetic stirring. Since the quick high-speed centrifuge may destroy the double emulsion and release catechins, the release kinetics was determined by free gelatin-A increase in the solution, which measured the dissociation of coacervate. During incubation, 1ml small intestinal juice mixture was taken out at 0.5, 1, 2, 4, 8, 12, 18, 22 min out and rapidly centrifuged at 13,000rpm for 1 min. The concentration of gelatin-A was measured at OD280 by Cary 50 Bis UV-Visible spectrophotometer (Varian Optical Instruments, Walnut Creek, CA). The incubation and sample taking steps were performed in quadruplicate. At the end of each coacervate dissociation, the incubation mixtures were centrifuged at 2,000 rpm for 5 min, and the final concentrations of catechins in the supernatant of small intestine juice were determined by HPLC.

3.2. Catechins Encapsulated by W/O/W Double Emulsion

3.2.1. Materials

Green tea extract, containing 98% catechins and 0.2% caffeine was obtained from Indfrag Ltd (India). Purity 2000 Starch was provided by National Starch & Chemical Corp. (Bridgewater, NJ, USA), and TIC PRETESTED® gum Arabic spray dry FCC powder was obtained from Tic Gums (Belcamp, MD, USA). Brominated oil with specific gravity 1.329 g/ml was given by Virginia Dare Inc. (Brooklyn, NY). Canola Oil (Hunt-Wesson Inc., Fullerton, CA) was purchased from a local supermarket. Ethanol and triacetin were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Poly-(ethylene glycol) of molecular weight 200 and 400, Tween 20, Tween 40, Tween 80,

Tween 85, Span 20, Span 80, Span 85 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water (18.3 MΩ) was used throughout the experiments.

3.2.2. Methods

3.2.2.1. Maximum Solubility of Catechins in Different Solvent

Tea catechins were dissolved in 10% ethanol in water, 10% PEG 200 in water, 10% PEG 400 in water or water-saturated triacetin under nitrogen flush. To get the maximum solubility, catechins were added and mixed with the above three solvents to reach the saturated concentrations. The solvent with the highest catechins solubilities was used for the next step.

3.2.2.2. Optimum HLB Value and Mixing Ratio for Catechins Solution in Canola Oil (W/O) Emsulsion

Tween 80, Tween 85, Span 20, Span 80, Span 85 were minxed according to table 2 to obtain emulsifiers with specific HLB values for water in oil (W/O) and oil in water (O/W) emulsions. 1 g of emulsifier A, B, C, D, E, F, or G was mixed with 9g of canola oil. The oil phase was homogenized under high-speed homogenizer (ULTRA-TURRAX T-25 basic, IKA Works, Inc., Willmington, USA) at 13,500 rpm, and 3g catechines in triacetin and catechins in ethanol/water (made from previous step) were added dropwise, and continuously stired for another 5 min. The samples were prepared in triplicate and stored at 4°C, 25°C, and 45°C to monitor the stability. The most stable emulsion formultaion based on percentage of phase separation was chosen for further study.

Canola oil (specific gravity 0.920 g/ml) was mixed with brominated oil at the ratio of 65.6%:34.4% to reach the final specific gravity of 1.06 g/ml. The resulted mixed oil was then added with 10% of emulsifier of the optimum HLB value. Subsequently, the

mixture of catechin/triacetin hydrophilic phase and oil phase at the ratios of 1:3, 1:4 and 1:5 were emulsified to form W/O emulsions. The optimum water to oil ratio was decided based on stability of the emulsion.

For the same HLB value, emulsifier mixtures containing different emulsifiers showed different emulsion ability. Emulsifiers P, Q, R, S, T and U from table 2 were tested in this W1/O emulsion to optimize the condition.

3.2.2.3. Optimum System for Catechins/Canola Oil in Water (W1/O in W2)

The stable W/O system was determined from previous step. Here is named as catechin-triacetin/canola-brominated oil W/O phase. This phase was further emulsified into water with 5% of emulsifier H, I, J, K, L, M, N or O; with 5% or 10% purity 2000 modified starch or gum Arabic; with 5% purity 2000 modified starch or gum Arabic and 5% emulsifiers H, I, J, K, L, M, N, O, Tween 80, Tween 40, or Tween 20. The W/O to W ratio used in this test was 1:3 and performed with homogenization at 9,500 rpm for 2 min. The system was selected based on stability at 4°C.

Once the optimum emulsion system was decided, W1/O to W2 phases at different ratios were tested. Ratios were selected as 1:3, 1:2 and 1:1.

3.2.2.4. Particle Sizes Reduction and Encapsulation Efficiency

The optimum emulsion formulation system was selected from the previous steps. Catechin was dissolved in triacetin first and further emulsified into canola-brominated oil containing 10% emulsifier1. The emulsion was performed under vertex for 180s, and then homogenized at 6,500 rpm, 9,500 rpm, 13,500 rpm, or 17,500 rpm for 5 min.After vertex for another 120s the emulsions were homogenized at 13,500 rpm for 5 min. The particle sizes of the emulsions were measured under inverted optical microscope (Nikon

TE 2000, Nikon Corporation, Japan) at 1hr, 4h, 8h, 12h and 24h after emulsion preparation.

The double emulsion was centrifuged at 6,500 rpm for 4 min to separate W1/O and W2 phases. The unencapsulated catechin concentration in the W2 phase was measured by HPLC to calculate encapsulation efficiency.

Table 2. The HLB values of different emulsifiers and their mixtures

Emusifier Percentage					HLB after mix	Emulsifier Label
Span 85 HLB 1.8	Span 80 HLB 4.3	Span 20 HLB 8.6	Tween 85 HLB 10.5	Tween 80 HLB 15		
20%	80%				3.80	A
	100%				4.30	B
	95%			5%	4.83	C
	90%			10%	5.30	D
	85%			15%	5.90	E
	80%			20%	6.44	F
	75%			25%	7.00	G
		75%		25%	10.20	H
		60%		40%	11.16	I
		45%		55%	12.12	J
		30%		70%	13.08	K
		15%		80%	14.04	L
			65%	35%	12.08	M
			50%	50%	12.75	N
			40%	60%	13.2	O
	70%	30%			5.59	P
	65%	35%			5.80	Q
	60%	40%			6.02	R
	55%	45%			6.23	S
	82.5%			17.5%	6.17	T
	87.5%			12.5%	5.64	U

3.3. Curcumin Encapsulation

3.3.1. Materials

Curcumin (85% pure, with 11% of demethoxycurcumin and 4% of bisdemethoxycurcumin as impurities) was a gift from Sabinsa Corp. (Piscataway, NJ, USA) and used without further purification; Medium chain triacylglyceride (MCT) (Neobee^{TM} 1053) was obtained from Stepan Corp. (Northfield, IL); Tween 20, Tween 40, Tween 80, Span 20, Span 80, and all other chemicals were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodes for IL-6 (sc-1265) IL-4 (sc-12723) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for cyclin D1 (13-4500) was from Zymed (South San Francisco, CA). Secondary antibody against goat (605-732-002) and secondary antibody against mouse (A-21058) were purchased from Rockland (Gilbertsville, PA) and Invitrogen (Carlsbad, CA), respectively. Milli-Q water (18.3 MΩ) was used throughout the experiments.

3.3.2. Methods

3.3.2.1. Preparation of Emulsion

MCT containing Span 20 was first heated to 100ºC under nitrogen flush to dissolve curcumin using magnetic stirring. After curcumin was fully dissolved in MCT, the oil was cooled down to room temperature, and immediately homogenized into water emulsified with Tween 40 with high-speed homogenizer (ULTRA-TURRAX T-25 basic, IKA Works, Inc., Willmington, USA) at 13,500 rpm for 5 min. Then the emulsions were transferred to Emulsiflex C-3 high-pressure homogenizer (Avestin Inc., Ottawa, Canada), and homogenized for three cycles. Emulsion prepared with the same procedure but without the incorporation of curcumin was used as the control.

3.3.2.2. Emulsion Optimization

The previous emulsion preparation steps were repeated with various emulsifiers. By combining different emulsifiers, for example, Span 20 or Span 80 with Tween 20, Tween 40, or Tween 80, respectively, emulsifiers of different HLB values can be achieved. The stability of emulsions was evaluated by comparing the appearance of phase separation during storage at room temperature. The most stable emulsion was chosen as the formulation for further anti-inflammatory research.

3.3.2.3. Particle Size Determination

After high-speed homogenization, the emulsions (20× dilution) were observed under inverted optical microscope (Nikon TE 2000, Nikon Corporation, Japan) with scale ruler. The average diameter and size distribution of emulsions after two stages of homogenization were determined by using dynamic light scattering 90 plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlatior (Brookhaven Instrument Corporation, New York, NY, USA). The light source of the particle size analyzer is a solid state laser operating at 658 nm with 30 mW power, and the signals were detected by a high sensitivity avalanche photodiode detector. All measurements were made at a fixed angle of 90˚ and temperature of 25.0 ± 0.1˚C. The emulsion suspensions were diluted 2500 times with water. Each measurement was in triplicate. The normalized field-field autocorrelation function *g(q,t)* is obtained from the intensity-intensity autocorrelation function, *G(q,t)*, via the Sigert relation (Stepanek, 1993):

$$\alpha\, g(q,t) = [G(q,t)/A - 1]^{1/2} \qquad (1)$$

where A is the experimentally determined baseline, α is the contrast factor which is less than 1, due to the fact that only a fraction of dynamic scattering intensity falls within the

correlator window and also the fact that a finite size pinhole is used in the experiment. For all particle size measurements, the measured baseline A is in agreement with the theoretically calculated baseline to 0.01%.

Two procedures were used to analyze the *g(q,t)* versus *t* data. The first data analysis method utilized in this paper was William-Watts (WW) single stretched exponential function given by

$$g(q,t) = \exp[-(t/\tau)^{\beta}] \tag{2}$$

Here β is a parameter that describes the polydispersity of diffusing particles. For monodisperse emulsion droplets, $\beta=1$ is expected; for a polydisperse system, $0<\beta<1$.

The diffusion coefficient D was calculated according to $D=\tau^{-1}q^{-2}$, where *q* is the amplitude of scattering vector defined as $q=(4\pi n/\lambda)\sin(\theta/2)$, n is the solution refractive index, λ is the laser wavelength and θ is the scattering angle. The diffusion coefficient *D* can be converted into mean emulsion droplet diameter d using the Stokes-Einstein equation:

$$d = \frac{kT}{3\pi\eta D} \tag{3}$$

where *k* is the Boltzmann constant, *T* is the absolute temperature, and η is the solvent viscosity.

The second method used was Cumulant analysis, where *g(q,t)* was decomposed into a distribution of decay rate $\Gamma(=1/\tau)$ given by

$$g(q,t) = \int G(\Gamma)e^{-\Gamma t}d\Gamma \tag{4}$$

The first two moments of the distribution *G(Γ)* are as follows:

$$\Gamma = Dq^2 \tag{5}$$

$$\mu_2 = (D^2 - D*^2)q^4 \quad (6)$$

Where $D*$ is the average diffusion coefficient. The polydispersity term defined in the Cumulant analysis is:

$$Polydispersity = \mu_2 / \Gamma^2 \quad (7)$$

Here *polydispersity* has no unit. It is close to zero for monodisperse or nearly monodisperse samples, and larger for broader distribution.

3.3.2.4. HPLC Assay

The concentration of curcumin was determined by using a HPLC system (Dionex Ultimate 3000) equipped with an auto-sampler, a Dionex Bio LC AD25 UV/Vis Detector (Dionex Corp., Sunnyvale, CA, USA), and a C-18 column (Luna, 3U, 4.6×150mm, Phenomenex, Torrance, CA, USA). The data were quantified with Dionex Chromeleon chromatography management system software. The mobile phase consisted of HPLC-grade water with 2% acetic acid (A) and acetonitrile (B). The solvent gradient was programmed linearly from 65% A: 35% B to 30% A: 60% B from 0 to 30 min, and back to initial concentration for an additional 1 min. The flow rate was 1.0mL/min, the detection wavelength was 425nm, and the injection volume was 10ul. A series of standard acetone solutions, 0.002%, 0.005%, 0.01%, 0.02%, 0.05%, and 0.1% of curcumin were diluted from 1% curcumin in acetone solution. Pure acetone was used for blank calibration. The standard curve of absorption peak area versus curcumin concentration was plotted and fitted by a linear function. The measurement of each stock and working solution was triplicated.

3.3.2.5. Encapsulation Efficiency

The Encapsulation efficiency was defined as the percentage of entrapped curcumin over the total curcumin in the system. Free curcumin was removed from freshly made emulsion using the following steps. 1.2g emulsion in an eppendorf tube was centrifuged at 6,500 rpm for 5 min and the supernatant was removed. 1mL water was added into the tube to loose the precipitate, and centrifuged again at 6,500 rpm for 8 min. The supernatant was discarded and the previous step was repeated. After the removal of supernatant and 1g of acetone was added into the tube to fully dissolve the precipitate. The solution was diluted 10 times with acetone for HPLC analysis.

3.3.2.6. Stability of Emulsions

The emulsions were stored in plastic tube at 4˚C. Following the procedures on measuring encapsulation efficiency described in 3.3.2.5, curcumin emulsion stability was evaluated after 1h, 28h, 60h, 84h, and 132h of storage.

3.3.2.7. Anti-inflammatory Activity

The procedure was described previously (Huang et al. 2006). Female CD-1 (4-5-week old) mice were purchased from Charles River Breeding Laboratories (Kingston, NY, USA). The mice were kept in our animal facility for at least 1 week before use. Mice were provided with water and Purina Laboratory Chow 5001 diet (Ralston-Purina, St. Louis, MO, USA) *ad libitum.* They were kept on a 12-h light, 12-h dark cycle.

Female CD-1 mice were orally administered of vehicle emulsion (emulsion containing no curcumin as control) or curcumin emulsions 10 min prior to the topical application of 10ul of acetone (control) or 1.0 nmole TPA in acetone on both ears. The mice were sacrificed after 6hater TPA treatment by cervical dislocation. Ear punches of 6

mm in diameter were taken and weighed. Data were averaged from 8 ears per group. Ear samples from each group were pooled to homogenize in PBS buffer, and quantified for the levels of inflammatory mediators using ELISA described as following.

Ear tissues were homogenized in a PBS containing 0.4M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1mM PMSF, 0.1mM benzethonium, 10mM EDTA and 20U aprotinin per ml. The homogenates were centrifuged at 12,000×g for 60 min at 4˚C. The supernatant was used for the determination of cytokine protein levels. A two-site sandwich ELISA was used for cytokines assays.

3.3.2.8. Enzyme-Linked ImmunoSorbent Assay (ELISA)

The levels of the cytokines, IL-1β and IL-6, were quantified using ELISA kit purchased from Biosource International (Camarillo, CA, USA). The IL-1β and IL-6 ELISA kits follow the same basic procedure. The capture antibody, diluted with PBS, was used to coat a 96-well plate overnight at room temperature. The plate was then washed, blocked (1% BSA, 5% sucrose in PBS with 0.05% NaN_3), and washed again. The standards were added to the plate leaving at least one zero-concentration well and one blank well. The diluted samples (1:3 – 1:8) were then added to the plate. After incubation for 2h, the plates were washed and the detection antibody was added. After incubation for another 2 h the plates were washed and Streptavidin-HRP was added. After 20-min incubation, the plates were washed, and H_2O_2 substrate and tetramethylbenzidine were added. After another 20-min incubation, the stop solution (2 N H_2SO_4) was added and the plates were read with a microplate reader at a wavelength of 450nm. Statistical analyses were performed using the Student's *t*-test.

3.3.2.9. Western Blot

Protein concentration was measured with a Protein Quantification Kit from Dojindo Molecular Technologies. The same amount of protein samples was then mixed with 2x SDS loading buffer, respectively, and treated in boiling water for 3 min. Twelve percent SDS-PAGE gels were prepared according to a standard protocol from Molecular Cloning (Sambrook, et al., 1989). After loading to gel, samples were resolved with 1x Tris-Glycine-SDS running buffer (Boston BioProducts, Worcester, MA) and then transferred to a nitrocellulose membrane with 1x transfer buffer (Boston BioProducts, Worcester, MA). The membrane was then blocked with the blocking buffer from Odyssey system (LI-COR biosciences, Lincoln, NE) for 1 h at room temperature. The primary antibody diluted in the same blocking buffer was added to detect the target band. After incubation at 4°C overnight, the membrane was washed with 1xTBST (0.05% Tween-20/Tris-buffered saline) and then added with the secondary antibody. Finally, the target band was recognized with an Odyssey infrared imager.

3.4. Enzyme Encapsulation

3.4.1. Materials

κ-Carrageenan and α-amylase (from bacillus amyloliquefaciens) were purchased from Sigma-Aldrich Co. (St. Louis, MO), Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA), potato starch was purchased from Fisher Scientific (Fairlawn, NJ).Iodine reagent was made by diluting 1.25g potassium iodide in 20ml DI water, followed by the addition of 125mg iodine. The volume was then adjusted to 25ml as stock. The working iodine reagent was diluted 100 times dilution of stock solution. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q water (18.3 MΩ) was used throughout the experiments.

3.4.2. Methods

3.4.2.1. Turbidimetric Titration.

κ-Carrageenan and α-amylase were dissolved separately in sodium chloride solution at appropriate concentrations. pH was adjusted to above 8 with 0.1M sodium hydroxide before mixing two solutions. Turbidity measurements were made on a Brinkmann PC910 colorimeter equipped with a 2.0cm path-length fiber optics probe. "Type I" titration was carried out at 25°C by adding 0.05M hydrochloric acid with a micrometer buret to the protein-polyelectrolyte mixed solutions, and the turbidity was recorded (100 - T%). A protein solution prepared under the same conditions in the absence of polysaccharide was used as the blank.

3.4.2.2. Preparation of κ-Carrageenan & α-Amylase Complex Coacervates

Equal volume of 0.5g/L κ-carrageenan and 1.0g/L α-amylase was mixed and the pH was adjusted to 3.3 with the addition of 0.05M hydrochloride dropwise. Particle size increases during titration was measured with 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). The coacervate was collected through centrifugation at 3,000 rpm for 10 min. The free α-amylase and coacervate-encapsulated amylase were treated with pH 3 acidic buffer for 30 min, followed by the removal of acid and the release of coacervates by adjusting pH to 8.

3.4.2.3. Determination of Encapsulation Efficiency and Enzyme Activity

coacervates of κ-carrageenan and α-amylase were formed at pH 3.2. After removing α-amylase/κ-carrageenan coacervate through centrifuge, free α-amylase in the solution was determined by Bio-Rad protein assay. Thus the encapsulation efficiency was calculated as percentage of coacervate encapsulated enzyme over the total enzyme. Next, the solution pH was adjusted to 6.9, which was the optimum catalytic pH of α-amylase.

Free or released enzyme was then added to 1% pre-gelatinized potato starch solution and digested at room temperature. 100ul sample was taken out in every 30 sec, and mixed with 1ml iodine reagent. The blue color of starch-iodine complex was measured at OD 620nm by Cary 50 Bis UV-Visible spectrophotometer (Varian Optical Instruments, Walnut Creek, CA). The digestion rate was determined with the disappearance of starch in solution.

Under the same conditions, digestion rates of the enzyme were studied at the concentrations of 0.2, 0.4, 0.6, 0.8 and 1g/L free and released encapsulated α-amylase. Lineweaver-Burke Plot was made by plotting 1/[starch concentration] against 1/[digestion rate]. According to Michaelis-Menten equation, $1/r = K_m/r_{max}[S] + 1/r_{max}$. Here K_m is the Michaelis-Menten constant and r_{max} is the maximal hydrolysis rate. K_m and r_{max} were determined to compare the enzyme kinetics of α-amylase before and after encapsulation and acid treatment.

Chapter 4

Results and Discussions

4.1. Tea Catechins Encapsulated by Coacervates

4.1.1. Turbidimetric Titration

A series of turbidmetric titrations were studied first to characterize the properties of gelatin-A and carrageenan coacervation. Food grade gelatin-A and four types of carrageenans, furcelleran, κ-carrageenan, ι-carrageenan and λ-carrageenan, were tested for the coacervate formation. Carrageenans contain sulfate groups, which give the polymer negative charges in solution. The more sulfate side chains a polysaccharide has, the more negative charges it presents, and the stronger the interaction it forms with proteins.

Firstly, the interactions between gelatin-A and four kinds of carrageenans were studied. Gelatin-A is a non-globular protein. During the coacervate formation, it forms more stable complexes with carrageenans than globular proteins. Coacervate formation between gelatin-A and κ-carrageenan was studied under different physicochemical conditions. At a 0.1M NaCl solution (result not shown), which is a common ionic strength for coacervation study. But at this ionic strength, iota and lammda carrageenan strongly interacted with gelatin-A even at pH above 12. As NaCl concentration was increased to 0.2M the interactions between protein and polysaccharide decreased, since the salt has a screening effect to the polymers and weakens the electrostatic interaction between proteins and carrageenans. At 0.2M NaCl solution, λ-/ι-carrageenan would not interact with gelatin A at above pH 9, which was essential for later encapsulation application. But furcelleran could not establish enough interactions with the protein at

this ionic strength. Hence, a 2:1 gelatin-A:furcelleran ratio was used for further comparison study.

In the turbidimetric titration curves shown in Figure 4, it is found that the interactions between carrageenans and gelatin-A started at pH above 7.5, which is higher than the isoelectric point (pI) of gelatin-A. At pH above pI, the whole protein is negatively charged, but some parts of the protein may be positively charged. These positive charges are like patches on the protein surface and can interact with the negative charges of carrageenan. As the pH decreased, the total positive charges on the protein increased. Thus, the interactions between protein and polysaccharide increased. At this stage, the interactions were between the positively-charged patches on protein and polysaccharide, and the total net charges of the complexes still remained negative. There was still some electrostatic repulsion between complexes, and the densities of these complexes were not high enough, therefore soluble complex was formed under this condition. Protein and polysaccharide could not form precipitate due to the electronic repulsions between protein and polysaccharide.

When the pH of the mixed solutions was adjusted to pI, the turbidity of the solution increased dramatically due to the global phase separation. Further decrease of pH caused the increase in positive charges on the protein. At pH 3 the turbidity curve reached a plateau, suggesting the formation of maximum gelatin-A/carrageenan complex coacervates. The formation of coacervate was not found to increase with the increase of positive charges.

The coacervate suitable for encapsulation should have high coacervate yield and controllable formation/dissociation process. Interactions between gelatin-A and λ-/ι-

carrageenan were too strong and the coacervate dissociation process was too fast, due to high sulfate content of these two carrageenans. The encapsulation and release process was difficult to control, which would result in low encapsulation efficiency. On the other hand, interactions between gelatin-A and furcelleran were relatively weak, resulting in low coacervate yield. Whereas the process was still manageable. We tested the coacervation of furcelleran and different amount of gelatin-A (Figure 5). The best protein to polysaccharide ration was 2:1 in this experiment. On the other hand, the yield reflected as turbidity was not satisfactory. In Figure 5, the salt concentrations were adjusted. As salt concentration decreased, the coacervate yield increased. When the salt concentration was lower than 0.1M, gelatin-A and furcelleran started interaction at pH above 10. When coacervate is used for encapsulation, they need to be dissociated completely before titration. In this furcelleran/gelatin-A system, we could only choose an ionic strength of 0.1M NaCl. However, at this strength, the coacervate yield is too low. Therefore, this system is not an ideal one for encapsulation.

