ENCAPSULATION OF NARINGENIN USING ZEIN NANOPARTICLES

by

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ABSTRACT OF THE THESIS

Encapsulation of Naringenin Using Zein Nanoparticles

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Naringenin (4',5,7-trihydroxyflavanone) is a flavanone known to have many health benefits including antitumor, antioxidant, anti-inflammatory, and hepatoprotective properties. It is naturally found in citrus fruits, predominantly in grapefruits. However, when naringenin is orally ingested, the health benefits are limited due to its low solubility in water, which causes low bioavailability. In an effort to improve the bioavailability of naringenin, various nanoparticle systems were investigated and optimized to counter this problem. Zein, a corn based protein, was chosen as the primary encapsulation material due to its capability of forming self-assembled nanoparticles and sustained release. To minimize the immunogenicity effects of zein, the nanoparticles were further treated with a polysaccharide to produce a more hydrophilic surface coating. Three polysaccharides, carboxymethyl cellulose (CMC), carboxymethyl chitosan (CMCH), and carboxymethyl dextran (CMD), were individually explored with the naringenin filled zein nanoparticles.

Optimized ratios with the highest load and the smallest particle size were identified for each nanoparticle system. The optimized ratios for each polysaccharide were as follows: 0.1N: 5Z: 15CMC, 3N: 5Z: 15CMCH, 3N: 5Z: 15CMD. MTT testing identified cytotoxicity thresholds for each formulation. Pure naringenin was found to be not cytotoxic at <50 μg/mL. The 0.1N: 5Z: 15CMC formulation was not cytotoxic at 10 fold dilution. For 3N: 5Z: 15CMCH and 3N: 5Z: 15CMD, each formulation was not cytotoxic at 20 fold dilution.

Across the polysaccharide sets, carboxymethyl chitosan was found to be smallest in particle size. Further investigation was done on this polysaccharide to see whether further reductions of particle size could be achieved. The addition of a calcium chloride coating reduced the particle size further compared to the original carboxymethyl chitosan ratio. This research showed that the combination of carboxymethyl chitosan with calcium chloride resulted in the most promising system of encapsulating naringenin with zein nanoparticles.

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Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Functional Foods and Consumer Trends

As consumers walk down the supermarket aisles, there are multiple factors that influence their decision in purchasing food. Beyond just availability, brand, packaging, and cost, consumers also look for factors such as taste, nutrition, quality, convenience and much much more which makes a very complex decision tree at the grocery store. Although there are many factors that influence this decision, what is evidently clear is the growing increase of consumer trends that cannot be ignored. Natural, organic, and gluten-free are some of these "better for you" trends that consumers are looking for with functional foods being an increasingly hot topic. So, what are functional foods? How do we define them and what are examples of functional foods?

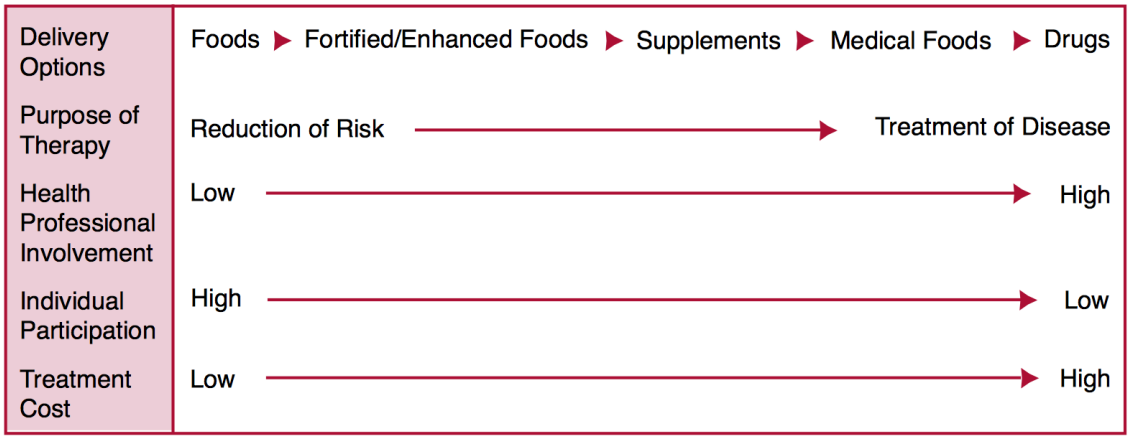
The International Food Information Council (IFIC) defines functional foods as "foods or beverages that may provide benefits beyond basic nutrition… and may consist of a variety of food components and provide additional health benefits that may reduce disease and/or promote optimal health" (IFIC, 2013). Some of the examples they include are fruits, vegetables, vitamins and minerals naturally or fortified in food, as well as dietary supplements (IFIC, 2013).