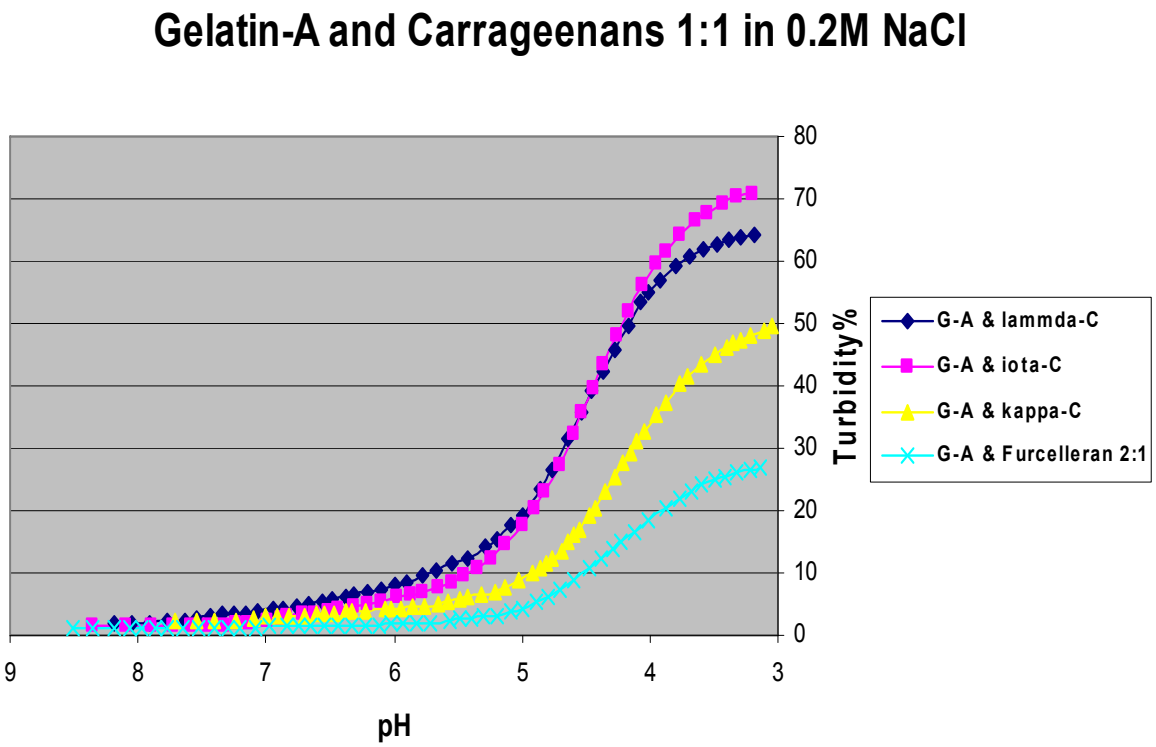


Figure 4. Turbidimetric titration curves of gelatin-A and four kinds of carrageenans (Furcelleran, kappa, iota, and lammda) in 0.2M NaCl solutions. The ratios of κ-carrageenan, ι-carrageenan, λ-carrageenan and gelatin-A were fixed at 1:1 whereas the ratio of furcelleran/gelatin-A was fixed at 1:2.

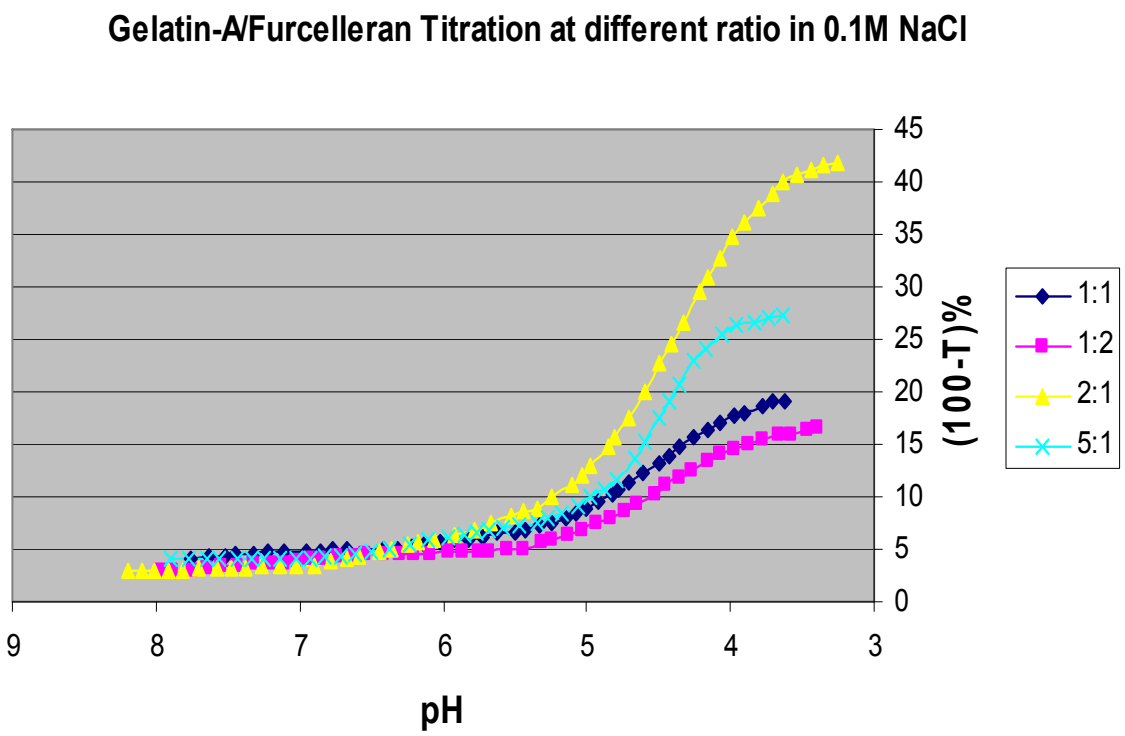


Figure 5. Turbidimetric titration curves of gelatin-A and furcelleran at different ratios in 0.1M NaCl solutions.

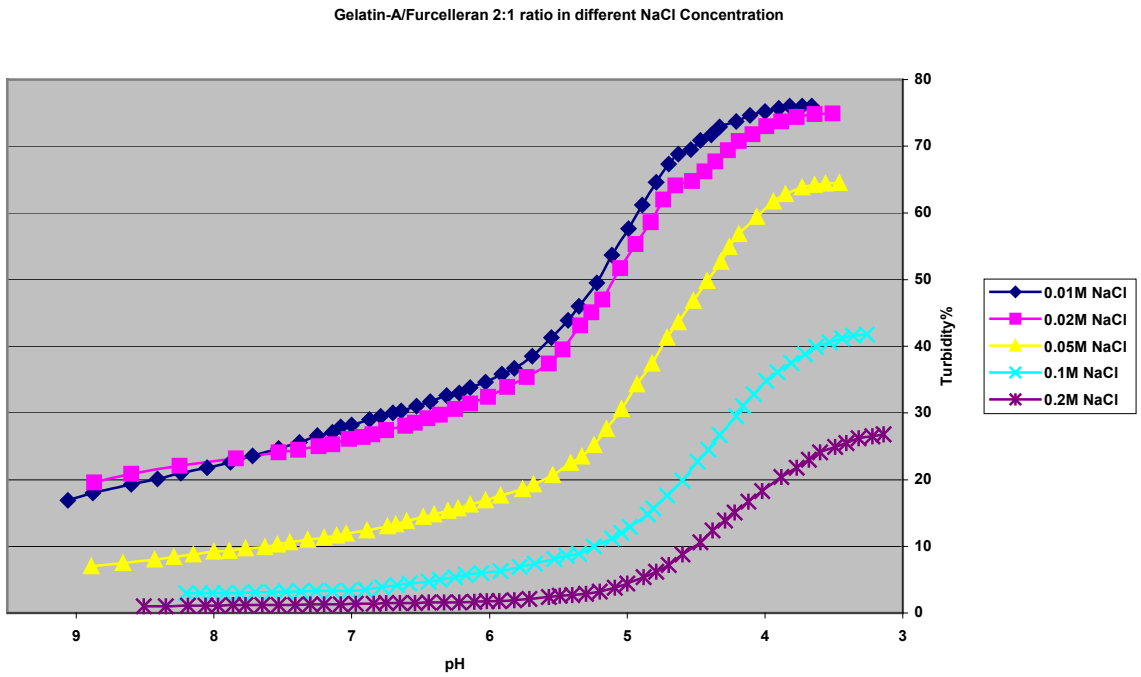


Figure 6. Turbidimetric titration curves of gelatin-A and furcelleran at different NaCl concentrations. The ratio of furcelleran/gelatin-A was fixed at 1:2.

Coacervates formed by gelatin-A and κ-carrageenan had unique properties. The conditions need to be optimized for encapsulation application. Figure 7 showed the turbidimetric titration curves of gelatin-A and κ-carrageenan in 0.1M NaCl solution at different ratios of 1:1, 2:1, 5:1 and 10:1. The ratios of 1:1 and 2:1 gave the highest turbidity, which corresponded to the highest yield. Thus, the complex of gelatin-A/κ-carrageenan with ratio 1:1 was selected for further study. The titration curves at different ionic strengths were further tested to study the effects on coacervate formation. The results were shown in Figure 8. One noted that the maximum yield of coacervate didn't change much when salt concentration was lower than 0.1M. However the turbidity decreased when the salt concentration was increased. A relatively low ionic strength was required to manage the coacervation process. Under low ionic strength ($I \leq 0.1M$), protein and polysaccharide started to form coacevate at pH between 5.0 and 6.0. On the other hand, the background complexation at low ionic strength could affect encapsulation. Therefore, the optimum coacervation condition for gelatin-A and κ-carrageenan was set at 1:1 ratio in 0.1M NaCl. Under this condition, the maximum coacervation occurred at pH 3.3.

Figure 9 and 10 were titration curves of pure gelatin-A and κ-carrageenan under different ionic strengths. It was found that the turbidity of either gelatin-A or κ-carrageenan was nearly independent upon pH.

It was mentioned early that the salt acted as a shield and screened the intermolecular interactions between macromolecules. Na^+ formed ion pairs with the negatively charged groups of carrageenan, and Cl^- formed ion pairs with the positively charged groups of gelatin-A. At low salt concentration, this effect was negligible. The

increase of salt concentration shifted the titration curves to a lower pH, as revealed in Figure 6 and 8. Meanwhile, the maximum coacervate pH decreased with the increase of salt concentration.

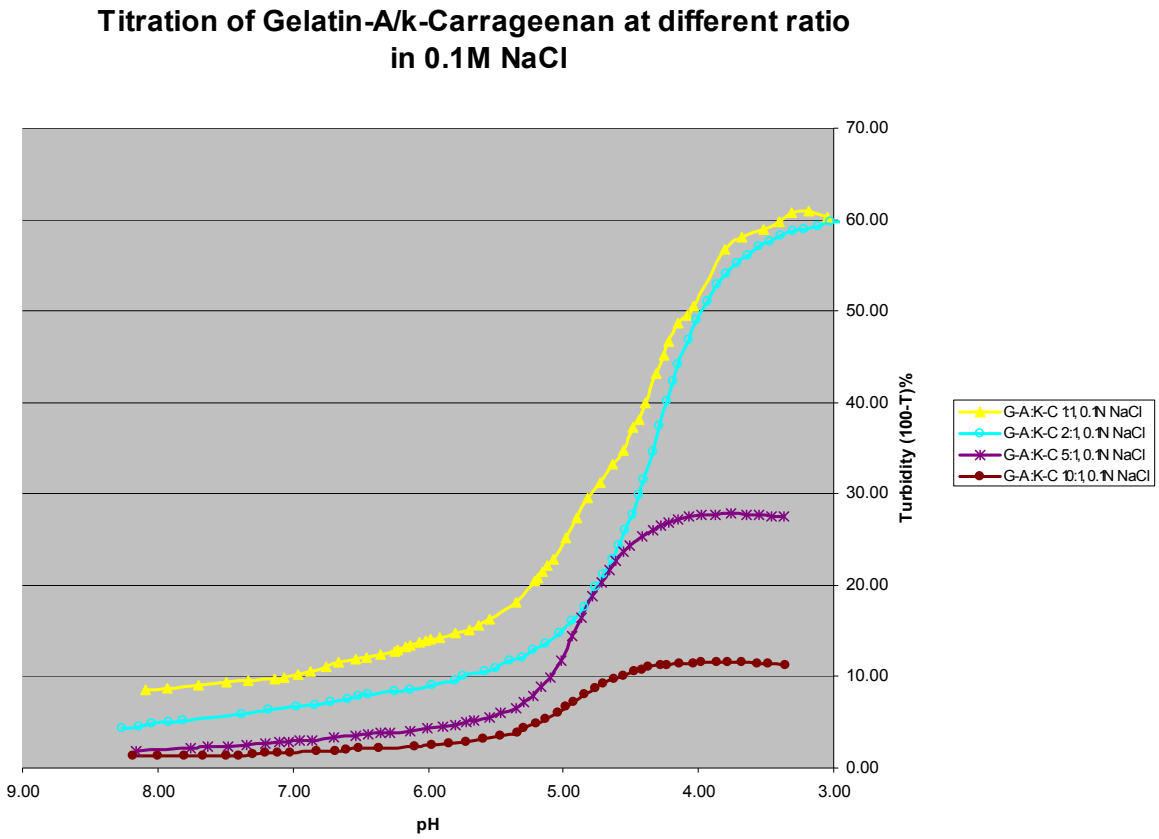


Figure 7. Turbidimetric titration curves of gelatin-A and kappa-carrageenan with different ratios in 0.1M NaCl solutions

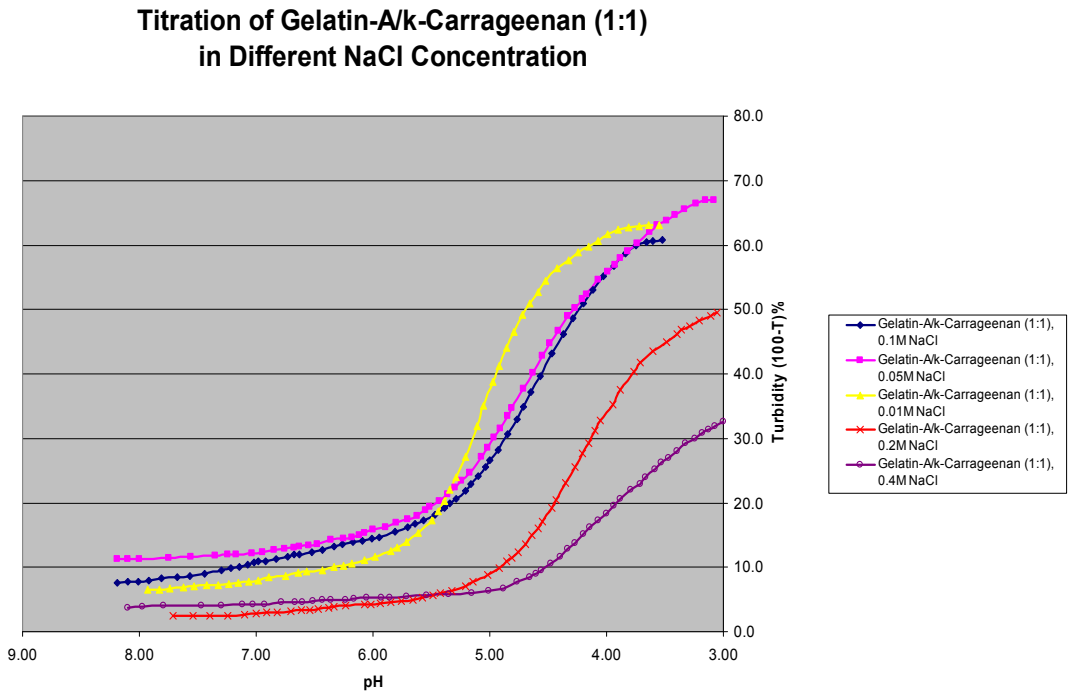


Figure 8. Turbidimetric titration curves of gelatin-A and kappa-carrageenan at 1:1 ratio in NaCl solutions of different ionic strengths.

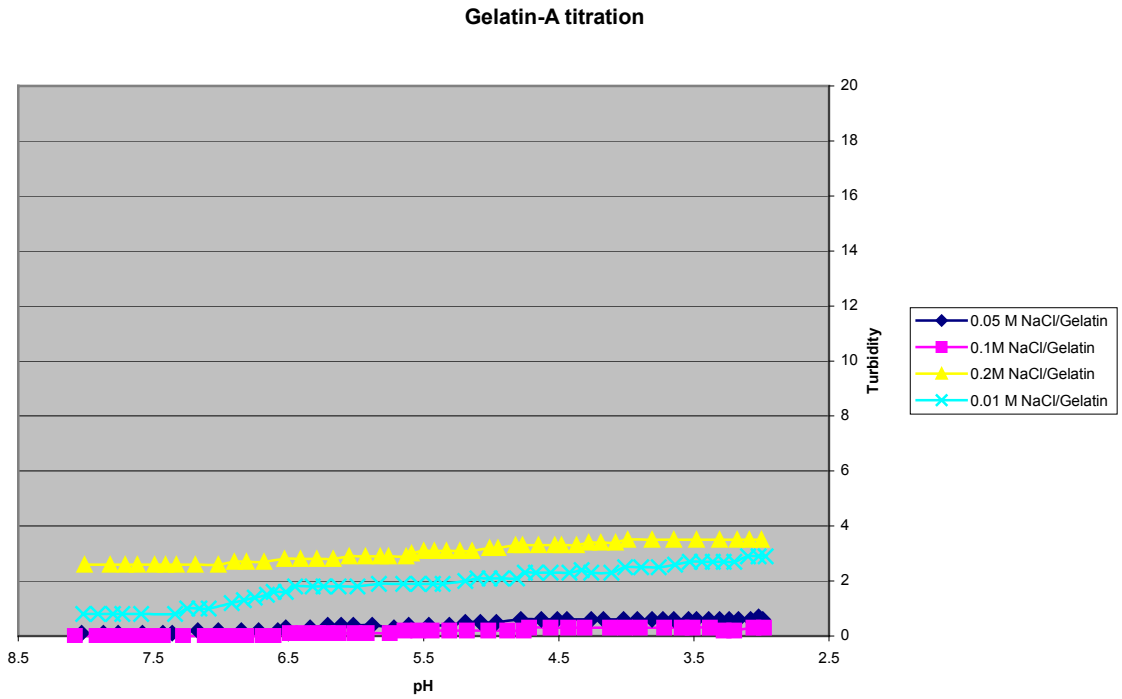


Figure 9. Effects of ionic strength on the titration curves of pure gelatin-A.

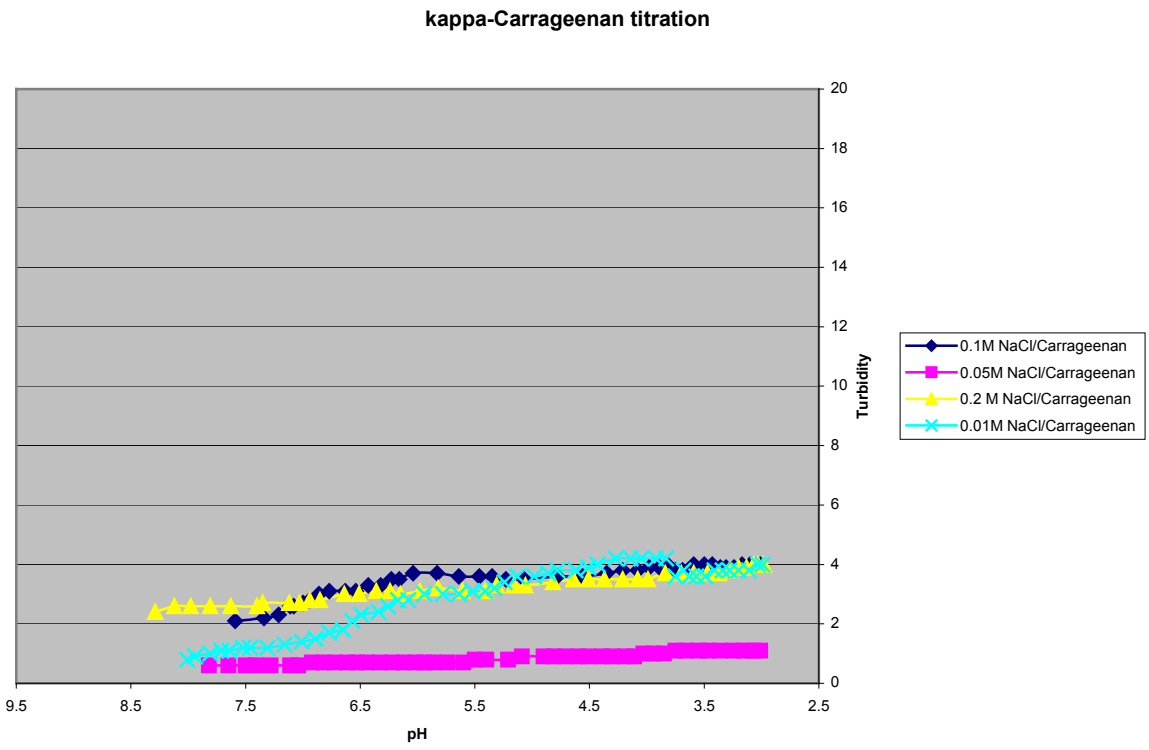


Figure 10. Effects of ionic strength on the titration curves of pure κ-carrageenan.

Under the optimum conditions, gelatin-A and κ-carrageenan started coacervation at pH about 5.6 (from Figure 7 and Figure 8). Below this point, macrophase separation occurred. This pH was close to the pI of gelatin-A. Above the pH value, gelatin-A and κ-carrageenan formed soluble complex. To study the soluble complex, we use Dynamic Light Scattering-based Particle Size Analyzer to measure the average diameters of the molecules. Figure 11 and 12 showed the sizes of pure gelatin-A and κ-carrageenan at different pH in 0.1M saline. Gelatin-A is a non-globular protein, therefore, the diameter of the molecules is bigger than globular protein. At pH range 3.5 to 8.5, the diameter of gelatin-A was to be around 40nm. At this pH range κ-carrageenan was about 70-75nm. The molecules of κ-carrageenan might associate together and form dimmer or trimmer at current concentration. When titrated the mixture of 0.5g/L gelatin-A and κ-carrageenan in 0.1M NaCl, the particle size started increase at about pH 8.5. This was the point when soluble complex started forming. When pH was below 7.7 the particles were too big to pass through the 0.2um filter. At pH around the transition point, it was difficult to control the pH change in small range. A little drop of diluted HCl will change pH dramatically.

In Figure 14, hydrodynamic diameter change during titration was compared with turbidity. A slight increase in the diameters of the particle at pH 8.5 did not affect turbidity. The soluble complex formed during titration. At pH 5.5, complex diameters started to increase dramatically. At pH 5, very large complexes formed, accompanied by increased turbidity. Compared the two curves, it showed that the diameter increase always occurred at pH higher than the turbidity increase. That meant when the particles increased to certain sizes, they could be detected by turbidity meter and precipitated.

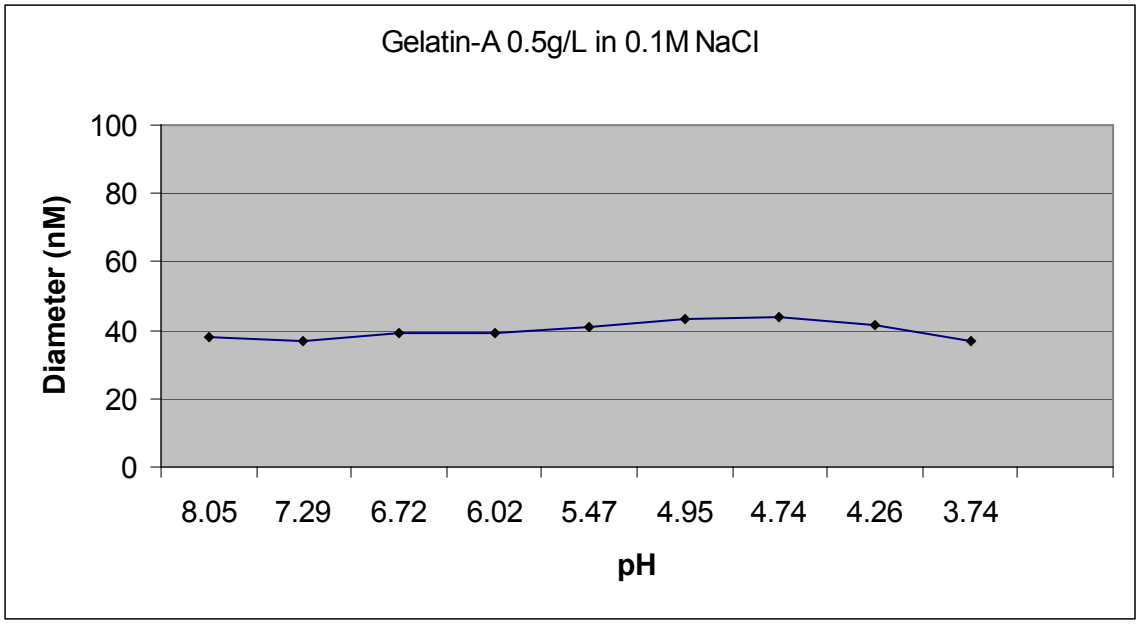


Figure 11. Gelatin-A molecular size at different pH in 0.1M NaCl, as measured by Dynamic Light Scattering Based Particle Size Analyzer

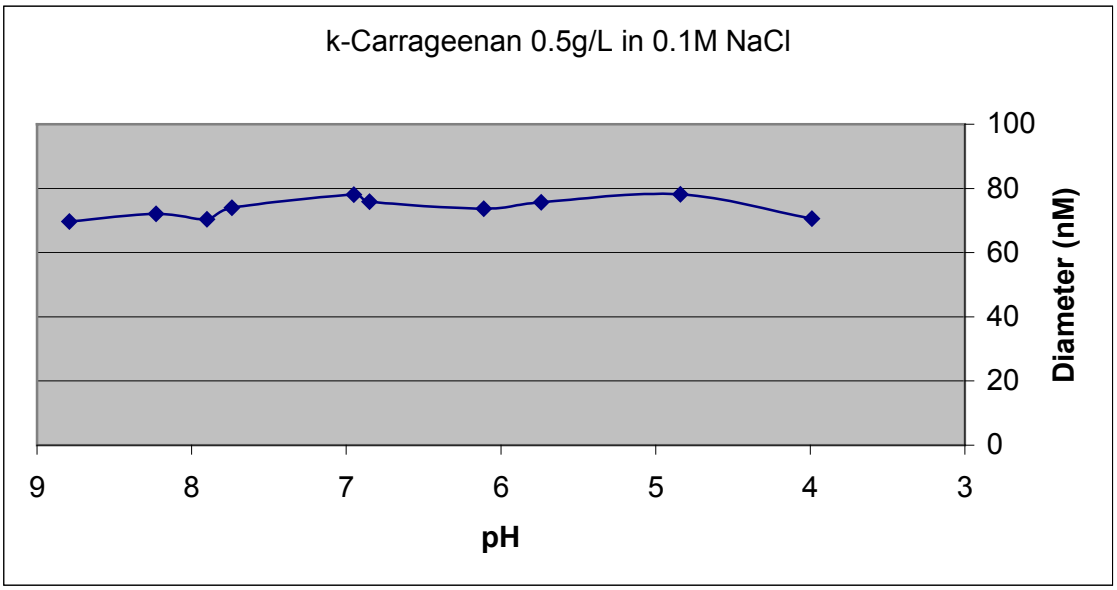


Figure 12. Kappa-carrageenan molecular size at different pH values in 0.1M NaCl, as measured by Dynamic Light Scattering Based Particle Size Analyzer

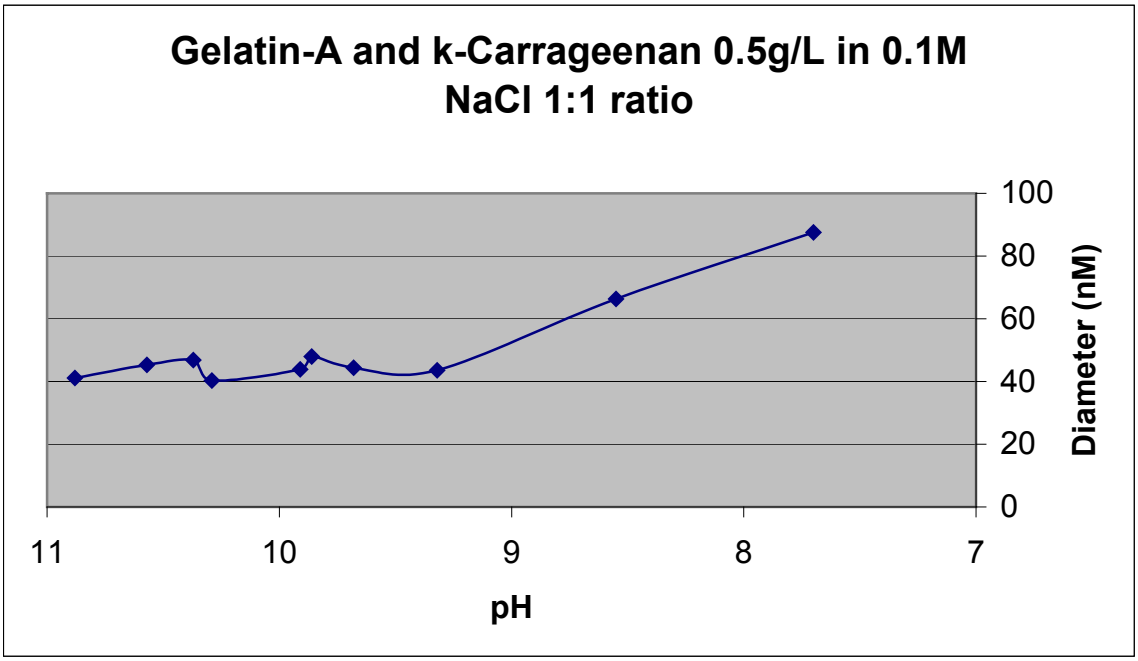


Figure 13. Effects of pH on the particle sizes of kappa-carrageenan and gelatin-A complexes at protein/polymer ratio of 1:1 in 0.1M NaCl, as measured by Dynamic Light Scattering Based Particle Size Analyzer

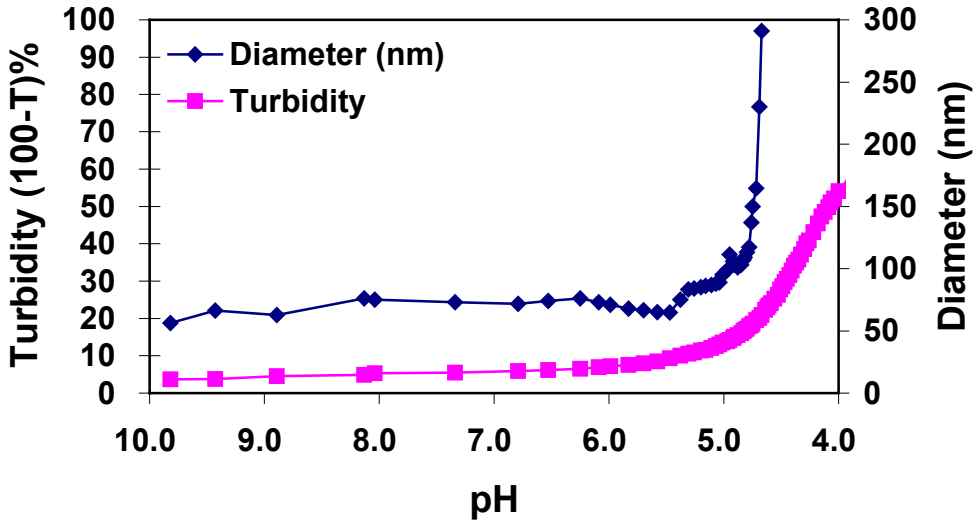


Figure 14. Comparison of hydrodynamic diameter change and turbidity during gelatin-A and kappa-carrageenan complex formation

To compare the current system with BSA and k-Carrageenan coacervation, particle sizes of soluble complex of BSA and k-Carrageenan had been measured. Particle sizes in Figure 15 showed average molecular diameter of BSA was 14.9nm at pH 3.5-8.5. When mixing with k-Carrageenan and titrating, soluble complex formed at around pH 7. BSA had isoelectric point of 4.6. Carrageenan formed soluble complex at lower pH with BSA than with gelatin-A. Also since BSA was a smaller molecule, the diameters of complexes of BSA/κ-Carrageenan were smaller than those of Gelatin-A and k-Carrageenan system.

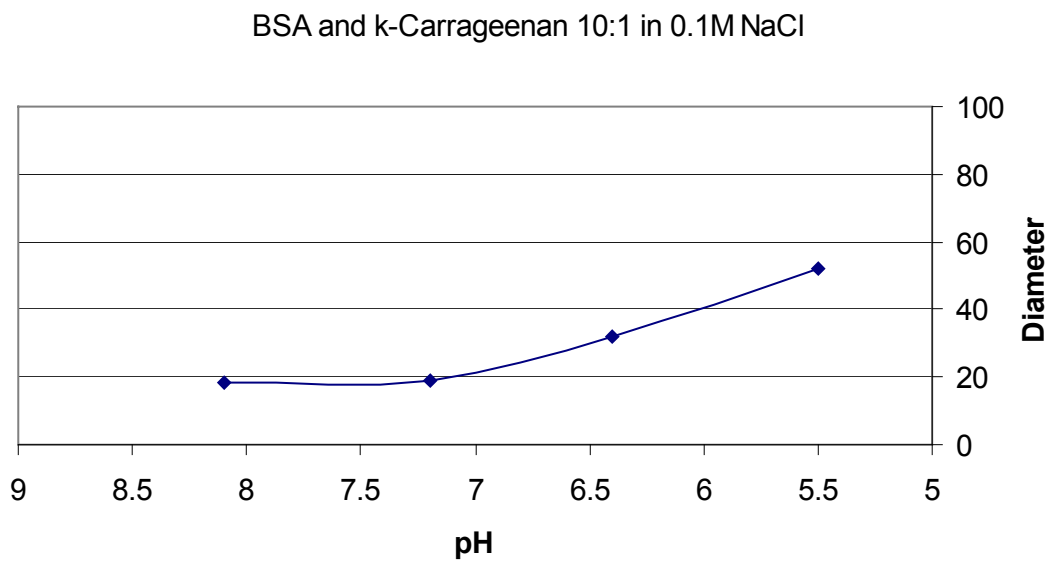


Figure 15. Effects of pH on the diameter of the complexes formed by kappa-carrageenan and BSA (at protein/polymer ratio of 1:10) in 0.1M NaCl, as measured by Dynamic Light Scattering Based Particle Size Analyzer

4.1.2. Coacervate Encapsulation

Tea catechins were dissolved in aqueous solution and then emulsified in oil. The oil phase would be further emulsified in water and encapsulated with coacervate. The process would form a water in oil in water (W/O/W) double emulsion.

After testing the emulsion stability of a series of emulsifiers, a HLB 4.7 emulsifier, sorbitan monostearate, was chosen for W/O emulsion. Here the aqueous phase referred as 5% tea catechins in 20/80 ethanol/water, and the oil was canola oil. To understand the system and emulsion process better, a Pseudoternary Phase Diagram was plotted against two phases and the emulsifier. The plot was specific to the system, if any one of the component changed, the whole plot would be shifted.

The plot of Figure 16 showed that, to form W/O emulsion, water content in the system should not exceed 25%. If the system had more than 25% of water, it either separated into two phases or formed liquid crystalline. If there was more than 70% of water, with certain amount of emulsifiers, the system would reverse to O/W emulsion. The plot helped to predict the formation of emulsion, and avoid the unwanted liquid crystalline, two-phase, or O/W regions. Therefore, the formulation containing 15% water, 5% emulsifier and 80% oil point was chosen to form stable W/O emulsion.

It has been reported (Rudhardt et al., 2003) that with the addition of emulsion stabilizers, liquid crystalline area and two-phase area could be reduced. In this way more water would be included in the emulsion.

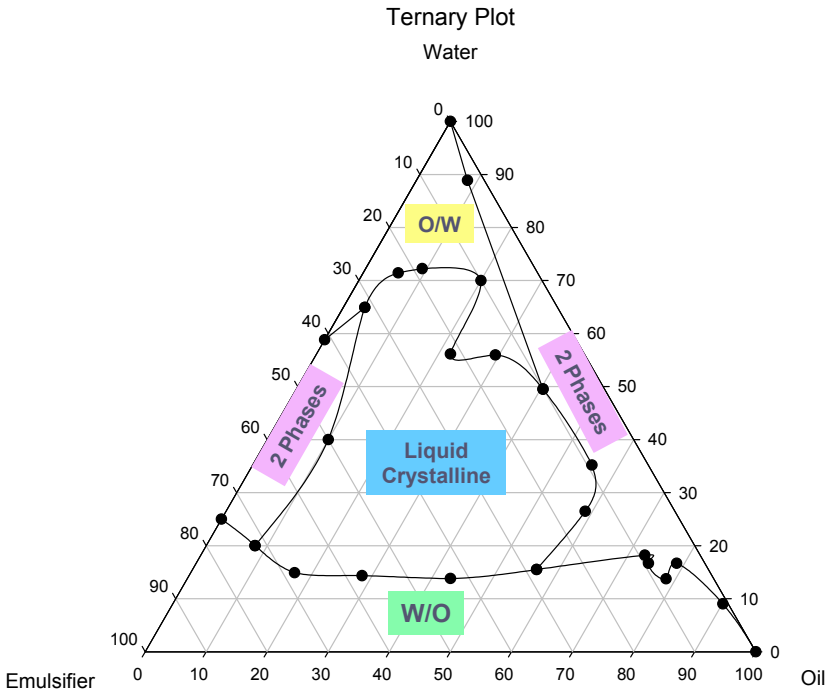


Figure 16. Pseudoternary phase diagram of the water in oil emulsion system, water phase – 5% catechins in the mixture of water and ethanol (H2O : ETOH = 80% : 20% v/v), Oil – canola oil, Emulsifier – sorbitan monostearate, HLB 4.7.

With the emulsion formulation was selected, the emulsion was prepared with high-speed homogenization. A picture of W/O emulsion was taken under optical microscope with 400 times amplification (Figure 17). The particle sizes of the emulsion were not very homogeneous. From the picture they might range from ~0.4 μm to 2 μm. This W/O system was stable for 72 h at 4˚C, and phase separation appeared after 3 days. Emulsion stabilizers or higher energy input could reduce the particle sizes and increase the stability.

The W/O emulsion was treated as one phase, and encapsulated into gelatin-A/κ-carrageenan coacervate. The coacervate also acted as stabilizer to wrap the oil particles into water during encapsulation. A double emulsion was thus formed. Figure 18 was the picture of the typical double emulsion taken under 400 times optical microscope. The size of the encapsulation particles ranged from 10 μm to 30 μm. In the picture, the inner dark dots were the aqueous phase containing catechins, which were the particles showed in Figure 17. Out of the aqueous phase was the oil. A dark shell that covered the oil phase was the coacervate. The coacervate was a solid layer, so it showed very dark under microscope.

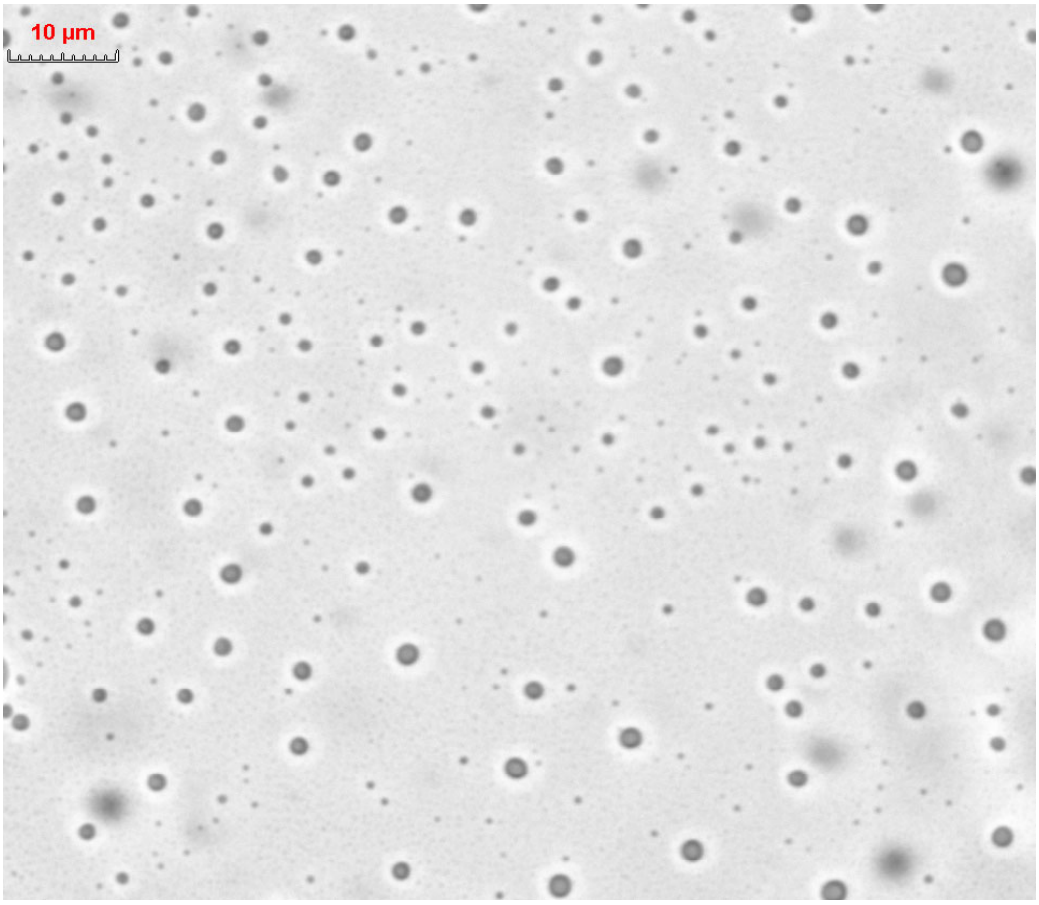


Figure 17. Emulsified tea catechin in aqueous phase in oil emulsion, as imaged by 400X optical microscope.

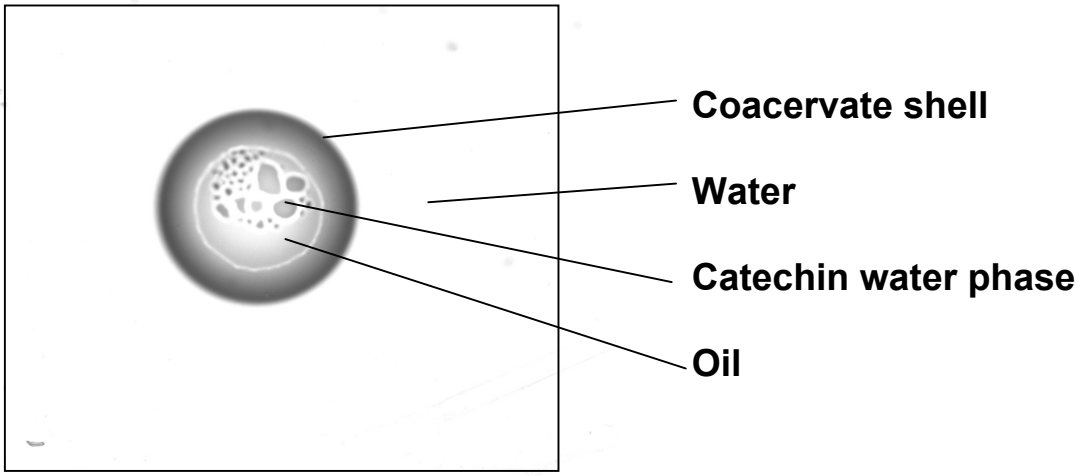


Figure 18. Water in oil in water double emulsion system, using coacervate to encapsulate tea catechins, as measured by 400X inverted optical microscope

4.1.3. Encapsulation Efficiency and *In vitro* Release

Encapsulated catechins were separated through centrifugation. Figure 19 showed encapsulated and unencapsulated catechins measured by HPLC. Both absorption peaks were at 26.533 min. The peak areas were calculated, and the results were shown in Figure 20. The encapsulated catechins concentration was 10.24mg/ml and the unencapsulated catechins concentration was 1.08mg/ml, which is equivalent to the encapsulation efficiency of 90.5%.

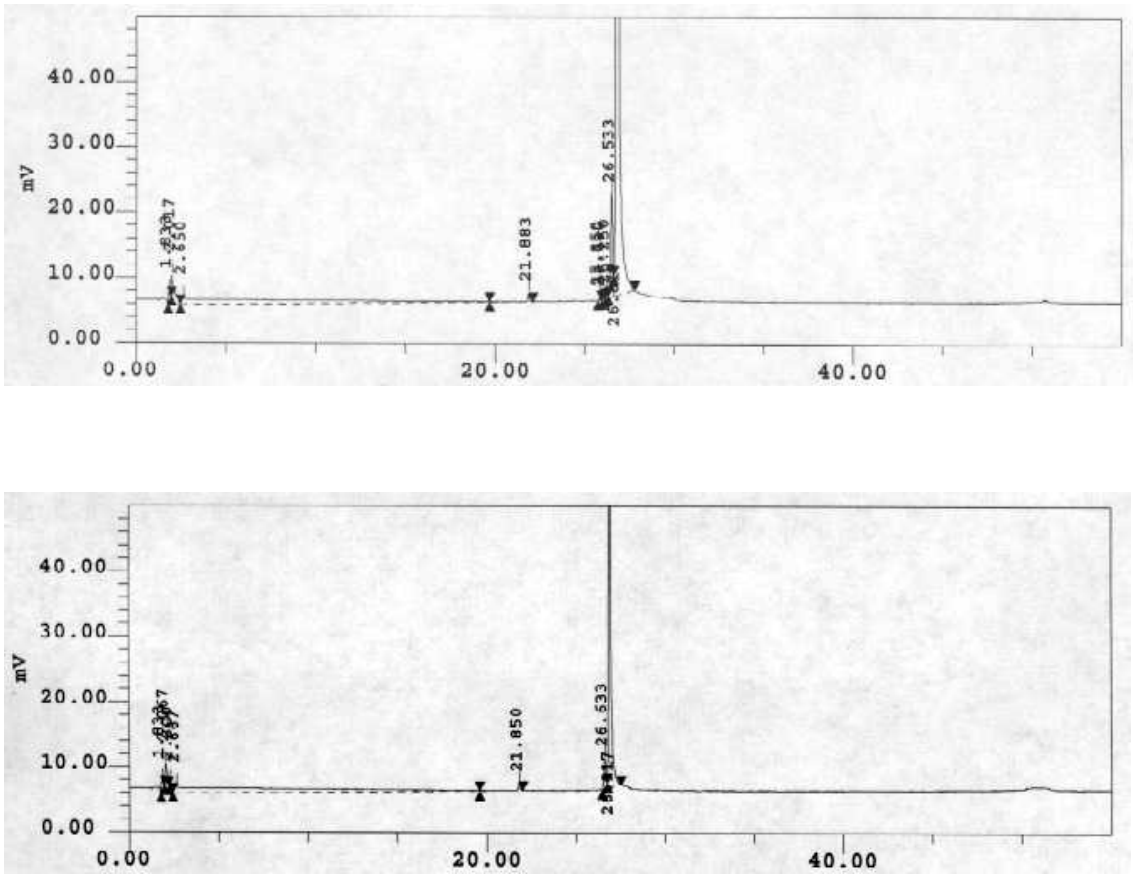


Figure 19. Top: Encapsulated catechins extracted with acetone and measured by HPLC, Bottom: Free unencapsulated catechins extracted with acetone and measrued by HPLC.