Other organizations also have similar definitions with slightly difference nuances. According to the Institute of Food Technologists (IFT), their Expert Panel defines functional foods as "foods and food components that provide a health benefit beyond basic nutrition (for the intended population)… [as they] provide

essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects" (IFT). Furthermore, the Expert Panel provides examples of functional foods such as fortified foods, enriched/enhanced foods, as well as dietary supplements (IFT).

Organizations might vary in their definitions of functional foods. However, they align that these foods have health benefits beyond basic nutrition and most consumers agree. A recent survey conducted by the IFIC suggests that 90% of consumers believe that some foods have health benefits beyond basic nutrition (IFIC, 2013). Consumers are overall more interested and educated about the foods they eat as well as the health benefits they provide than ever before. The numbers will only continue to rise as 86% are interested in learning more about these health benefits (IFIC, 2013). This doesn't come as a surprise with the increase of scientific knowledge of food and increase in healthcare costs, consumers are looking for other avenues where they can find health benefits beyond the traditional doctor's office. Figure F1 shows a visual summary of the current role of functional foods in healthcare and its delivery options. With the advancement of science and technology, the options will continue to grow and provide consumers variations of functional foods.

F1. Role of Functional Foods in Health Care Continuum



Citation: IFT

To keep consumer interest as well as a competitive edge, Consumer Packaged Goods (CPG) companies have jumped on the functional food band wagon. The increasing consumer need is continuously striving to be satisfied by these companies to increase the nutrition and health benefits of their products and brands. Antioxidants and flavanols are only a couple of these added benefits some of which would not be expected to be naturally found within some products. An example of this would be the recent addition of Omega-3 and Omega-6 fatty acids to dairy products including whole milk. In a recent study done with American consumers, results showed that only 1 out of 2 people believe they get enough of their daily intake of Omega-3 fatty acids (IFIC, 2013). Naturally found in fish, these fatty acids could also be found in supplement form. However, for those consumers who have concern whether they're getting enough fatty acids in their diet, the new milk variety with added Omega-3 and Omega-6 provides added benefit, convenience, and all without any unpleasant tastes or odors. Foods with these

additional benefits provide convenience and more nutrition to consumers than ever before with new technologies and advances in the food industry enabling these opportunities.

1.2 Nanotechnology

One of the tools that can transform the food we eat and take functional foods to the next level is Nanotechnology. Nanotechnology can be defined as the "the creation of functional materials, devices, and systems through the manipulation of matter at a length scale of ~1 – 100nm" (Srinivas, et al., 2010). Different physical properties emerge at the nanoscale (10^{-9} meter) which provides unique opportunities across multiple industries such as food, nutrition, packaging, and medicine (Srinivas, et al., 2010). Some of the many benefits of nanotechnology in food include increased bioavailability, thermal stability, and water solubility which can provide multiple prospects in functional foods (Huang, et al., 2010).

1.3 Rationale

This research investigates nanoencapsulation of naringenin using zein nanoparticles with the use of polysaccharides and calcium chloride. Naringenin is the primary flavonoid focus as it has numerous health benefits but is poorly absorbed when orally consumed. Mostly found in grapefruits, one would need to eat about 40 grapefruits a day to see naringenin's beneficial effect (Allison, 2010). Beyond the impractical side of consumption, too much of a good thing might not be so good. According to the United States Department of Agriculture (USDA), a

serving size, which is ½ a medium sized grapefruit (½ cup), contains 9 grams of sugar which quickly adds up to 720 grams of sugar for 40 grapefruits and may not be recommended to certain consumers especially with diabetes (USDA, 2012). Encapsulating naringenin provides the convenience to consumers and increases the bioavailability that would not be naturally attained. Using food-grade or Generally Recognized as Safe (GRAS) ingredients such as zein, carboxymethyl cellulose, carboxymethyl chitosan, carboxymethyl dextran, and calcium chloride, provides the opportunity to be safely consumed in food.