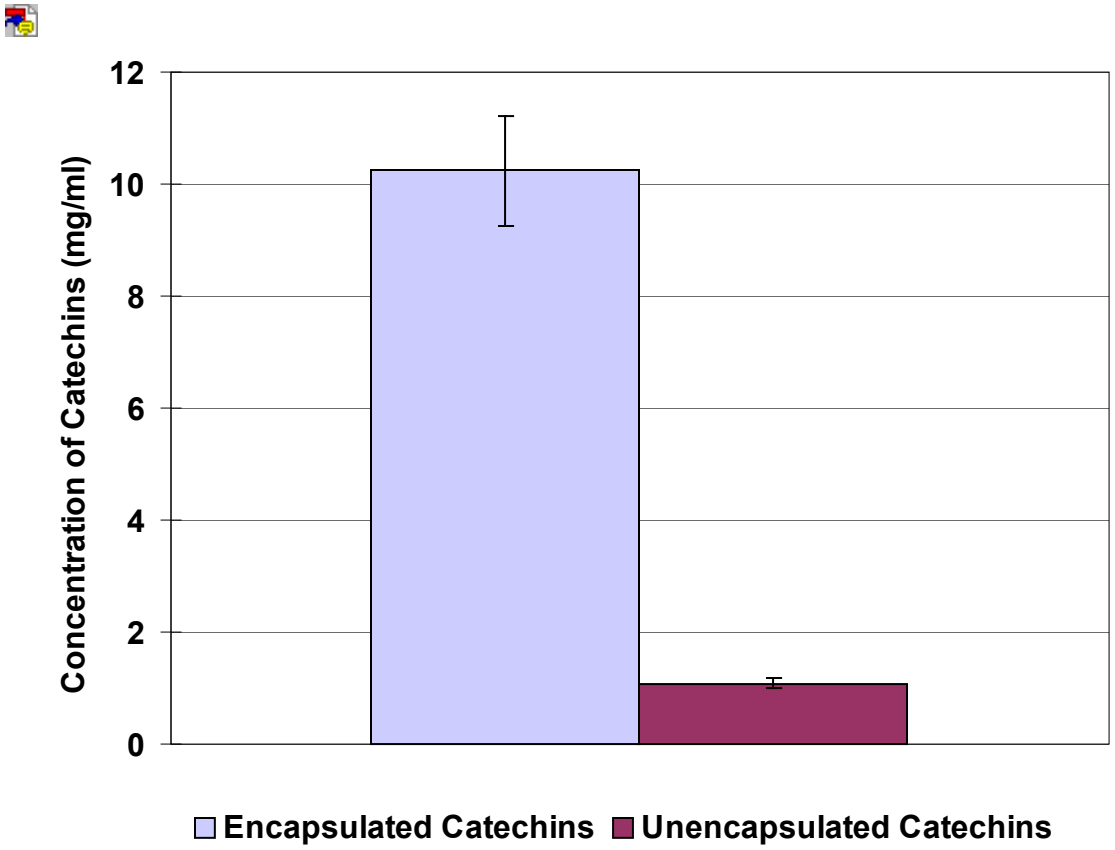


Figure 20. Catechin encapsulation efficiency calculated from Figure 19.

Encapsulated catechins were incubated in artificial gastric juice at 37ºC for 2 h, according to the normal retention time of food in stomach. No catechins were detected by HPLC in the continuous water phase. The coacervate was very stable under stomach acidic condition (pH 1.5). Pepsin in stomach theoretically should be able to digest protein, but the current result indicated it could not digest coacervated gelatin. Encapsulated catechins were then incubated in artificial small intestinal juice at 37ºC for 4 h, which is the normal retention time of food in small intestine. After 20 min no coacervate could be visually observed in the solution. The pH of small intestine juice was 8.5 and coacervates dissociated quickly at this pH. The release kinetic was measured, and showed in Figure 21, which indicated the complete dissociation happened between 18 to 22 min of incubation. As soon as the W/O phase exposed to small intestine juice, lipase and bile salt would start acting on oil.

It has been reported (Porter et al., 2008) that hydrophilic drug encapsulated by oil or liposome was absorbed through lipid absorption pathway. Our current study showed that coacervate could controlled-release catechins at the absorption location, small intestine. Therefore, catechins would not be rapidly flushed out as a polar drug before absorption. The release occurred within a short period, in this way the local concentration of catechins was increased. The pH-triggered release rate of coacervate in small intestine could be managed by applying proteins of different pIs or polysaccharides with different charge densities in coacervate.

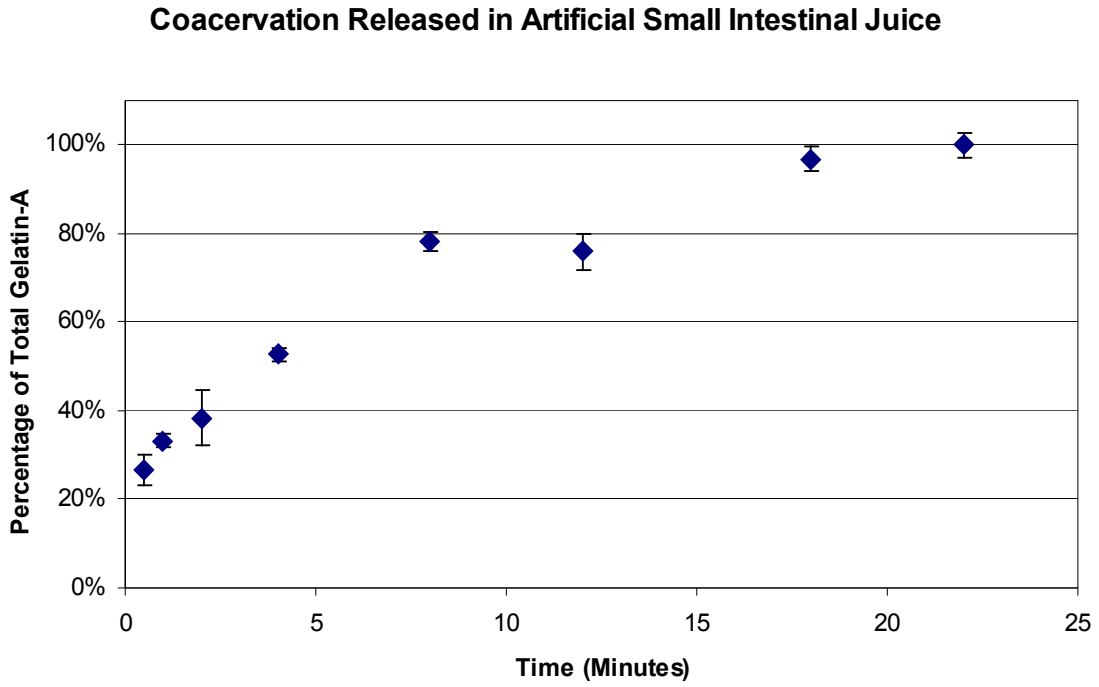


Figure 21. Release kinetics of coacervate formed by gelatin-A and κ-carrageenan prepared at 0.1M NaCl in artificial small intestinal juice at 37°C.

4.2. Catechins Encapsulated by W/O/W Double Emulsion

Using coacervate to encapsulate catechins was a good application, but it involved many sample preparation steps and had low overall yield. Large amount of water had to be removed in the end to achieve microencapsulated catechins products. Therefore, a simpler double emulsion method was explored to increase the encapsulation yield and efficiency.

4.2.1. Maximum Solubility of Catechins in Different Solvents

The maximum solubility of catechins in different hydrophilic solutions was measured. In 10g of each solvent, the solubility of catechins at room temperature was different. About 1.5g catechins could be dissolved in 10g 10% ethanol solution; about 2.0g catechins could be dissolved in 10g of 10% PEG 200 solution; about 0.2g catechins could be dissolved in 10g of 10% PEG 400 solution; and about 1.5g catechins could be dissolved in 10g water saturated triacetin.

Catechins had great solubility in pure ethanol, but to reduce the toxicity of alcohol in animal study, 10% ethanol in water was selected. Triacetin was food grade solvent with high polarity. Triacetin is slightly soluble in water, but not miscible with oil. At room temperature, water-saturated triacetin contained about 4% of water. The result showed more than 10% of the solubility of catechins in 10% ethanol, water-saturated triacetin and 10% PEG 200. In 10% PEG 200 it had almost 20% solubility. Other solvents, including 10% glycerol in water, tripropionin and tributyrin had also been tested. But catechins are not soluble in these solvents. Based on the result, 10% ethanol, water-saturated triacetin and 10% PEG 200 were possible candidates for further study.

4.2.2. Optimum HLB Value and Mixing Ratio for Catechins Solution in Canola Oil (W/O) Emulsion

Normally for water in oil emulsion, the emulsifier's HLB should in the range of 3.5-8. Emulsifiers A to G have been tested for W/O emulsion for a) catechins in 10% ethanol, b) catechins in 10% PEG 200, c) catechins in water-saturated triacetin. These were a series of emulsifier facilitated W/O emulsions. The emulsifiers were mixed in a way that the final HLB values increased by about 0.5. Through the series of tests, the rough suitable HLB number for the systems could be decided. The separation starting times under different temperatures were summarized in Table 3: Under the same mixing condition the latest separation system was the most stable one. The experiment was performed in triplicate, and the suitable HLB values were identified as: a) 5.3 for catechins in 10% ethanol; b) 5.9 for catechins in 10% PEG 200; and c) 5.9 for catechins in water-saturated triacetin. No brominated oil was added in this step, so phase separation was easy to be identified. In the later step, more emulsifier combinations and more delicate changes in HLB number would be examined to choose the most suitable emulsifier.

The suitable HLB values for each system were determined. It varied with the surface properties of both hydrophilic and hydrophobic phase. It was found during the research that the suitable HLB not only varied with the solvents, but also depended on catechins' concentration in polar phase. The concentration affected the overall polarity of hydrophilic phase. For current study 10% catechins in solvent was selected for all the systems. Furthermore, in the stability study of W/O/W, we found that fully saturated catechins inner phase would result in instable multiple emulsions. The content of the

hydrophilic inner phase leaked to outside easily. This might be due to the high osmotic pressure difference between W1 and W2 phases (Mezzenga et al., 2003) or the surface property change of the polar phase.

Table 3. Stability of W/O emulsions with different emulsifiers

Emulsifier Label	HLB	Temp.	Emulsion Seperation Starting Time
A	3.80	4°C	20min
		20°C	20min
		45°C	<10min
B	4.30	4°C	20min
		20°C	20min
		45°C	<10min
C	4.83	4°C	40min
		20°C	30min
		45°C	10 min
D	5.30	4°C	a)12hr b)6hr c)6hr
		20°C	a)5hr b)3hr c)3hr
		45°C	a)2hr b)45min c)45min
E	5.90	4°C	a)8hr b)12hr c)18hr
		20°C	a)3hr b)6hr c)12hr
		45°C	a)30min b)2hr c)2hr
F	6.44	4°C	a)30min b)4hr c)12hr
		20°C	a)20min b)2hr c)4hr
		45°C	a)<10min b)30min c)45min
G	7.00	4°C	20min
		20°C	20min
		45°C	<10min

Emulsions with different inner phase: a) 10% catechins in 10% ethanol solution, b) 10% catechins in 10% PEG 200 solution, and c) 10% catechins in water saturated triacetin. If not specified, the result referred to all three systems.

As mentioned in literature review part, emulsifiers with HLB 3.5 to 8.0 stabilize water in oil emulsion. However, each individual system has its favorite HLB value. Emulsifier of this optimum HLB can create the most stable emulsion. In table 3, it showed stability result of different HLB emulsifiers tested in the emulsion system. Three inner phases, which we called W1, had been selected. Seven emulsifiers had been tested on the stability of W1/O emulsion. Seven emulsifier's HLB ranged from 3.80 to 7.00. To show the result rapidly, the emulsions were only homogenized for 5 min at 13,500 rpm. The W/O emulsion stabilized by 3.80, 4.30, 4.83 and 7.00 emulsifiers separated quickly. That means they were not the suitable emulsifiers for the system. Emulsifiers with HLB value 5.30, 5.90 and 6.44 could create relatively stable emulsion. HLB 5.30 was suitable for 10% catechins in 10% ethanol / canola oil emulsion. We found that HLB 5.90 was favorite to 10% catechins in 10% PEG 200 / canola oil emulsion, and 10% catechins in water saturated triacetin / canola oil emulsion. The stable emulsions could last for more than 12 hours.

Three relatively stable W/O emulsions were further emulsified into water as preliminary tests (results not shown). When catechins in 10% ethanol/canola-brominated oil were further emulsified in water, the system was not very stable. The system would not be stable for more than 48 h no matter how to adjust the condition. This might be due to the high mobility of the small molecules, ethanol and water, in W1 phase. To improve the stability of this system, emulsion stabilizers could be used in hydrophilic phase. Since this research was no intended to explore the stabilizers, 10% ethanol phase would not be used for further study. During the formation of double emulsion of catechins in 10% PEG 200 / canola-brominated oil / water, emulsion crystallization could not be avoid. During

the formation of emulsion, the whole system could suddenly become creamy solid. It was not a desirable state for encapsulation application. Crystallization is a very complicated phenomenon especially when several phases and several emulsifiers were involved. So catechins in 10% PEG 200 was not selected for the inner polar phase at current study.

Then we tested 10% catechin in water saturated triacetin / canola-brominated oil / water emulsion. It was relatively stable and handle easily. Brominated oil is commonly used to increase specific gravity of oil phase. Canola oil has specific gravity of 0.920 g/ml. The specific gravity of pure water is 1.00 g/ml, and triacetin is 1.156-1.158 g/ml. To match the W1 phase specific gravity, a combination of brominated oil and canola oil was prepared as in the method section. It has been tested in this study that addition of brominated oil in the oil phase won't affect the suitable HLB value for the system. With addition of brominated oil stability of the system can change from one day to four days.

Here further studies were performed on the emulsifier for W1/O emulsion. The previous step results of W1/O emulsion were based on mix of Span 80 and Twin 80. Then a different set of emulsifiers were mixed by Span 80 and Span 20 (P, Q, R and S in Table 2). But the result turned out combinations of Span 80 and Span 20 could not stabilize the current W1/O system.

As HLB 5.90 was selected among a broad HLB range, a detailed look on narrower ranged emulsifiers (U, E and T in Table 2) mixed by Span 80 and Twin 80 were examined for the system. They all generated the similar result, which was 3 days stability at 20°C or 7 days stability at 4°C. So the middle HLB number (5.90) emulsifier was finally picked.

The components of the system had been fixed from the previous results. Then the ration or percentage of each component was going to be studied. According to the literatures, 10% emulsifier is a commonly used level in W/O emulsion. Catechins in water-saturated triacetin were emulsified in canola-brominated oil, which contains 10% emulsifier E (HLB 5.9). Then a range of different ratios of W1 to O had been tested. The ratios were 1:3, 1:4 and 1:5. At 1:3 W1/O ratio, phases started separation at 12 h; at 1:4 W1/O ratio, it started at 72h; at 1:5 W1/O ratio, it started at 72h at room temperature but at lower rate of phase accumulation. To get a stable emulsion and also maximize catechins concentration, a 1:4 W1/O ratio was selected.

From the result of this step W1/O emulsion condition was temporarily fixed as 10% catechins dissolved in water-saturated triacetin and then emulsified in canola brominated oil mix, which contained 10% HLB 5.90 emulsifier. The ratio of W1 to O was 1 to 4.

4.2.3. Optimum System of Catechins/Canola Oil in Water (W1/O in W2)

5% in a commonly used concentration of emulsifier in O/W emulsion in the research. According to literature, which was reviewed in early section, emulsifiers with HLB 8-16 facilitate O/W emulsion. Eight emulsifiers (H to O in Table 2) were prepared, HLB ranging from 10.2 to 13.2. On the other hand gum Arabic and purity 2000 modified starch were well known as polysaccharide emulsifiers. They are unique polysaccharides and have multiple functions in stabilizing O/W emulsion.

With 5% H, I, J, K or L emulsifier, only three emulsifiers (I, J, K) could generate stable emulsions. The system emulsified HLB 11.16 lasted for 8 h, HLB 12.12 lasted for

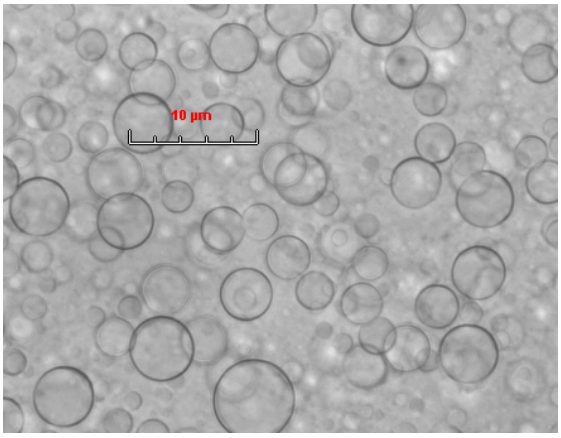
24 h, and HLB 13.08 last for 24 h at 4°C. Then narrowed the range of HLB value, but combined different emulsifiers (M, N, O) to test. They all gave the similar 24 h stability.

Then polysaccharides were introduced. 10% purity 2000 and 10% gum Arabic were used as emulsifiers and tested to emulsify the O/W system separately, but none of them worked. Then 5% polysaccharide was combined with 5% M, N, O, L, Tween 80, Tween 40 or Tween 20, and emulsified the same system. The result showed 5% polysaccharide with 5% L or Tween 80 could stabilize the system for 24 h. But surprisingly, using 5% gum Arabic with 5% Tween 40, the system was stable for 72 h at 4°C.

Then oil to water ratio was adjusted to get the most stable emulsion. Used 5% gum Arabic with 5% Tween 40, but varied W1/O to W2 ratio at 1:3, 1:2 and 1:1. The most stable emulsion was at 1:2 ratio, and lasted for more than 1 week at 4°C.

4.2.4. Particle Sizes and Encapsulation Efficiency

The right emulsifier and the right phase ratios ensure stability of emulsion system. The outside mechanic force contributes to control the particle sizes of the emulsion. Normally more mechanic force input results in smaller emulsion particles. It takes longer time for smaller emulsion particles to aggregate and separate. Particle sizes of each level of emulsion in the multiple emulsion system all contribute to the stability. The following results in Figure 21 showed the influence of force to the particle sizes of each emulsion.



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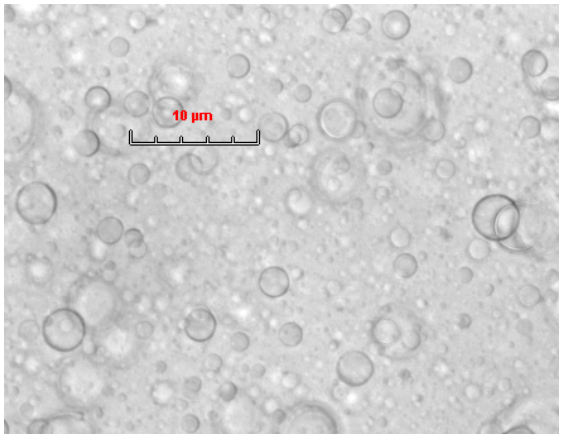

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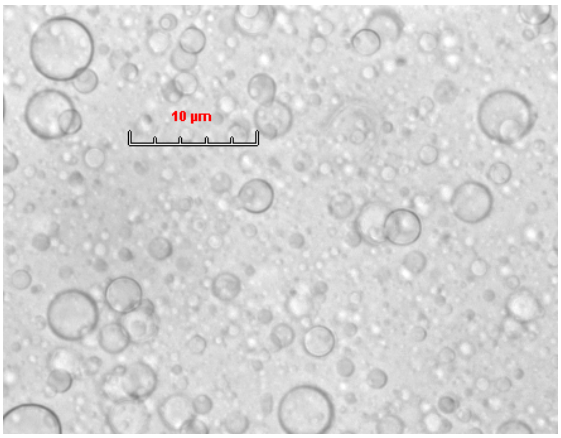

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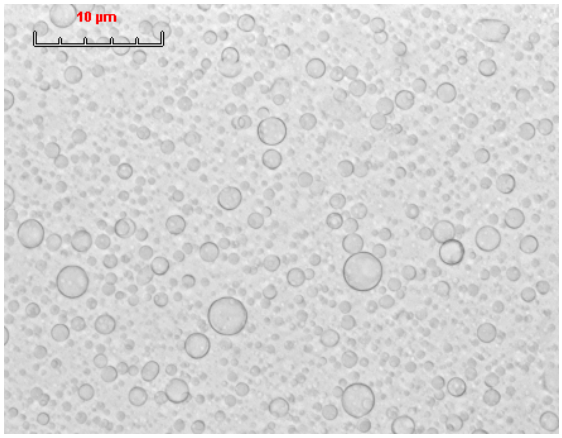

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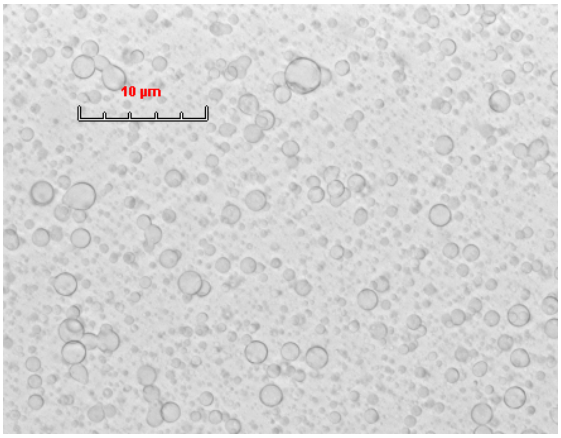

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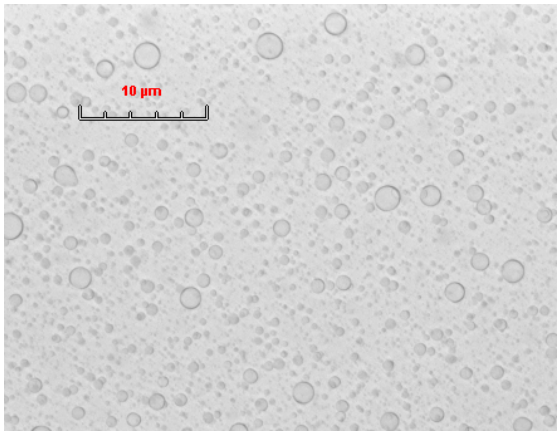

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Figure 22. Pictures were taken under optical microscope (400 times) at one hour after W1/O emulsions had been made. 1. vertex for 180s; 2. homogenize 6,500 rpm for 5min; 3. homogenize 9,500 rpm for 5min; 4. homogenize 13,500 rpm for 5min; 5. homogenize 17,500 rpm for 5 min; 6. vertex for 120s then homogenize 13,500 for 5 min.

In Figure 21, the pictures were taken under optical microscope (400X amplification) at one hour after W1/O emulsions were formed. A calibrated ruler was added to the picture to measure the particle sizes. The processing conditions were 1. vertexed for 180s; 2. homogenized at 6,500 rpm for 5min; 3. homogenized at 9,500 rpm for 5min; 4. homogenized at 13,500 rpm for 5min; 5. homogenized at 17,500 rpm for 5 min; 6. vertexed for 120s and then homogenized at 13,500 rpm for 5 min. From visual observation, the particle sizes in #1 ranged 2-5 micron, in #2 ranged 0.5-4 micron, in #3 ranged 0.5-4 micron, in #4 ranged 0.2-3 micron, in #5 ranged 0.2-2 micron, in #6 ranged 0.2-2 micron. Further analysis of the particle sizes would be performed under picture analysis software, and it would give the average size and size distribution. The photo result showed higher mechanic force input, which referred as higher homogenize speed here, could generate smaller particle size. But when homogenizing reached a certain speed, the particle sizes wouldn't get smaller with speed increase. Longer homogenizing time or high pressure homogenizing might help at this stage.

The picture showed in Figure 22 was taken under 400X optical microscope. The W1/O emulsion was made as the previous step and further emulsified into water. The picture clearly showed catechins W1/O/W2 double emulsion, that several small W1/O emulsion particles were encapsulated into big O/W2 emulsion. Further work is needed to increase the emulsion efficiency and decrease the particle sizes.

The encapsulation efficiency of the double emulsion system was 94.46% It was determined by measuring unencapsulated catechins in solution.