In addition to its safety and use in the food industry, zein was chosen as the primary encapsulation method due to its unique physical properties including insolubility in water, resistance to microbes, and potential delivery system for hydrophobic compounds (Li, et al., 2012). Zein is a material that is also widely accessible which is convenient for application purposes in the food industry (Li, et al., 2012). This research used zein's insolubility in water to form precipitation, in this case self-assembled nanoparticles, through a method known as liquid-liquid dispersion (Luo, et al., 2014, Liang, et al., 2015). The polysaccharides (carboxymethyl cellulose, carboxymethyl chitosan, and carboxymethyl dextran) were also chosen for their safety and use in the food industry to optimize the zein nanoparticles and provide improved physiochemical properties (Luo, et al., 2012). Finally, calcium chloride, another food-grade and GRAS component commonly used in the food industry, was also chosen for further improvement of the physiochemical properties for the optimized nanoparticle system (Luo, et al., 2012).

1.4 Hypothesis and Specific Aims

The hypothesis of this research is that zein and the use of polysacchides will provide smaller particle sizes compared to pure naringenin. Furthermore, the use of calcium chloride will provide additional benefit in creating smaller particle sizes versus just the zein/polysaccharide nanoparticles alone.

To test these hypotheses, there will be two specific aims to investigate the nanoencapsulation of naringenin:

1. **Investigation of the optimal polysaccharide**

   Three polysaccharides will be studied (carboxymethyl cellulose, carboxymethyl chitosan, and carbuxymethyl dextran) with the zein nanoparticle system to determine which polysaccharide would be the best candidate to encapsulate naringenin. Particle size analysis as well as cytotoxicity will be used to determine potential candidacy**.**

2. **Investigation of the addition of calcium chloride**

   Once the optimum polysaccharide is determined, further optimization of the system will be studied to determine whether calcium chloride can improve the physical properties of the system. Particle size analysis and zeta potential will be used to determine these qualities.

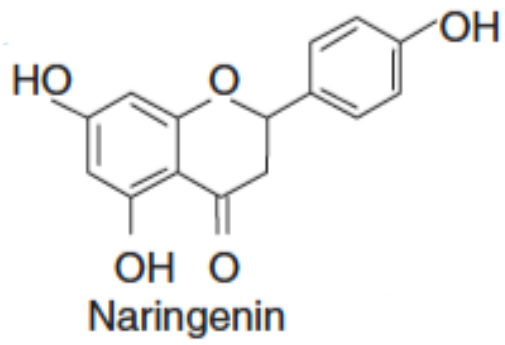
2. Background

2.1 Naringenin

2.1.1 Classification

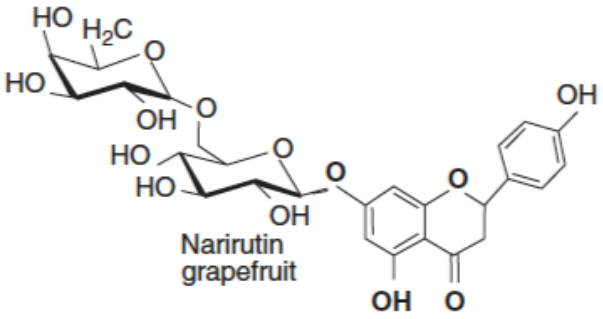
Naringenin (4',5,7-trihydroxyflavanone) is a flavanone which is a type of flavonoid (Peterson, et al., 2006). It is characterized by a hydroxyl group in the 7th and 4' position (Erlund, 2004). Its glycosides, narirutin (naringenin-7-rutinoside) and naringin (naringenin-7-neohesperidoside), replace the hydroxyl group in the 7th position with a sugar (Bugianesi, et al., 2002). For narirutin, the sugar moiety is 6-O-L-rhamnosyl-D-glucoside and for naringin it is 2-O-α-L-rhamnosyl-D-glucoside (Erlund, 2004). Figures F2, F3, and F4 show the chemical structure of naringenin and its glycosidic forms.

F2. Structure of Naringenin



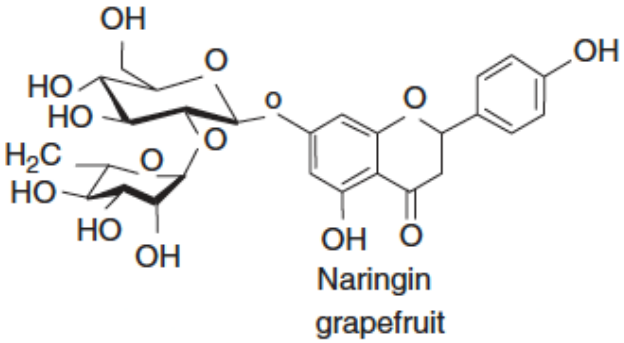
Citation: Peterson, et al., 2006

F3. Structure of Narirutin



Citation: Peterson, et al., 2006

F4. Structure of Naringin



Citation: Peterson, et al., 2006

2.1.2 Sources

Naringenin is commonly found in citrus fruits including grapefruits, oranges, lemons, and limes (Vallverdú-Queralt, et al., 2012, Peterson, et al., 2006, Erlund, 2004). It has also been found in non citrus fruits particularly tomato (Bugianesi, et al., 2002). The structures of naringenin vary depending on its natural source. In citrus fruits, it is commonly found in its glycosidic forms, which are narirutin and

naringin (Bugianesi, et al., 2002). While in tomatoes, naringenin is found as the aglycone form in the skin of the tomato (Bugianesi, et al., 2002).

Concentrations of narginenin vary greatly across these fruits. The concentration of naringenin in a red tomato range between 0.8 to 4.2 mg/100 g (Davies, et. al., 1981, Paganga, et al., 1999). In oranges, narirutin is the major glycoside ranging between 1.5 to 4.2 mg/100 g (Erlund, 2004). In lemons and limes, the total of naringin and narirutin range between 0.0 to 4.8 mg/100 g and 0.0 to 1.0 mg/100 g respectively (Peterson, et al., 2006). However, the highest concentration is found in grapefruits where it can reach up to 60.87 mg/100 g (Peterson, et al., 2006).

2.1.3 Health Benefits

Naringenin's chemical structures that are commonly found in the human diet have important biological effects. Studies have shown many health benefits, including anti-oxidant, anti-inflammatory, anti-fibrogenic, and hepatoprotective effects (van Acker, et al., 2000, Jeon, et al., 2002, Manthey, et al., 2001, Lee, et al., 2004). Naringenin has also been shown to have effects on sex hormone metabolism, one of which is the binding to estrogen receptors (Ruh, et al., 1995, Rosenberg, et al., 1998, Déchaud, et al., 1999, Yoon, et al., 2001, Kuiper, et al., 1998). It has prevented obesity effects in LDL receptor-null mice that were given a high-fat diet and lowered plasma and hepatic cholesterol concentrations in rats that were fed a high-cholesterol diet (Mulvihill, et al., 2009, Lee, et al., 1999). Naringenin showed potent inhibitor effects on metabolizing enzymes in in vitro experiments in mice, as well as producing BDNF-dependent anti-depressant-like effects (Yi, et al., 2014, Ueng, et al.,

1999). It has also been shown to slow down the spread of the hepatitis C virus by interfering with the secretion of the virus from infected cells as well as inhibiting tumor growth of multiple cancer cells lines (Nahmias, et al., 2008, Kanno, et al., 2005).