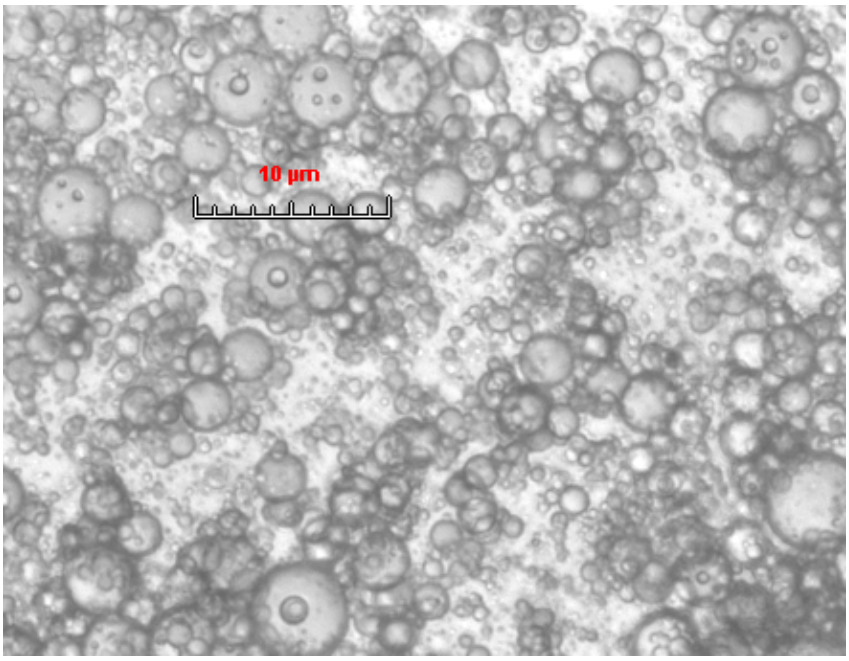


Figure 23. W/O/W emulsion of catechins. Picture was taken under 400X optical microscope.

4.3. Curcumin Encapsulation, *in vitro* and *in vivo* tests

4.3.1. Curcumin Encapsulation

4.3.1.1. Emulsion Optimization

Curcumin is not soluble in water or oil at room temperature. It is soluble in acetone, ethyl acetate and other organic solvent. Curcumin could be dissolved (less than 1%) in heated oil, whereas the solid separated from the oil after cooling down. MCT had shorter fatty acid chains than regular oil, which resulted in higher polarity of MCT. So curcumin had higher solubility in MCT (C6-C10) than oil (C16-C20). Emulsifiers could help to solubilise curcumin by reducing the surface energy. Each emulsifier has its unique molecular structure and shows different physicochemical property during emulsion formation (Krog, 1997; Faergemand and Krog, 2006). It is important to select a suitable emulsifier for each particular system. Normally a low HLB value (3.5-7) emulsifier is mostly soluble in oil and stabilize W/O emulsion; a high HLB value (9-16) emulsifier mostly soluble in water and stabilize O/W emulsion; a intermediate HLB value (7-9) emulsifier doesn't have particular preference to water or oil and normally act as wetting or spreading agent (McClements, 2005).

10% Span 20 (intermediate HLB) or Span 80 (low HLB) was added in MCT. During heating, curcumin dissolved in both and formed clear solutions. But after chilling to room temperature, curcumin crystallized and separated from the span 80 solution. The span 20 solution became opaque at room temperature but was stable. No phase separation happened even after months of storage at room temperature or at 4˚C. Thus, medium HLB value emulsifier Span 20 (HLB 8.6) could help curcumin to dissolve in MCT. Further emulsified curcumin-Span 20-MCT phase into water with help of several high

HLB value emulsifiers, which facilitated O/W emulsion. The results turned out that in this system Tween 40 (HLB 15.6) created more stable emulsion than Tween 20 (HLB 16.7) or Tween 80 (HLB 15.0).

A series of optimization experiments on 1) combination of different HLB value emulsifiers; 2) percentage of each component, finally gave the formulation as 3% or 4.5% curcumin dissolved in MCT (containing 10% Span 20) and emulsified into water (containing 5% Tween 40) at 1:2 ratio. The final concentrations of curcumin in the emulsion systems were 1% and 1.5%, respectively.

4.3.1.2. Particle Sizes

The particle sizes of curcumin emulsion were measured by using particle size analyzer after homogenizing at 6,500 rpm for 5 min, 13,500 rpm for 5 min, or 3 rounds of high-pressure homogenizing at 13,500rpm for 5 min. The particle sizes after each processing were listed in table 4. High-speed homogenizing generated micron-size emulsion with a broad size distribution. Higher speed meant more energy input, which produced smaller and more homogeneous emulsion. The picture of emulsion, processed under 13,500 rpm for 5 min, were taken under optical microscope (400X) as shown in Figure 23.

Combined high-speed and three cycles of high-pressure homogenization, the emulsion particle sizes were dramatically reduced. They were almost invisible under optical microscope. The resolution of optical microscopes is limited by light diffraction. The theoretical resolution limit is approximately 200nm (Cowhig, 1974). Since the particles were too tiny to be resolved, no picture could be taken for this stage of emulsion. Measured by DLS, the mean particle diameters were nm and 187.3nm and

214.5nm for 1% and 1.5% curcumin, respectively. The size distributions were narrow, which meant the emulsions were homogeneous in the nano size. A previous study in our groups generated a curcumin emulsion with >700nm size (Wang et al., 2008). By adjust the conditions, particle sizes were reduced dramatically. Smaller sizes might increase the stability and bioavailability. As discussed later on encapsulation efficiency we knew curcumin was not 100% encapsulated in the oil phase. Free curcumin was observed as crystal in the emulsion under optical microscope. The crystals were spindle-shaped with sizes roughly about 0.5-1 micron. In 1.5% curcumin emulsion, there were more free curcumin crystals than 1% emulsion. Presence of the crystals might give larger average particle size of the emulsions. Thus, the particle sizes of the curcumin emulsions were measured after removing the free curcumin with 5 min centrifuge at 6,500 rpm. The mean particle diameters were 150.5nm and 148.4nm, and polydispersities were 0.253 and 0.223 for 1% and 1.5% curcumin emulsion, respectively. This result should reveal the real emulsion particle sizes.

Figure 25 and 26 were cumulant analysis and single stretched exponential fit of the particle sizes of 1.5% nano-emulsified curcumin, respectively. The data were acquired from dynamic particle size analyser. The mean emulsion diameters from both data fitting methods were comparable. For 1.5% curcumin, the particle diameters were 148.4nm and 163.5nm for cumulant analysis and single stretched exponential fit, respectively.

Table 4. Curcumin emulsion mean particle diameters under different emulsion conditions, measured by Dynamic Light Scattering Particle Size Analyzer.

	1% curcumin	1.5% curcumin
13,500 rpm	2194.6 nm	2284.3 nm
135,00 rpm + high-pressure	187.3 nm	214.5 nm
13,500 rpm + high-pressure + free curcumin removal	150.5 nm	148.4 nm

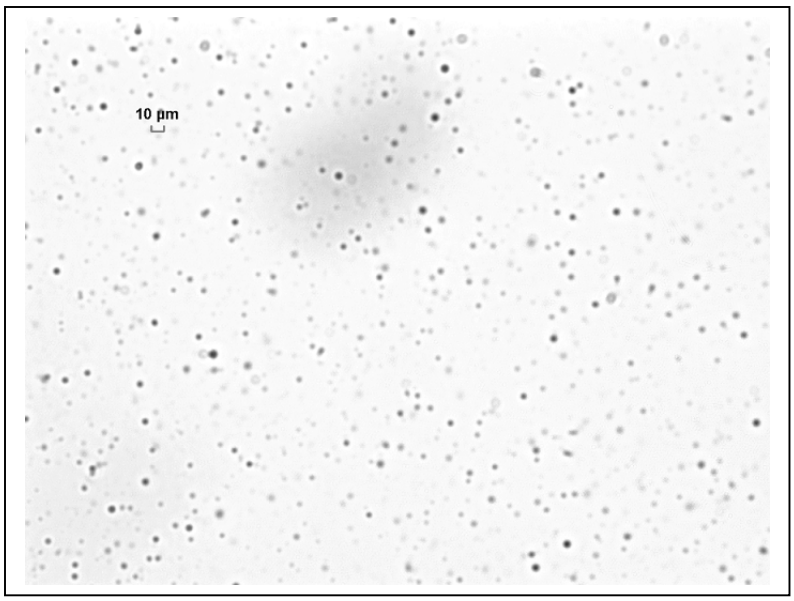


Figure 24. Curcumin emulsion, processed with 13,500rpm for 5 min, picture was taken under 400X optical microscope.

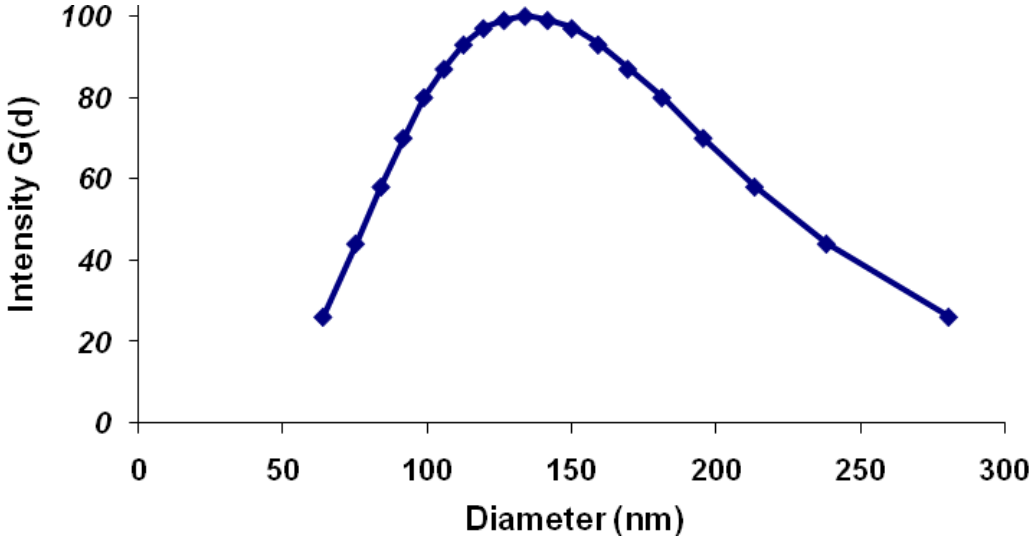


Figure 25. Particle sizes cumulant analysis of 1.5% curcumin nano-emulsion.

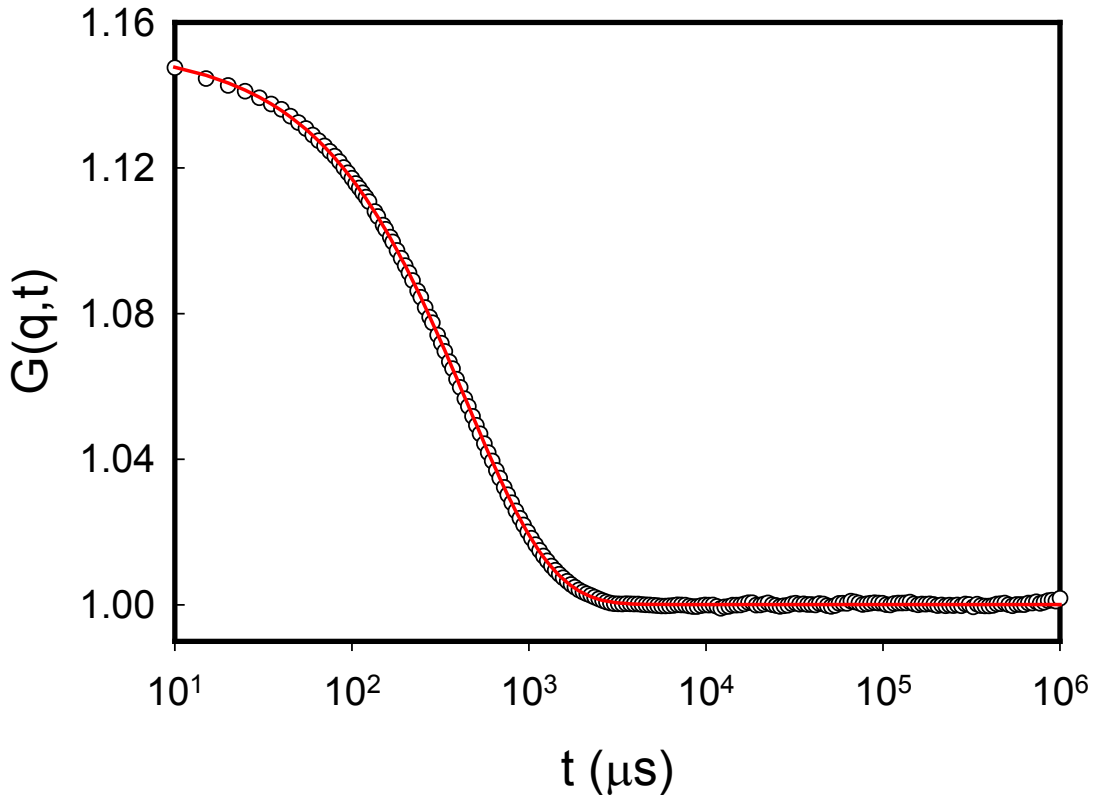


Figure 26. Single stretched exponential fit of particle size correlation functions of 1.5% curcumin nano- emulsion, in which the circles are experimental data and the solid line is the fitting result.

4.3.1.3. Encapsulation Efficiencies and Stabilities

Curcumin is not soluble in water. Even with help of emulsifiers, the solubility is still extremely low. The unencapsulated curcumin presented as solid crystal in water phase. Encapsulated and unencapsulated curcumin could be separated as liquid and solid, respectively. To avoid unnecessary phase separation droved by centrifuge a low speed and short time were used. After a couple of rounds of centrifuge and wash, insoluble free curcumin was collected as precipitate. By using this method, 1% curcumin emulsion was proved to have 77.49% encapsulation efficiency, whereas it was 71.50% for 1.5% curcumin emulsion. The numbers were started to measure 1hr after the formation of emulsion.

Curcumin was 100% dissolved in MCT. During O/W homogenizing, a small amount of curcumin dissociated from the oil, formed crystal and precipitated in water phase. It had been observed that too much energy input of high-speed homogenization would result in curcumin dissociation from oil phase. Processed under the same speed (13,500rpm) but doubled the time of high-speed homogenizing (to 10 min), the encapsulation efficiencies decreased to 70.08% and 64.67% for 1% and 1.5% curcumin emulsions, respectively. It was about 10% reduction. On the other hand, higher homogenizing speed also dissociated curcumin from oil. While with energy input smaller than experiment condition, 13,500 rpm/5 minute, the efficiency didn't improve, which implied the massive dissociation started when the energy input exceeded a certain limit. Whereas, high-pressure homogenizing (within reasonable pressure and rounds) didn't affect the efficiency or destroy the encapsulation.

The same procedure was used to evaluate the stability of the emulsion during storage. Table 5 showed the result measured by HPLC. The encapsulation efficiency declined over time. The most loss of encapsulation was on the first day. Then about 1% of total curcumin would release from the emulsion each day. Concerning the stability of the emulsion, all *in vitro* or *in vivo* tests discussed later were performed on the same day when emulsion was made.

The current curcumin emulsions were not formulated as product development. It could keep reasonable stability within a week. With more optimization on emulsifiers, phase-ratio, homogenizing process, and emulsion stabilizers, the emulsion could achieve higher encapsulation efficiency and longer stability. Some natural emulsion stabilizer is under testing to increase the stability. Polysaccharides, such as gum Arabic, modified food starch and gum Tragacanth, can be used independently as emulsifiers or conjunctly with other surfactants as emulsifiers and stabilizer (Tan, 2004). Thickening agent can applied in O/W emulsion to increase the viscosity of the aqueous phase (Mitchell and Ledward, 1986). Nature or food grade polymers, such as polysaccharides and proteins are often used for this purpose (McClements, 2005).

Table 5. Curcumin nano-encapsulation stability under 4°C storage condition, measure by HPLC.

	1% curcumin emulsion	1.5% curcumin emulsion
1 hour	77.5%	71.5%
28 hours	65.8%	63.0%
60 hours	64.4%	61.2%
84 hours	62.8%	60.2%
132 hours	61.1%	58.6%

4.3.2. Oral Bioavailability

TPA-induced mouse ear inflammation model was used as a quick test of anti-inflammatory activity of drug. A single oral administration of curcumin nano-emulsion before TPA treatment could inhibit TPA-induced edema of mouse ears. Inflammation in ears was evaluated by measuring average weight of ear punches. The reduced inflammation showed as lighter weight of ear punches. Two dosages, 0.7 ml of 1% curcumin emulsion and 0.7 ml of 1.5% curcumin emulsion were fed to mice by oral gavages. The curcumin amounts in the two doses were 7mg and 10.5 mg per mouse. Figure 27 showed average weight of ear punches after 6 h of TPA treatment. Both doses of nano-emulsified curcumin could inhibit inflammation completely.

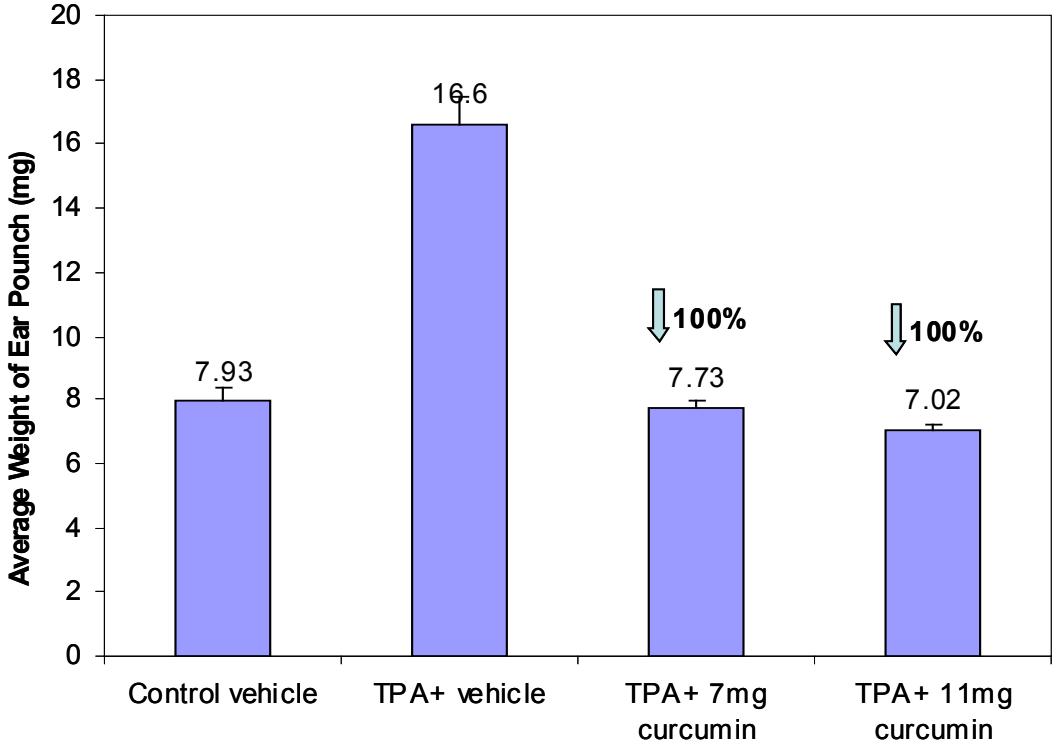


Figure 27. Inhibitory effect of oral administration of nano-curcumin emulsion on TPA induced ear inflammation in CD-1 mice.

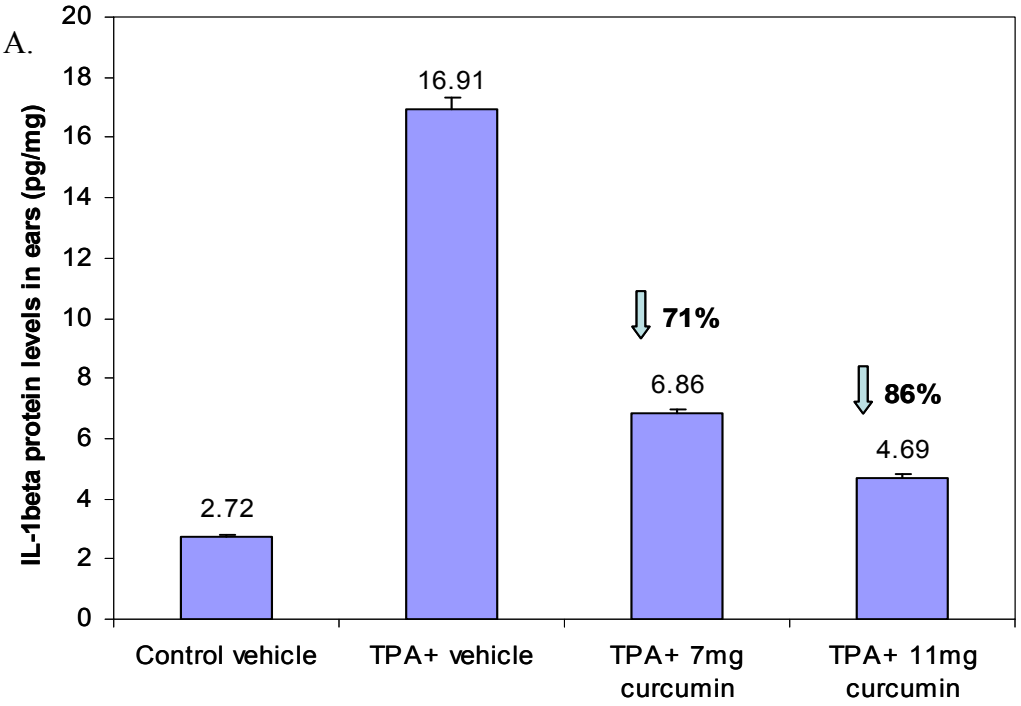
Topical application of TPA to ears of the mice induced inflammation and also up-regulated the expression of pro-inflammatory cytokines, and matrix metalloproteinases (MMP) levels in ear. Oral administration of 0.7 ml of 1% curcumin emulsion and 0.7 ml of 1.5% curcumin emulsion could block TPA-induced up-expression of IL-6 by 63% and 84%, IL-1beta by 71% and 86%, and MMP-9 by 61% and 91% (Figure 28 A, B and C), respectively. Another cell cycle regulated protein cyclin D1 induced by TPA on mouse ear was evaluated through Western Blotting analysis method. Figure 29 showed clearly that oral administration of the two doses of nano-emulsified curcumin could inhibit the expression of cyclin D1 and the higher dose, 10.5 mg curcumin, repress the expression more. Further protein quantification could be done on image analysis software.

It has been reported (Garcea et al., 2004) curcumin was poorly available in blood after oral administration of curcumin/water emulsified with Tween 20. An earlier experiment (result not shown) demonstrated that oral intake oil-dissolved curcumin didn't inhibit TPA-induced mouse ear inflammation. Whereas, the strong anti-inflammatory activity of current formulation directly confirmed that nano-encapsulated curcumin was bioavailable. Nano-encapsulated curcumin was absorbed in small intestine, transferred to ears through circulation system, and acted on the inflammation site.

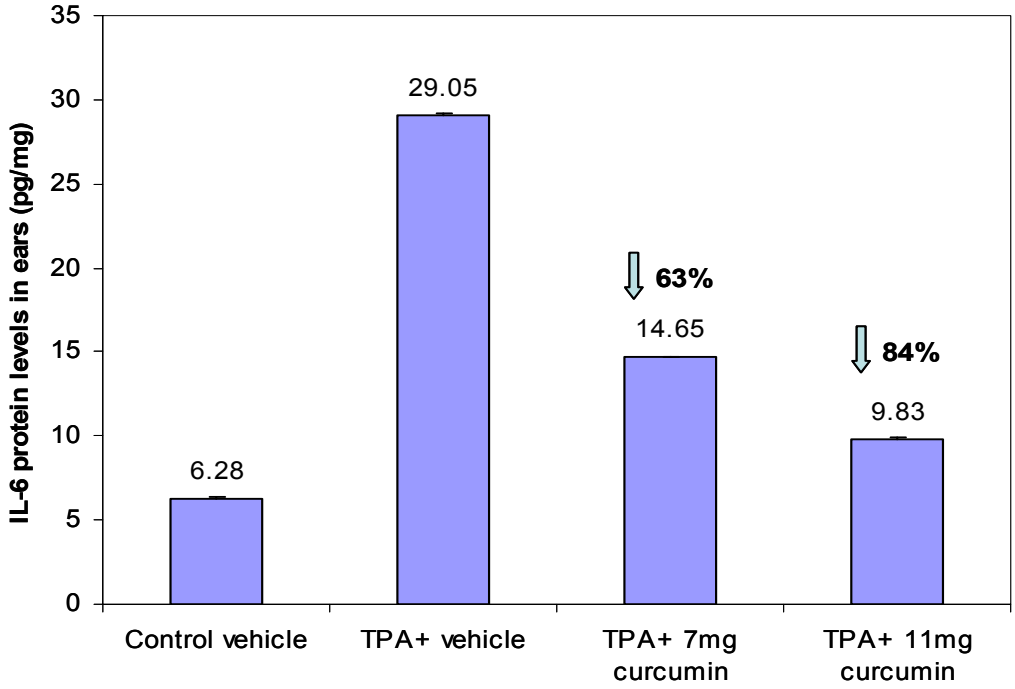
The oral delivery system should be able to carry the drugs through the harsh pH, ionic and enzymatic environments in the gastrointestinal tract (Charman, 1992). The possible mechanisms of absorption of lipid-based delivery system have been reviewed in the introduction part. Furthermore, nano-sized delivery system may present slightly different mechanisms and exhibit higher and more rapid delivery efficacy. Nano-sized particles are able to adhere to biological surface, such as the epithelial gut wall

(Kawashima, 2001). In this study we minimized the emulsion into less than 200nm size. Thus the nano-size curcumin emulsion might adhere to the epithelial wall or at least have higher diffusion rate in small intestine. Curcumin was then absorbed through lipid absorption pathway and entered the circulation, which reduced the first-pass metabolism. In this way, curcumin bypassed the inactivation (conjugation and modification) in liver, and was transferred to act on the inflammation site. To confirm the assumption and get better understanding of the mechanism more in vitro and in vivo tests are needed, such as caco-2 cell culture, duodenum elution study, and curcumin metabolite structure identification from the blood. To demonstrate the bioavailability, a pharmacokinetics study is also necessary.

A.


IL-1beta protein levels in ears (pg/mg)
20
18
16
14
12
10
8
6
4
2
0
16.91
71%
6.86
86%
4.69
2.72
Control vehicle
TPA+ vehicle
TPA+ 7mg curcumin
TPA+ 11mg curcumin

B.


IL-6 protein levels in ears (pg/mg)
35
30
25
20
15
10
5
0
29.05
63%
14.65
84%
9.83
6.28
Control vehicle
TPA+ vehicle
TPA+ 7mg curcumin
TPA+ 11mg curcumin

C.

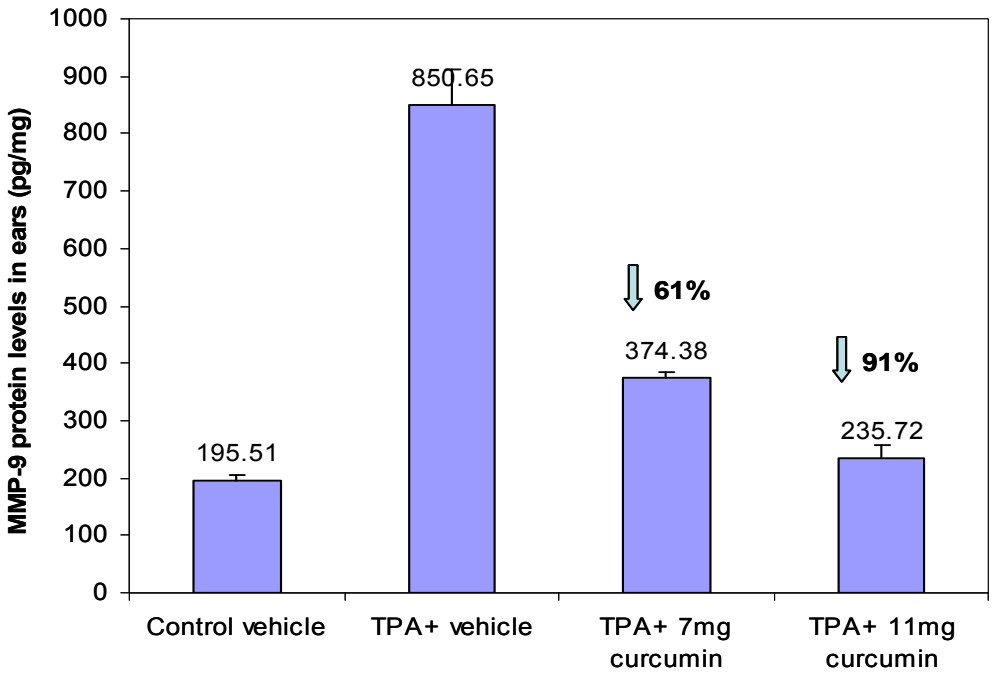


Figure 28. Inhibitory effect of oral administration of nano-curcumin emulsion on TPA induced formation of A. IL-1beta, B. IL-6, C. MMP-9, protein levels in ears of CD-1 mice.

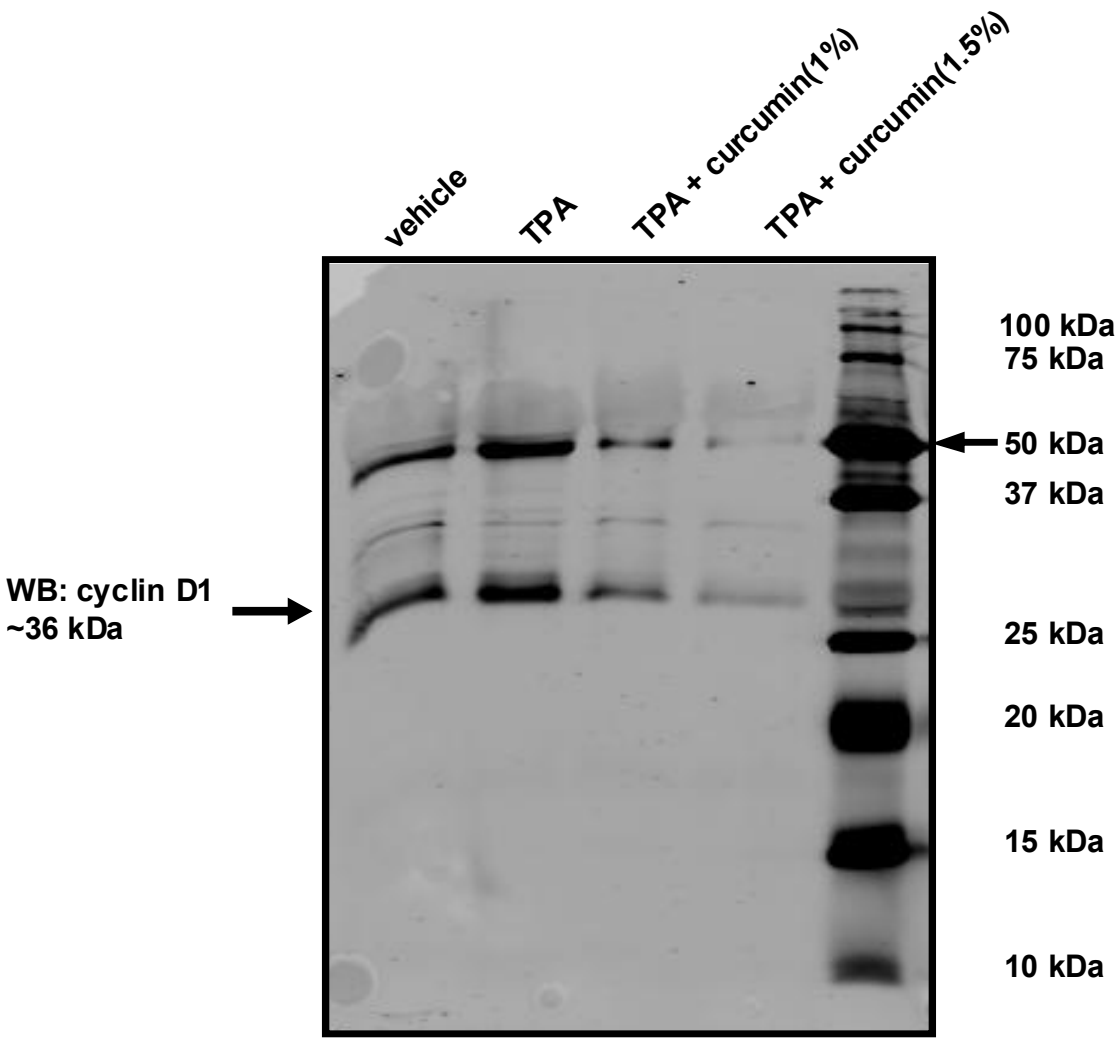


Figure 29. Oral administration of curcumin nano-emulsion could repress cyclin D1 inducted by TPA in CD-1 mouse ear. One hundred μg/well protein samples were subjected to immunoblotting for cyclin D1. The primary antibody was diluted in the Odyssey blocking buffer (1:1000).

4.4. Enzyme Encapsulation

4.4.1. Complex Coacervation of κ-Carrageenan and α-Amylase

In this research κ-carrageenan was used to coacervate with α-amylase (EC. 3.2.1.1) and controlled release of α-amylase. Some initial studies had been conducted revealing that κ-carrageenan was a great candidate for coacervation encapsulation (result showed in section 4.1). We tested lambda, iota and kappa carrageenan in formation of coacervates with protein. λ- and ι-carrageenan had higher charge densities than κ-carrageenan, thus they strongly interacted with protein, which resulted in difficulty in dissociation. Whereas, κ-carrageenan was less charged and performed very well during coacervate complexation and dissociation.

Firstly, we studied the coacervation characteristics of κ-carrageenan/α-amylase coacervate. Figure 30 showed turbidimetric titration curves of 0.5g/L κ-carrageenan and 1.0g/L α-amylase at 1:1, 1:2, 1:3 ratios, and at an ionic strength of 0.1M NaCl. The titration started from above pH 8, and the curve showed a sudden increase in turbidity at around pH 4.4. At high pH, both κ-carrageenan and α-amylase were both negatively charged. Thus, the molecules repulsed to each other. As pH decreased the protein, α-amylase, gradually gained positive charges. The positively charged amino acid groups, which were like positive patches on the proteins, started interacting with κ-carrageenan. When the pH decreased more, more electrostatic interaction established. The isoelectrical point (pI) of α-amylase was 4.2. At pH below pI, α-amylase and κ-carrageenan interacted strongly and started precipitation. This experiment also identified the best coacervation ratio of carrageenan and amylase, which was the most coacervate yield ratio. The maximum yield was found at 1:2 ratio (0.5g/L κ-carrageenan : 1.0g/L α-

amylase), which was much higher than 1:1 ratio, but almost the same as 1:3 ratio. Considering the encapsulation efficiency, 1:2 ratio was chosen for the further study. More detail about encapsulation efficiency would be discussed later with Figure 34.

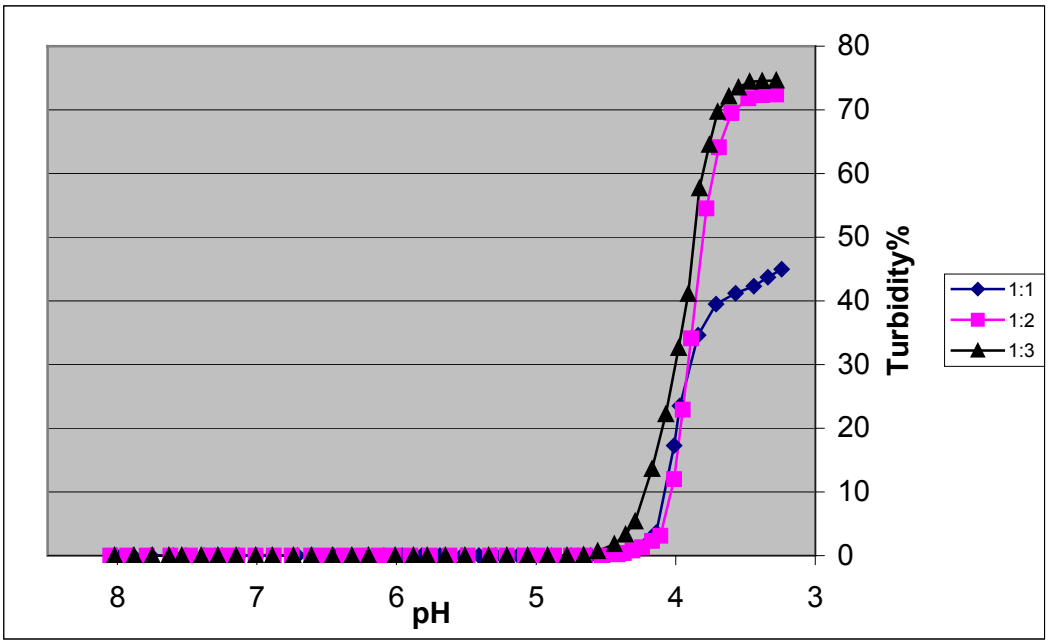


Figure 30. Turbidimetric titration curves 0.5g/L κ-carrageenan and 1.0g/L α-amylase at 1:1, 1:2, 1:3 ratio in 0.1M NaCl. The best ratio of carrageenan to amylase was 1:2.

Experiment in Figure 31 was to select the best ionic strength condition for coacervation. It was conducted as 0.5g/L κ-carrageenan and 1.0g/L α-amylase at 1:2 ratio in different ionic strength solution, 0.01M, 0.05M and 0.1M NaCl. Salt had a screening effect on the charges of the molecules and would weaken protein-polysaccharide interactions. At lower ionic strength, complex coacervation started formation at higher pH and yielded larger amount of coacervate. However, if the ionic strength was too low, protein and polysaccharide might have very strong interactions, which would be difficult to handle. In Figure 31, all three curves formed nicely. Therefore, we chose the highest yield condition to maximize the encapsulation efficiency. In 0.01M NaCl solution, the maximum coacervate formed at pH 3.2.

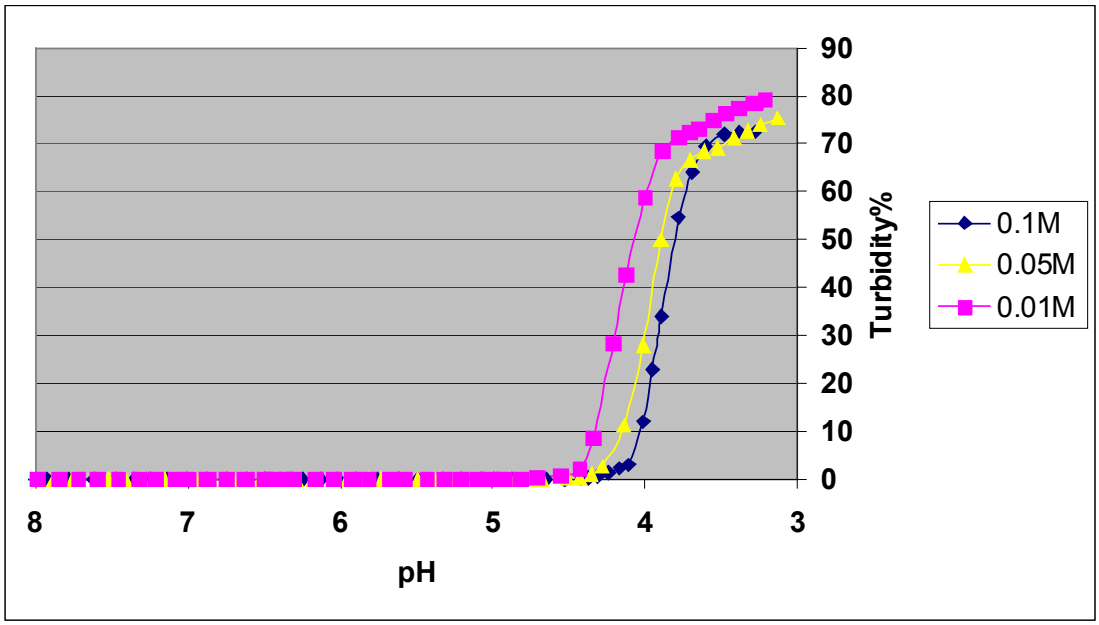


Figure 31. Turbidimetric titration curves for 0.5g/L κ-carrageenan and 1.0g/L α-amylase at 1:2 ratio in 0.01M, 0.05M and 0.1M NaCl. In 0.01M NaCl maximum coacervates form at pH 3.2.

4.4.2. Particle Sizes of α-Amylase and κ-Carrageenan Soluble Complex

Figure 32 showed Dynamic Light Scattering (DLS) measurement of particle size of κ-carrageenan and α-amylase during titration. The unassociated molecules have particle sizes about 70nm. As mentioned previously, at pH above pI some amino acids were already positively charged. The positively charged amino acids interacted loosely with carrageenan, which was negatively charged. Therefore, soluble complex between κ-carrageenan/α-amylase formed. At this stage, the turbidity change was not detectable, however, particle sizes and light reflection intensity (counts) increased. Figure 32 showed at pH around 5.5 the particle sizes started increasing, which indicated soluble complex formation. Figure 33 placed particle sizes together with turbidimetric titration curve. Particle sizes hiked at pH 5.5, and the turbidity increased at pH 4.4. Between pH 5.5 and 4.4, the two polymers formed soluble complex.

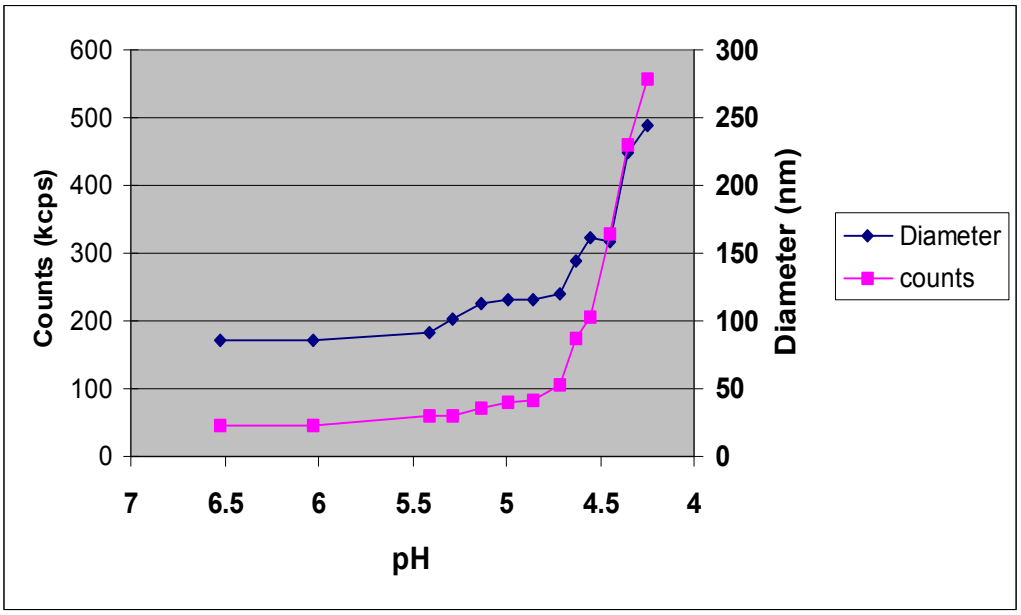


Figure 32. Particle sizes and intensity counts of κ-carrageenan and α-amylase during titration measured by Dynamic Light Scattering Particle Size Analyzer. Sizes and counts started increasing at pH 5.5.

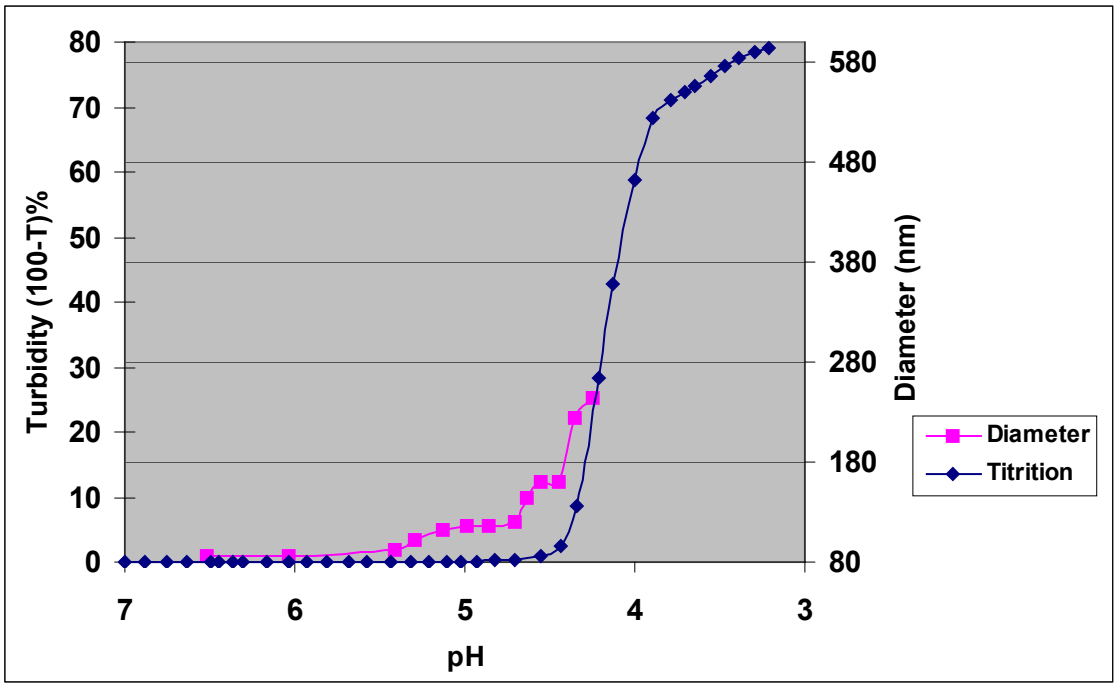


Figure 33. Particle sizes measured by DLS verses turbidity measured by colorimeter during titration showed soluble complexes formed at pH 5.5 and turbidity increased at pH 4.4.

4.4.3. Encapsulation Efficiency

Encapsulation efficiency was determined by Bio-Rad protein assay for protein content. The efficiencies of encapsulated and free enzyme were defined as:

Encapsulation Efficiency % = Encap. Enzyme / Total Enzyme

Free Enzyme % = Non-Encap. Enzyme / Total Enzyme

Table 6 showed encapsulation efficiency and yield of the enzyme at κ-carrageenan to α-amylase ratios of 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:4 and 1:5. The highest efficiency reached at 1:1, 1:1.5 and 1:2, as 99.27%, 99.5%, and 99.3%, respectively. When amylase ratio increased, the efficiency decreased. The encapsulation yield was defined as the amount of coacervated α-amylase. The yield increased with the amount of enzyme input. In 10ml solution at 1:5 ratio, 0.0432g amylase could be encapsulated, which was 87% efficiency. However, in current study, we tried to balance the efficiency and yield of the encapsulation. Thus, 1:2 ratio was chosen for the following experiments. The coacervation method was a very effective tool to encapsulate enzyme. The efficiency of 99.3% was much higher than other reported enzyme encapsulation or immobilization methods.

Table 6. Encapsulation yield and efficiency of α-amylase coacervated with κ-carrageenan at pH 3.2. 0.5g/L κ-carrageenan to 1.0g/L α-amylase ratios were 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:4 and 1:5. The highest encapsulation efficiencies were at 1:1, 1:1.5, 1:2. Considering the yield 1:2 was chosen as working ratio.

ratio	**Protein Added (g)**	**Encap Protein, Yield (g)**	**Encap Efficiency**
1:1	0.01	0.00993	99.27%
1:1.5	0.015	0.0149	99.50%
1:2	0.02	0.0199	99.30%
1:2.5	0.025	0.0240	96.11%
1:3	0.03	0.0283	94.41%
1:4	0.04	0.0363	90.64%
1:5	0.05	0.0432	86.34%

4.4.4. Enzyme Activity and Kinetics

Three α-amylase solutions were prepared as described in Method part, the first one was free amylase without any treatment; the second was coacervated with κ-carrageenan, treated with pH 3 for half an hour and then released from coacervate; and the third was the free enzyme exposed to pH 3 for half an hour and then adjusted back to pH 6.9. Amylase activity was defined as hydrolysis speed of starch. Figure 34 showed the activities of three α-amylases. The speed was measured by disappearance of starch during reaction. The curve could tell that α-Amylase released from coacervates maintained almost the same catalytic activity as the control enzyme. Whereas, α-amylase without encapsulation but exposed to acid for 0.5 hour, lost most of its activities. The result clearly indicated that coacervation protect amylase from acid denaturation.

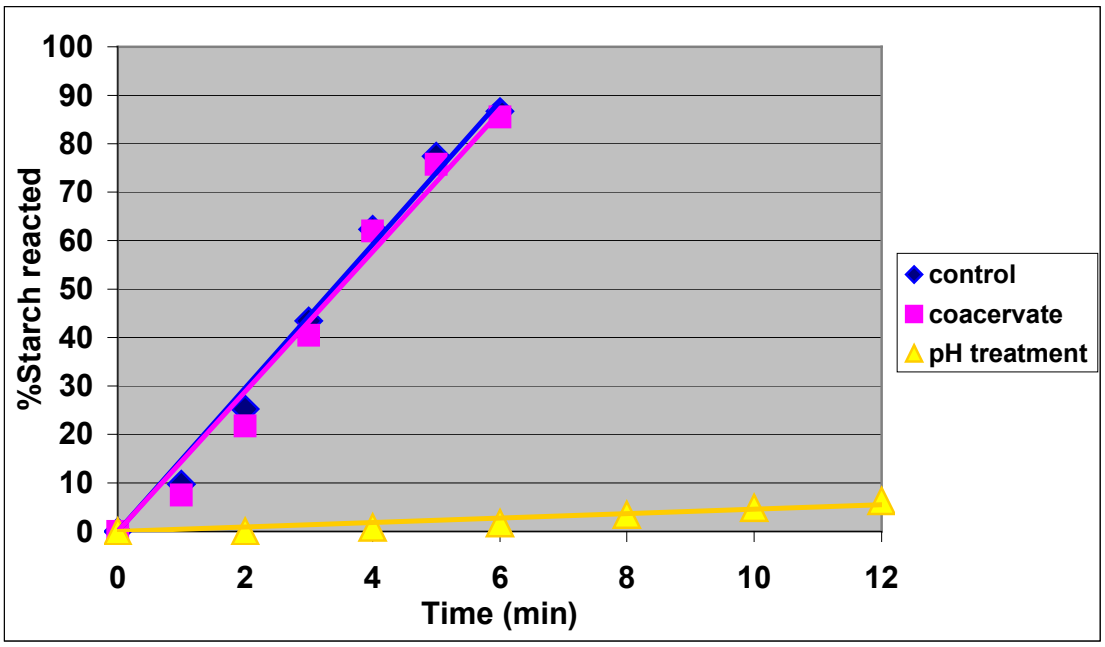


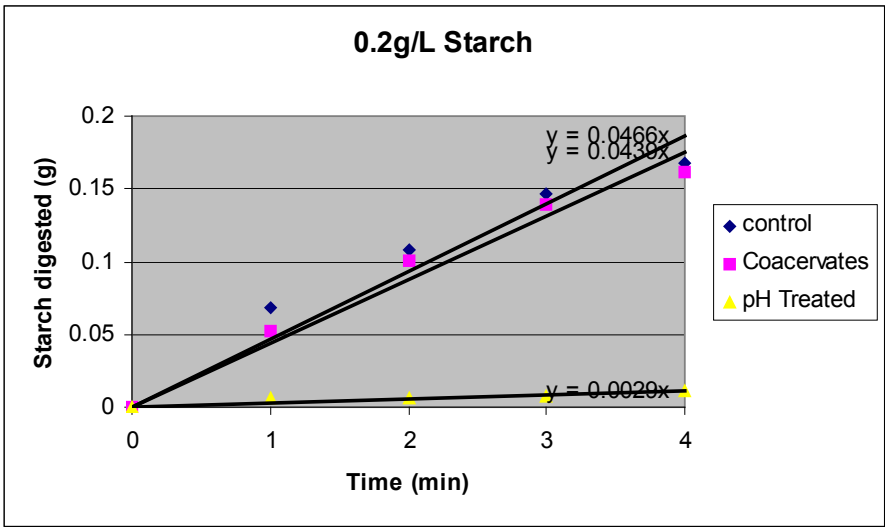
Figure 34. Starch digestive rate of 1. native α-amylase, 2. α-amylase released from coacervate, and 3. acid treated α-amylase.

Enzyme kinetics confirmed further of the amylase activity. Five concentrations of the substrate, starch, had reacted with three amylases. Figure 35 showed all five substrate concentrations, which presented similar result as Figure 34. Lineweaver-Burke plots in Figure 36 were developed from the data collected in Figure 35 by plotting 1/[Starch] against 1/rate. K_m, the Michaelis-Menten constant, and r_{max}, the maximal hydrolysis rate were then calculated from the Lineweaver-Burke plots with the following equation, $1/r = K_m/r_{max}[S] + 1/r_{max}$. Km of native enzyme was 1.08g and Vmax was 0.31g/(min · g enzyme), Km of encapsulated enzyme was 1.07g and Vmax was 0.28g/(min · g enzyme), and Km of the acid treated enzyme was 0.04g and Vmax was 0.0036g/(min · g enzyme),. At 0.8g/L and 1.0g/L starch, acid treated enzyme's activity was too low to measure. Also in the L-B plots the acid treated enzyme curve was far away from the other two, thus it was not presented in Figure 36. Encapsulated α-amylase had lost very little amount of activity, and exhibited similar enzyme kinetics as the native amylase, but the acid treated α-amylase was almost completely denatured. These evidences showed that coacervate encapsulation was an effective method to encapsulate enzyme and protect enzyme from acidic denaturation. The maintained activity of released α-amylase also suggested that coacervation process did not affect the catalytic binding sites of the enzyme. It indicated that the enzyme was kept its three dimensional structure during coacervation. Further confirmation from protein structure spectroscopy might be necessary.

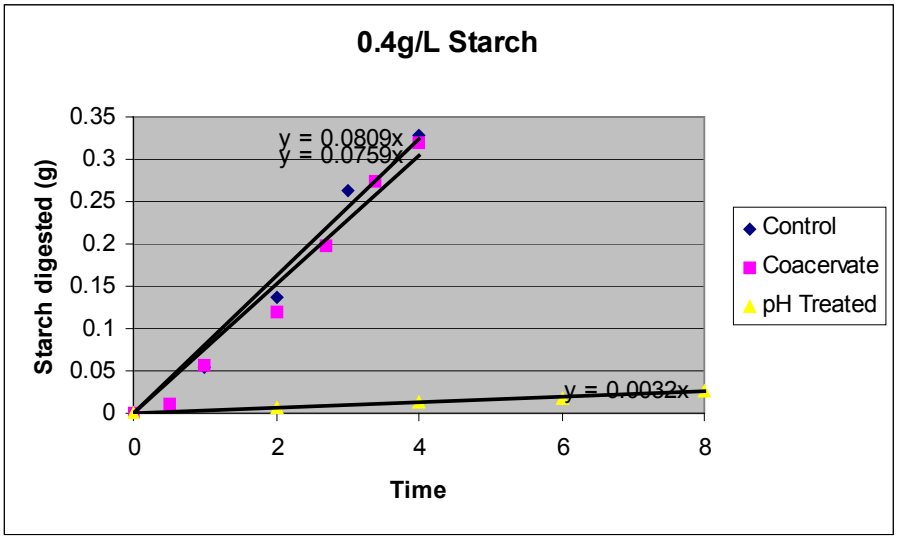
Heat was another denaturation factor. We had tried to study the enzyme activity after heating, but coacervate became a very stable co-polymer and could not release. During heating besides established electrostatic interactions, hydrogen bonding and van

der Waals interaction might form between molecules. With such strong interactions, the protein and polysaccharide could not be dissociated by only adjusting pH. Furthermore, other chemical denaturing factors could be investigated later.

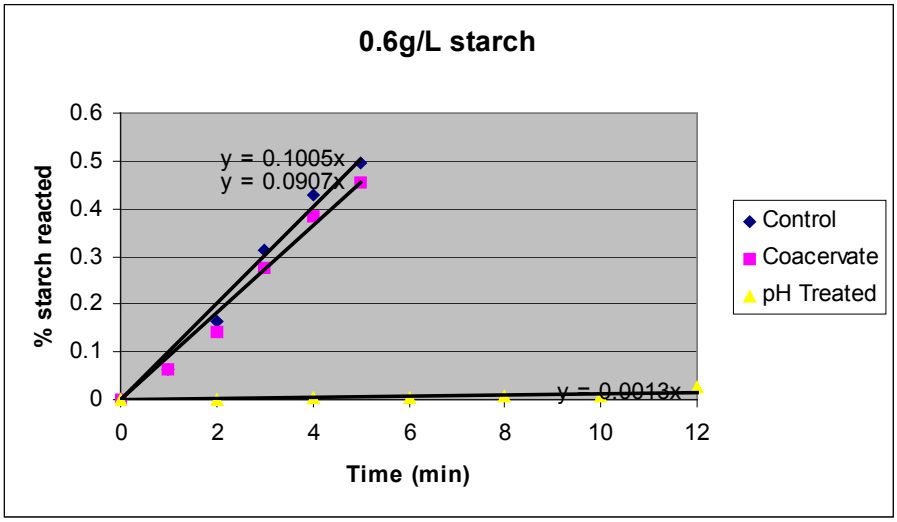
A.


0.2g/L Starch
Starch digested (g)
Time (min)
y = 0.0466x
y = 0.0438x
y = 0.0029x
control
Coacervates
pH Treated

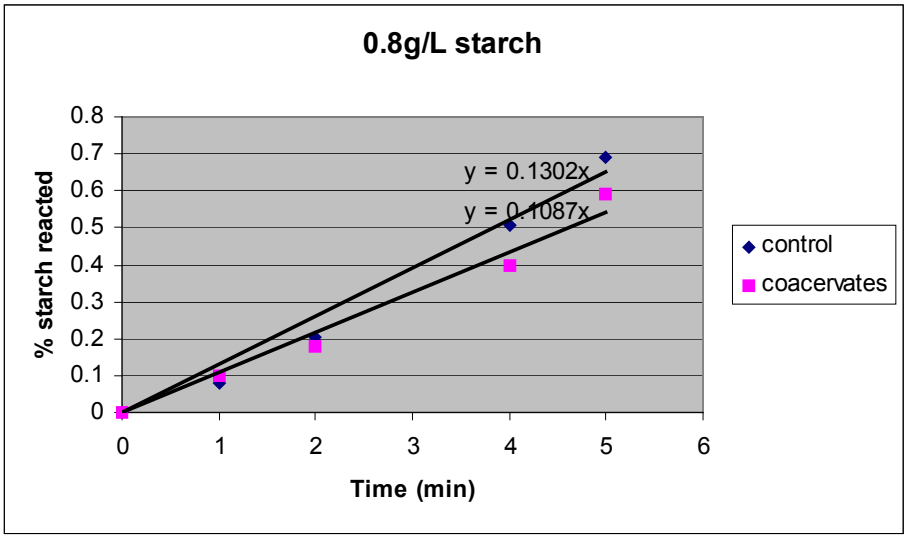
B.


0.4g/L Starch
Starch digested (g)
Time
y = 0.0809x
y = 0.0759x
y = 0.0032x
Control
Coacervate
pH Treated

C.


0.6g/L starch
% starch reacted
Time (min)
y = 0.1005x
y = 0.0907x
y = 0.0013x
Control
Coacervate
pH Treated

D.



E.

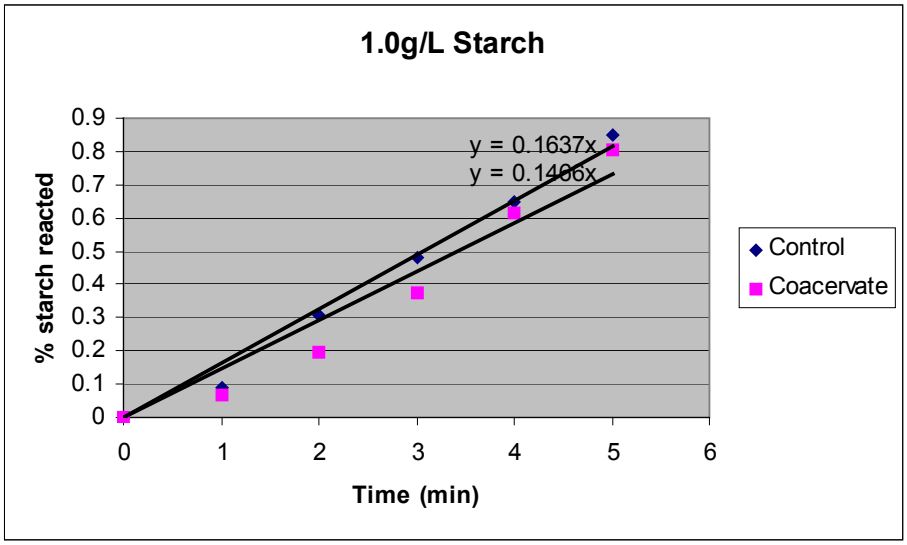


Figure 35. Enzyme kinetics of 1. native α-amylase, 2. α-amylase released from coacervate, and 3. acid treated α-amylase, at different substrate concentration, A. 0.2g/L, B. 0.4g/L, C. 0.6g/L, D. 0.8g/L and E. 1.0g/L starch.

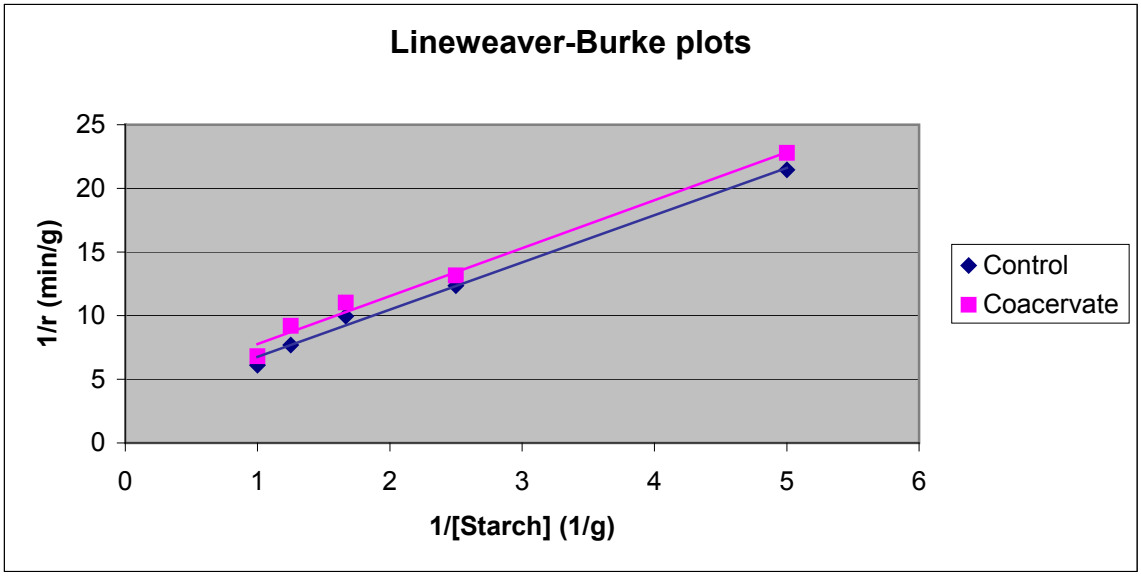


Figure 36. Lineweaver-Burke plots of native α-amylase and α-amylase released from coacervate, developed from enzyme kinetics plots in Figure 6. The curve of acid treated α-amylase was not shown on this figure.

Chapter 5

Conclusions and Future Works

5.1. Catechins Emulsion

Catechins were successfully encapsulated in two double emulsion systems. The first system was using gelatin-A and k-carrageenan coacervate as encapsulation materials. The formation process and coacervate properties had been understood at the beginning. Nano size W1/O catechins emulsion was encapsulated in coacervate at pH 3.2, in 0.1M NaCl. The encapsulation efficiency was 90.5%, yet this method gave low encapsulation yield. The encapsulation was stable in stomach juice, and catechins could be released rapidly into small intestine juice *in vitro*. The second system was to dissolve catechins in water-saturated triacetin and emulsified into canola/brominated oil and further emulsified into water with gum Arabic and surfactants. The formation process of this system was relatively simple and could reach 1-1.5% catechins in the final emulsion. Some conditions affecting particle sizes had been studied. Higher homogenizing speed and longer time could produce smaller particles. This system could reach as high as 94.5% encapsulation efficiency. All materials used in the current studies were food grade, which was friendly for nutraceuticals.

More conditions should be explored to reduce the particle sizes and increase the homogeneity. If the particle sizes are reduced, the stability of the system will be improved accordingly. In the future studies, the emulsion can be fed to TPA-induced ear inflammation mouse model to check its activity in the body. If it shows inhibition on mouse ear edema, a set of pro-inflammatory targets will be checked for the inhibitory effects to the biomarkers. The targets includes tumor necrosis factor α, interleukin-1β,

interleukin-6, nuclear factor kappa B, cellular adhesion molecules, cyclooxygenase-2, 5-lipoxygenase, and phospholipase A2. Further more, blood concentration and organ distribution of catechins or their metabolites can be studied.

5.2. Curcumin Emulsion

Curcumin was dissolved in MCT and further emulsified in water. Processed with high-pressure homogenizer, the average particle sizes of curcumin emulsion could be reduced to about 150nm. Encapsulation efficiencies of the systems were 77.5% for 1% curcumin emulsion and 71.5% for 1.5% emulsion. The system had been fed to mice and showed inhibition of TPA-induced mouse ear edema by 100%, and significantly inhibited pro-inflammatory factors IL-1beta, IL-6, MMP-9, and cyclin D1 dose-responsively.

What can be done next include increasing stability, increasing encapsulation efficiency, and elucidating metabolism pathway of nano-emulsified curcumin. Emulsion stabilizers might be used to enhance the stability and efficiency. Caco-2 cell culture can be used to demonstrate the absorption mechanisms. More oral *in vivo* tests are needed to illustrate the pharmacokinetics, and absorption, distributeion, metabolism and excretion (ADME) of nano-emulsified curcumin.

5.3. Enzyme Encapsulation

α-Amylase was encapsulated with coacervate, which could protect the enzyme from acid denaturation. High encapsulation efficiency, 99.3%, was achieved with current conditions. The maintained activity of enzyme also showed that coacervation process would not affect the enzyme catalytic function, indicating the three dimensional structure of enzyme was not touched. The current encapsulation method may have broad use in industry to recover the enzyme and prevent chemical denaturation of enzyme.

References

Ahmad, N., Katiyar, S. K., and Mukhtar, H. Cancer chemoprevention by tea polyphenols, in: C. Ionnides (Ed.), Nutrition and chemical toxicity, Wiley, West Sussex, UK, 1998, 301-343.

Anand, P., Kunnumakkara, A. B., Newman, R. A. and Aggarwal, B. B. Bioavailability of curcumin: problems and promises. Molecular Pharmaceutics 2007, 4 (6), 807-818.

Antonov, Y. A., Lashko, N. P., Glotova, Y. K., Malovikova, A., and Markovich, O. Effect of the structural features of pectins and alginates on their thermodynamic compatibility with gelatin in aqueous media. Food hydrocolloids 1996, 10(1), 1-9.

Becher, P., Encyclopedia of Emulsion Technology, V1, Basic Theory, New York, Marcel Dekker Inc., 1983

Becher, P., Encyclopedia of Emulsion Technology, V2, Application, New York, Marcel Dekker Inc., 1985.

Benichou, A., Aserin, A., and Garti, N. Protein-polysaccharide interations for stabilization of food emulsions. Journal of Dispersion Science and Technology. 2002, 23(1-3), 93-123

Bisht, S., Feldmann, G., Soni, S., Ravi, R., Karikar, C., Maitra, A., and Maitra, A. Polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"): a novel strategy for human cancer therapy. Journal of Nanobiotechnology 2007, 5, 3.

Bungenber de Jong, H. G. In La Coacervation Complexe et son importance en biologie. Faure-Fremiet, E., Ed., Paris; Hermann et Cie, 1936, 1,1.

Bungenber de Jong, H. G. In Colloid Science; Kruyt, H. R., Eds.; Elsevier: Amsterdam, 1949; Vol. II, Chapters Viii and X.

Burgess, D. J. and Carless, J. E., J. Microelectrophoretic studies of gelatin and acacia for the prediction of complex coacervation. Colloid Interface Sci., 1984, 98 (1), 1-8.

Burgess, D. J. and Carless, J. E., Manufacture of gelatin/gelatin coacervation microcapsules. International Journal of Pharmaceutics 1985, 27, 61-70.

Burgess, D. J., and Carless, J. E. In Coulombic Interactions in Macromolecular Systems. Eisenberg, A. and Bailey, E. F., Eds.; Washington, DC; ACS Symposium Series 302, 1986, 251.

Burgess, D. J. Complex coacervation: micro-capsule formation. In Macromolecular Complexes in Chemistry and Biology. Dubin, P. L., Bock, J., Davis, R., Schulz, D. N., and Thies, C., Eds.; Berlin: Springer Verlag, 1994, 281-300.

Charalambous, G., and Doxastakis, G., Food Emulsifiers: Chemistry, technology, functional properties and applications. Amsterdam: Elsevier Science; 1989

Charman, W. N. Lipid vehicle and formulation effects on intestinal lymphatic drug transport. In Lymphatic Transports of Drugs, Charman, W. N., and Stella, V. J., Eds; CRC Press, Coca Raton, 1992, 113-179.

Chen, J. J., Ye, Z.-Q., and Koo, M. W. L. Growth inhibition and cell arrest effects of epigallocatechin gallate in the NBT-II bladder tumour cell line. BJU International 2004, 93(7), 1082-1086.

Chilvers, G. R. and Morris, V. H. coacervation of gelatin-gellan gum mixtures and their use in microencapsulation. Carbohydrate Polymers 1987, 7, 111-120.

Chun, A. H. C., Joslin, R. S., and Martin, A. N. The high hydrophile-lipophile balance of gum, Part I, Drug and Cosmetics Industry 1958, 82, 164.

Cournarie, F., Savelli, M., Rosilio, V., Bretez, F., Vauthier, C., Grossiord, J., and Seiller, M. Insulin-loaded W/O/W multiple emulsions: comparison of the performances of systems prepared with medium-chain-triglycerides and fish oil. European Journal of Pharmaceutics and Biopharmaceutics 2004, 58 (3), 477-482.

Cowhig, J. The World Under the Microscope. New York: Bounty, 1974.

Dalgleish, A. G., and O'Byrne, K. J. Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer. Advanced Cancer Research 2002, 84, 231-276.

Daniels, R., and Mittermaier, E. M. Influence of pH adjustment on microcapsules obtained from complex coacervation of gelatin and acacia. Journal of Microencapsulation 1995, 12(6), 591-599.

Deasy, P. B. Microencapsulation and Related Drug Process, Marcel Dekker: New York and Basel, 1984.

Donati, I., Holtan, S., Mørch, Y. A., Borgogna, M., Dentini, M. and Skjåk-Bræk, G. New hypothesis on the role of alternating sequences in calcium-alginate gels, *Biomacromolecules* 2005, 6, 1031-1040.

Dong, C., and Rogers, H. A. Acacia-gelatin microencapsulated liposomes: preparation, stability, and release of acetylsalicylic acid. Pharmaceutical Research 1993, 10(1), 141-146.

Faergemand, M., and Krog, N. Interactions of emulsifiers with other components in foods. Food Science and Technology (Boca Raton, FL, United States) 2006, 154 (Ingredient Interactions (2nd Edition)), 389-421.

Fang, J-Y., Lee, W-R., Shen, S-C., and Huang, Y-L. Effect of liposome encapsulation of tea catechins on their accumulation in basal cell carcinomas. Journal of Dermatological Science 2006 May v42 (2): 101-109.

Farinati, F., Cardin, R., Russo, V. M., Busatto, G., Franco, M., and Rugge, M. Helicobacter pylori CagA status, mucosal oxidative damage and gastritis phenotype: a potential pathway to cancer? Helicobacter 2003, 8, 227-234.

Friedman, M. and Jurgens, H. S. Effect of pH on the stability of plant phenolic compounds. J Agric Food Chem. 2000 Jun; 48 (6):2101-10.

Fujiki, H., Suganuma, M., Imai, K., Nakachi, K., Green tea: cancer preventive beverage and/or drug. *Cancer Letters* 2002 (188): 9-13.

Gallarate, M., Chirio, D., Trotta, M., and Carlotti, E., Deformable liposomes as topical formulations containing α-tocopherol. Journal of Dispersion Science and Technology 2006 27:703-713.

Gallarate, M., Disperse systems as topical formulations containing α-tocopherol Journal of Drug Delivery Science and Technology 2004 14:6 471-477.

Gao, Y-T., Shen, P-Y., Wang, B-H., Lu, S-D., Huang, G-C., and Ho, L-S. Controlled release effect of insecticide microcapsules and their results in common household insect pest control. Journal of Microencapsulation 1984, 1(4), 307-315.

Garcea, G., Jones D. J. L., Singh, R., Dennison, A. R., Farmer, P. B., Sharma, R. A., Steward, W. P., Gescher, A. J. and Berry D. P. Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. British Journal of Cancer 2004 (90): 1011-1015.

Gerlat, P. Beverage stabilizer. In Food Product Design 2006, October.

Gibbs, B. F., Kermasha, S., Alli, I., and Mulligan, C. N. Encapsulation in the food industry: a review. International J. of Food Sci. and Nutrition, 1999, 50, 213-224.

Goddard, E. D. Polymer/surfactant interaction. J. Soc. Cosmet. Chem., 1990, 41, 23-49.

Gupta, S., Saha, B., and Giri, A. K. Comparative antimutagenic and anitclastogenic effects of green tea and black tea: a review. Mutation Research 2002, 512, 37-65.

Hansen, P. M. T., Hidalgo, J., and Gould, I. A. Reclamation of whey protein with carboxymethylcellulose. Journal of Dairy Science 1971, 54(6), 830-834.

Hidalgo, J., and Hansen, P. M. T. Selective precipitation of whey proteins with carboxymethylcellulose. Journal of Dairy Science 1971, 54(9), 1270-1274.

Holder, G. M., Plummer, J. L., and Ryan, A. J. The metabolism and excretion of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) in the rat. Xenobiotica 1978, 8 (12), 761-768.

Hong, J., Bose, M., Ju, J., Ryu, J-H.., Chen, X., Sang, S., Lee, M-J. and Yang, C.S. Modulation of arachidonic acid metabolism by curcumin and related β-diketone derivatives: effects on cytosolic phospholipase A2, cyclooxygenases and 5-lipoxygenase. Carcinogenesis Advance Access 2004, 25(9), 1671-1679.

Hou, W., and Papadopoulos, K. Y. W/O/W and O/W/O globules stabilized with Span 80 and Tween 80. Colloids and Surfaces A: Physicochemical and Engineering Aspects 1997, 125, 181-187.

Hsu, C. H., and Cheng, A. L. Clinical studies with curcumin. Adv. Exp. Med. Biol. 2007, 595, 471-480.

Huang, M-T., Ghai, G., and Ho, C-T. Inflammatory process and molecular targets for anti-inflammatory nutraceuticals. Comprehensive reviews in food science and food safety 2004, 3, 127-139.

Huang, M-T., Liu Y., Ramji, D., Lo C-Y., Ghai, G., Dushenkov, S., and Ho, C-T. Inhibitory effects of black tea theaflavin derivatives on 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and arachidonic acid metabolism in mouse ears. Mol. Nutr. Food Res. 2006, 50, 115-122.

Huang, M-T., Newmark, H. L. and Frankel, K., Inhibitory effects of curcumin on tumorigenesis in mice. J. Cellular Biochem. 1998, 67 (S27), 26-34.

Imeson, A. P., Ledward, D. A., and Mitchell, J. R., On the nature of the interactions between some anionic polysaccharides and proteins. Journal of the Science of Food and Agriculture 1977, 28, 669-672.

Imerson, A. P., Watson, P. R., Mitchell, J. R. and Ledward, D. A. Protein recovery from blood plasma by precipitation with polyuronates. Journal of Food Technology. 1978, 13, 329-338.

Israelachvili, J. The science and applications of emulsions: an overview. Colloids and Surfaces A: Physicocyhemical and Engineering Aspects 1994, 91, 1-8.

Jizomoto, H., Kanaoka, E., Sugita, K., and Hirano, K. Gelatin-acacia microcapsules for trapping micro oil droplets containing lipophilic drugs and ready disintegration in the gastrointestinal track. Paramaceutical Research 1993, 10(8), 1115-1122.

Katiyar, S. K. and Mukhtar, H. Tea in chemoprevention of cancer: epidemiologic and experimental studies. International Journal of Oncology 1996, 8, 221-228.

Kanke, M., Simmons, G. H., Weiss, D. L., Bivins, B. A., and Deluca, P. P. Clearance of 141 Ce-labeled microspheres in specific organs following intravenous and intra-arterial administration in beagle dogs. Journal of Pharmaceutical Science 1980, 69, 755-762.

Kawashima, Y. Nanoparticulated systems for improved drug delivery. Advanced Drug Delivery Reviews, 2001, 47, 1-2

Kayes, J B. Pharmaceutical suspensions: micro electrophoretic properties. J. Pharm. Phamacol. 1977, 29 (3): 163-8.

Kelly, R., Gudo, E. S., Mitchell, J. R., and Harding, S. E. Someobservations on the nature of heated mixtures of bovine serum albumin with an alginate and apectin, Carbohydrate Polymers 1994, 23, 115-120.

Khanna, N. M. Turmeric – nature's precious gift. Current Science. 1999, 76, 1351-1356.

Khopade, A. J., and Jain, N. K. Emulsion as drug delivery system. In Advances in Controlled and Novel Drug Delivery. Jain, N. K. Ed,; India, 2001, 381.

Kim, M. K., Choi, G. J., and Lee, H. S. Fungicidal property of Curcuma longa L. rhizome-derived curcumin against phytopathogenic fungi in a greenhouse. J. Agric. Food Chem. 2003, 51 (6), 1578-1581

Kinsella J. E., and Whitehead D. M., Proteins in whey: chemical, physical, and functional properties. Adv Food Nutr Res. 1989; 33:343-438.

Kokufuta, E., Shimizu, H., and Nakamura, I. Salt linkage formation of poly (diallydimethylammonium chloride) with acidic groups in the polyion complex between human carboxyhemoglobin and potassium poly (vinyl alcohol) sulfate. Macromolecules, 1981, 14, 1178-1180.

Krog, N. H., and Sparso, F. V. Food emulsifiers: Their chemical and physical properties. In S. Friberg, K. Larsson, and J. Sjoblom, Eds., Food Emulsions (4th ed.), 2004, New York; Marcel Dekker, Chapter 2.

Krog, N. Food emulsifiers and their chemical and physical properties. Food Science and Technology (New York, NY, United States) 1997, 81 (Food Emulsions, (3rd Edition)), 141-188.

Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M. C. Potential anticancer activity of turmeric (Curcuma longa). Cancer Letter 1985, 29 (2), 197-202.

Lahaye, M. Developments on gelling algal galactans, their structure and physico-chemistry. Journal of applied Phycology 2001, 13, 173-184.

Lambert, J. D., Hong, J., Kim D. H., Mishin, V. M., and Yang, C. S. Piperine enhances the bioavailability of the tea polyphenol (-) Epigallocatechin-3-gallate in mice. The Journal of Nutrition 2004, 134, 1948-1952.

Lambert, J. D., Sang, S., Hong, J., Kwon, S.-J., Lee, M.-J., Ho, C.-T., and Yang, C. S. Peracetylation as a means of enhancing in vitro bioactivity and bioavailability of epigallocatechin-3-gallate. Drug Metabolism and Disposition Fast Forward 2006, 34, 2111-2116.

Lambert, J. D., and Yang, C. S. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 2003, V523-524, 201-208

Lenk, T. and Thies, C. Complex coacervation of acid-precursor gelatin with a polyphosphate. In Coulombic Interactions in Macromolecular Systems. Eisenberg A., Bailey, F. E., Eds, American Chemical Society: Washington DC, 1987, Chapter 8.

Li, L., Ahmed, B., Mehta, K., Kurzrock, R. Liposomal curcumin with and without oxaliplatin: effects on cell growth, apoptosis and angiogenesis in colorectal cancer. Mol. Cancer Ther. 2007, 6 (4), 1276-1282.

Li, L., Braiteh, F. S., Kurzrock, R. Liposome-encapsulatied curcumin: invitro and invivo effects on proliferation, apoptosis, signaling, and angiogenesis. Cancer 2005, 104 (6), 1322-1331.

Liu, A., Lou, H., Zhao, L., and Fan, P. Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. Journal of Pharmaceutical and Biomedical Analysis 2006, 40 (3), 720-727.

Lu, Y-P., Lou, Y-R., Xie, J-G., Peng, Q-Y., Liao, J., Yang, C. S., Huang, M-T. Topical applications of caffeine or (-)-epigallocatechin gallate (EGCG) inhibit carcinogenesis and selectively increase apoptosis in UVB-induced skin tumors in mice Pharmacology 2002 99 (19) 12455-12460.

Lucas-Abellan, C., Fortea, I., Lopez-Nicolas, J. M., and Nunez-Delicado, E. Cyclodetrins as resveratrol carrier system. 2007 104 (1): 39-44 .

Manach, C., Williamson, G., Morand, C., Scalbert, A. and Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. American Journal of Clinical Nutrition 2005: 81 (suppl): 230S-42S.

Matsumoto, S. Development of W/O/W-type dispersion phase inversion of concentrated W/O emulsions. Journal of Colloid Interface Science 1983, 94, 362-368.

Matsumoto, S., Koh, Y., and Michiura, A. Preparation of W/O/W emulsions in an edible form on the basis of phase inversion technique. Journal of Dispersion Science and Technology 1985, 6, 507-520.

Matsumoto, S. W/O/W-type multiple emulsions. In Nonionic Surfactants. Physical Chemistry. Schick, M. J., Ed, Marcell Dekker, Inc., New York, 1987, 549.

Matsumoto, S., and Kang, W. W. Formation and applications of multiple emulsions. Journal of Dispersion Science and Technology, 1989, 10(4-5), 455-482.

Mattison, W., Brittain, I. J., and Dubin, P. L. Protein-polyelectrolyte phase boundaries. Biotechnology Progress 1995, 11(6), 632-637.

McClements, D. J. Theoretical Analysis of Factors Affecting the Formation and Stability of Multilayered Colloidal Dispersions. Langmuir 2005, 21 (21), 9777-9785.

McGhie, T. K., Ainge, G. D., Barnett, L. E., Cooney, J. M. and Jensen, D. J. Anthocyanin glycosidates from berry fruit are absorbed and excreted unmetabolized by both human and rats. Journal of Agriculture and Food Chemistry 2003, 51, 4539-4548

Miller, M., Chen, S., Woodliff, J., and Kansra, S., Curcumin (diferuloylmethane) inhibits cell proliferatin, induces apoptosis, and decreases hormone level and secretion in pituitary tumor cells. Endocrinology 2008, 149 (8), 4158-4176

Mine, Y., Shimizu, M., and Nakashima, T. Preparation and stabilization of simple and multiple emulsions using a microporous glass membrane. Colloids and Surfaces B: Biointerfaces, 1996, 6, 261-268.

Mitchell, J. R., and Ledward, D. A. Functional Properties of Food Macromolecules. 1986, 440 pp.

Mukhtar, H., and Ahmed, N. Tea polyphenols: prevention of cancer and optimizing health. American Journal of Clinical Nutrition 2000, 71, 1698S-1720S.

Nakagawa, K. and Miyazawa, T. Absorption and distribution of tea catechin, (-)-epigallocatechin-3-gallate, in the rat. Journal of Nutritional Science and Vitaminology (Tokyo), 1997, 43(6), 679-684.

Nguyen, T. Q. Interactions of human hemoglobin with high-molecular-weight dexran sulphate and diethylaminoethyl dextrin. Makromol. Chem., 1986, 187, 2567.

Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederiksen, H., Enslen, M., Barron, D., Horcajada, M., and Williamson, G. Bioavialability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. The Journal of Nutrition 2006 136: 404-408.

Noguchi, H. Interactions of proteins with polymeric materials. Biochimica et Biophsica Acta 1956, 22, 459-462.

Noguchi, H. Interactions of serum albumin and synthetic polyelectrolytes in various buffer systems. Journal of Physical Chemistry 1959, 64, 185-187.

O'Byrne, K. J. and Dalgleish, A. G. Chronic immune activation and inflammation as the cause of malignancy. British Journal of Cancer 2001, 85, 473-483.

O`Byrne, K. J., Dalgleish, A. G., Browning, M. J., Steward, W. P., and Harris, A. L. The relationship between angiogenesis and the immune response in carcinogenesis and the progression of malignant disease. European Journal of Cancer 2000, 36, 151-169.

Okochi, H., and Nakano, M. Basic studies on formulation, method of preparation and characterization of water-in-oil-water type multiple emulsions containing vancomycin. Chemical and Pharmaceutical Bulletin 1996, 44(1), 180-186.

Onyeji, C. O., and Adesegun, S. A. Influence of viscosity on nitrofurantoin absorption from W/O/W emulsions. Indian Journal of Pharmaceutical Sciences 1995, 57(4), 166-169.

Onyeji, C. O., and Omotosho, J. A. Bioavailability of nitrofurantoin from multiple W/O/W emulsions in man and the influence of the oil phase of the emulsion. Indian Journal of Pharmaceutical Sciences 1993, 55(1), 14-18.

Onyeji, C. O.; Omotosho, J. A.; Ogunbona, F. A. Increased gastrointestinal absorption of griseofulvin from W/O/W emulsions. Indian Journal of Pharmaceutical Sciences 1991, 53, 256-258.

Overbeek, J. T. J., and Voorn, M. J. Phase separation in polyelectrolyte solutions. Theory of complex coacervation. Journal of Cell Component Physiology. 1957, 49(S1), 7.

Palma, M., Pineiro, Z., and Barroso, C. G. Stability of phenolic compounds during extraction with superheated solvents. J Chromatogr A. 2001 Jul 6;921 (2):169-74.

Pan, M-H., Huang, T-M., and Lin, J- K. Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metabolism and Disposition 1999, 27 (4), 486-494.

Pharham, P. Elements of the immune systems and their roles in defense. The Immune System, Chapter 1. New York, Garland Publishing, 2000, 1-13.

Park, J. M., Muhoberac, B. B., Dubin, P. L., and Xia, J. Effects of protein charge heterogeneity in protein-polyelectrolyte complexation. Macromolecules, 1992, 25(1), 290-295.

Pen, G., Wargovich, M. J., and Dixon, D. A. Anti-proliferative effects of green tea polyphenol EGCG on Ha-Ras-induced transformation of intestinal epithelial cells. Cancer Letters 2006, 238(2), 260-270.

Petkowicz, C. L. O., Reicher, F. and Mazeau, K. Conformational analysis of galactomannans: from oligomeric segments to polymeric chains, Carbohydr. Polym. 1998, 37, 25-39.

Piculell, L. and Lindman, B. Association and segregation in aqueous polymer/polymer, polymer/surfactant and surfactant/surfactant mixtures: similarities and differences. Advanced Colloid Interface Science. 1992, 41, 149-178.

Piculell, L., Bergfeldt, K. and Nisson, S. Factor determining phase behavior of multi-component polymer systems. In Biopolymer Mixtures. Harding, S. E., Hill, S. E., and Mitchell, J. R., Eds.; Nottingham; Nottingham University Press, 1995, 13-36.

Porter, C. J. H., and Charman, W. N. Uptake of drugs into the intestinal lymphatics after oral administration. Advanced Drug Delivery Reviews 1997, 25, 71-98

Porter, C. J. H., Pouton, C. W. Cuine, J. F., and Charman, W. N. Enhancing intestinal drug solubilisation using lipid-based delivery sytems. Advanced Drug Delivery Reviews 2008, 60, 673-691

Porter, C. J. H., Trevaskis, N. L., and Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drug. Nature Reviews. Drug Discovery 2007, 6 (3), 231-248

Ravindranath, V. and Chandrasekhara, N. Absorption and tissue distribution of curcumin in rats. Toxicology 1980, 16 (3), 259-265.

Salager, J. L. Formulation concepts for the emulsion maker. In Pharmaceutical Emulsions and Suspensions. Drug and Pharmaceutical Sciences Series, Nielloud, F. and Marti-Mestres, G., Eds, New York, Marcell and Dekker Inc. 2000, V 105, 19-71.

Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning, 2nd edition, Cold Spring Harbor Laboratory Press, 1989.

Sanchez, C. and Paquin, P. S. Protein and protein-polysaccharide microparticles. In Food proteins and their applications; Damoran, S., Paraf, A., Eds.; Marcel Dekker: New York; 1997; Chapter 17, 503-528.

Schimitt, C., Sanchez, C. Desobry-Banon, S. and Hardy, J. Structure and technofunctional properties of proein-polysaccharide complexes: a review. Critical Reviews in Food Science and Nutrition 1998, 38(8), 689-753.

Seifriz, W. Studies in emulsion. Journal of physical Chemistry, 1925, 29, 738

Serov, A. V., Antonov, Y. A., and Tolstoguzov, V. B. Isolation of lactic whey proteins in form of complexes with apple pectin. Nahrung 1985, 1, 19-30.

Sharma, O.P. Antioxidant activity of curcumin and related compounds. Biochem. Pharmacol. 1976, 25 (15), 1811-1812.

Shima, M., Tanaka, M., Kimura, Y., Adachi, S., and Matsuno, R. Enhancement in transport of a hydrophilic marker through intestinal epithelial cell (Caco-2) monolayer by W/O/W multiple emulsion containing C8TG. Food Hydrocolloids 2005, 19(2), 321-328.

Shively, M. L., and Thompson, D. C. Oral bioavailability of vancomycin solid-state emulsions. International Journal of Pharmaceutics 1995, 117 (1), 119-122.

Shukla, A. Gulumian, M., Hei, T. K., Kamp, D., Rahman, Q., and Mossman, B. T. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. Free Radical Biology and Medicine 2003, 34, 1117-1129.

Singh, R. P. and Agarwal, R. Flavonoid antioxidant silymarin and skin cancer. Antioxidants and Redox Signaling 2002, 4(4), 655-663.

Sluyter, R. and Halliday, G. M. Infiltration by inflammatory cells required for solar-stimulated ultraviolet radiation enhancement of skin tumor growth. Cancer Immunology, Immunotherapy 2001, 50, 151-156.

Srimal, R. C., and Dhawan, B. N. Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent. J. Pharm. Pharmacol. 1973, 25 (6), 447-452.

Stainsby, G. Proteinaceous gelling systems and their complexes with polysaccharides, Food Chemistry, 1980, 6, 3-14.

Stauffer, C. E. Emulsifers. St. Paul, MN; Eagen Press, 1999.

Stepanek, P. Data analysis in dynamic light scattering. In: W. Brown Ed., Dynamic Light Scattering: The Method and Some Applications, Oxford University Press Inc., Oxford, 1993, 177-241

Suguira, S., Nakajima, M., Yamamoto, K., Iwamoto, S., Oda, T., Satake, M., and Seki, M. Journal of Colloid Interface Science 2004, 270, 221.

Suresh, D., and Srinivasan, K. Sutdies on the in vitro absorption of spice principles – curcumin, capsaicin and piperine in rat intestines. Food Chemistry and Toxicology 2007, 45 (8), 1437-1442

Tan, C-T. Beverage emulsions. Food Science and Technology (New York, NY, United States) 2004, 132 (Food Emulsions (4th Edition)), 485-524.

Thies, C. Microcapsules as drug delivery devices. Critical Reviews in Biomedical Engineering 1982, 8, 335-383.

Tiebackx, F. W., Gleichzeitige Ausflockugn zweier Kolloide. Z. Chem. Ind Kolloide 1911, 8, 198.

Tirkkonen, S. Turakka, L., and Paronen, P. Microencapsulation of indomethacin by gelatin-acacia complex coacervation in presence of surfactants. Journal of Microencapsulation 1994, 11(6), 615-626.

Tiyaboonchai, W., Tungpradit, W., and Plianbangchang, P. Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. International Journal of Pharm. 2007, 337 (1-2), 299-306

Tolstoguzov, V. B. Functional properti9es of protein-polysaccharide mixtures. In Functional proterrties of Macromolecules. Mitchell, J. R. and Ledward, D. A., Eds.; London; Elsevier Applied Science Publishers, 1986, 385-415.

Tolstoguzov, V. B. Functional properties of food proteins and role of protein-polysaccharides interaction. Food Hydrocolloids 1991, 4, 429-468.

Tolstoguzov, V. B. Protein-polysaccharide interactions. In Food Proteins and Their Applications; Damodaran, S, Paraf, A., Eds.; Marcel Dekker, Inc. ; New York, 1997, 171-200.

Tsoga, A., Richardson, R. K. and Morris, E. R. Role of cosolutes in gelation of high methoxy pectin. Part 1. Comparison of sugars and polyols, *Food Hydrocolloids,* 2004, 18, 907-919.

Tso, P. Intestinal lipid absorption. In Physiology of the gastrointestinal tract, Johnson, L. R. Chief Ed.; Raven Press, New York, 1994, 1867-1908.

Tsushida, E. and Abe, K. Interactions between macromolecules, in solution and intramolecular complexes. In Advances in Polymer Science. Berlin; Springer-Verlag, 1982, 45(2), 1.

Van Kuijk-Meuwissen M. E. M. J., Junginger, H. E., Bouwstra, J. A. Interactions between liposomes and human skin in vitro, a cofocal laser scanning microscope study. Biochimica et Biophysica Acta – Biomembranes 1998, 1370, 31-39

Villanueva, R. D., Mendoza, W. G., Rodrigueza, M. R. C., Romero, J. B., and Montano, M. N. E. Structure and functional performance of gigartinacean kappa-iota hybrid carrageenan and solieriacean kappa-iota carrageenan blends. Food Hydrocolloids, 2004 (18-2): 283-292.

Vyas, S. P., and Khar, R. K. Targeted and Controlled Drug Delivery: Novel Carrier Systems. 1st Ed, CBS Publishers and Distributors, New Delhi, 2002, 303-.330.

Wahlstrom, B., and Blennow, G. A. A study on the fate of curcumin in the rat. Acta Pharmacol. Toxicol. (Copenhagen) 1978, 43 (2), 86-92.

Wallace, J. M. Nutritional and botanical modulation of the inflammatory cascade - eicosanoids, cyclooxygenases, and lipoxygenases - as an adjunct in cancer therapy. Integrative Cancer Therapies 2002, 1(1), 7-37.

Wang, X., Jiang, Y., Wang, Y-W., Huang M-T., Ho C-T., and Huang Q. Enhancing anti-inflammation activity of curcumin through O/W nanoemulsions. Food Chemistry 2008, 108, 419-424.

Wenzel, E., Soldo, T., Erbersdobler, H., and Somoza, V. Bioactivity and metabolism of trans-resveratrol orally administrated to Wistar rats. Mol. Nutr. Food Res. 2005, 49, 482-494.

Xia, J., and Dubin, P., L. Protein-polyelectrolyte complexes. IN Macromolecular Complexes in Chemistry and Biology. Dubin, P. L., Bock, J., Davis, R., Schulz, D. N., and Thies, C., Eds.; Berlin; Springer-Verlag, 1994, 247-271.

Xia, J., Mattison, K., Romano, V., Dubin, P. L., and Muhoberac, B. B. Complexation of trypsin and alcohol dehydrogenase with poly (diallyldimethylammonium chloride). Biopolymers 1997, 41(4), 359-365.

Yang, C. S., Landau, J. M., Huang, M-T., and Newmark, H. L., Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* 2001 (21): 381-406.

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Wang, Xiaoyong, Jiang, Yan, and Huang, Qingrong (2007) "Encapsulation technologies for preserving and controlled-release of enzymes and phytochemicals" In Encapsulation and Controlled Release Technologies: Practical Applications in Food Systems, edited by Lakkis, Jamileh, Blackwell Publishing: Ames, Iowa, 135-148.

Wang, X. Y.; Jiang, Y.; Wang, Y. -W.; Huang, M. T.; Ho, C. T.; and Huang, Q. R. (2008) "Enhancing anti-inflammation activity of curcumin through O/W nanoemulsions", Food Chemistry, 108, 419-424