2.1.4 Interactions

Multiple studies have investigated naringenin in grapefruit juice and its interactions with medicines. One study showed naringenin in grapefruit juice to have an inhibitory effect on the human cytochrome P450 isoform CYP1A2 which can change pharmacokinetics and pharmacodynamics of certain drugs (Fuhr, et al., 1993). Naringenin has also been shown to be an inhibitor of CYP3A4 (Ghosal, et al., 1996). Studies have shown an increase in plasma concentrations of drugs that have been metabolized by CYP3A4 when administered together with naringenin in grapefruit juice (Dresser, et al., 2000, Lilya, et al., 2000). Another study claims that naringenin present in grapefruit juice is an esterase inhibitor, which is shown to mediate the pharmacokinetic drug interaction with several prodrugs (Li, et al., 2007). Overall, findings show there are interactions between grapefruit juice and certain drugs. Subsequently, prescription bottles for those drugs are labeled to avoid consumption of grapefruit juice during the course of the treatment to avoid adverse effects.

2.1.5 Bioavailability and Pharmacokinetics

As with most other compounds, naringenin has higher bioavailability intravenously versus when consumed orally (Choudhury, et al., 1999). However, taking naringenin intravenously is not as convenient or consumer friendly as consuming it by mouth. Naringenin is consumed throughout the world easily and effortlessly through its multiple natural sources. A study estimated that the average intake of naringenin in Finland is about 8.3 mg per day (Kumpulainen, et al., 1999). Research has been done on the bioavailability and pharmacokinetics of naringenin with these natural sources. In one study, the peak plasma concentrations for naringenin were 0.6 ± 0.4 μmol/L and 6.0 ± 5.4 μmol/L from orange juice (including 151 μmol/L of naringenin) and grapefruit juice (including 1283 μmol/Lof naringenin), respectively (Erlund, et al., 2001). Results from another study showed that after ingesting 3.8 mg of naringenin in a meal containing 150 mg of cooked tomato paste, the peak plasma concentration was 0.12 ± 0.03 μmol/L after 2 hours of consumption (Bugianesi, et al., 2002).

2.1.6 Metabolism

Some studies have investigated the metabolism of naringenin when ingested orally. These studies showed that the glycosidic group is separated by an enzyme in the intestine (Choudhury, et al., 1999, Jang, et al., 1996). This process happens before glucuronidation and before absorption of the aglycones (Choudhury, et al., 1999, Jang, et al., 1996).

2.1.7 Previous Encapsulations of Naringenin

There have been several studies that have encapsulated naringenin. In one study, naringenin-loaded monomethoxy poly(ethylene glycol)-poly (ε-caprolactone) nanoparticles were created into buccal tablets with mucoadhesive polymers (Wang, et al., 2014). These naringenin buccal tablets improved the release of naringenin as well as made it release faster (Wang, et al., 2014). Another study investigated naringenin nanoparticles made with Eudragit® E using the nanoprecipitation method (Yen, et al., 2009). Results of that study showed higher release rate and improved solubility of naringenin (Yen, et al., 2009). Naringenin was also encapsulated in lipid-based onion-type multilamellar vesicles (Kerdudo, et al., 2014). Although naringenin was only encapsulated less than 10%, the adsorbtion on the multilamellar vesicle was over 60% (Kerdudo, et al., 2014).

2.2 Zein

2.2.1 Background

Zein is a highly hydrophobic protein naturally found in corn (Luo, et al., 2014). It is shown be safe for consumption and has the regulatory GRAS status. Furthermore, it has many other positive qualities including biocompatibility, biodegradability, and low toxicity (Luo, et al., 2014). Due to these many features, zein is a great candidate for nanoparticles. It has been used to encapsulate bioactive compounds in both the food and pharmaceutical applications such as daidzin, paclitaxel, Vitamin D3, indol-3-carbinol, and 3,3'-diindolylmethane (Zou, et al., 2013, Liang, et al., 2015, Luo, et al., 2012, Luo, et al., 2013).

2.2.2 Creation of Zein Nanoparticles

Zein has the capability to form self-assembled nanoparticles. One of the methods of creating these zein nanoparticles is through liquid-liquid dispersion, which was used in this study with some modifications (Luo, et al., 2014). Zein is completely dissolved in an aqueous alcohol solution and looks transparent in color (Luo, et al., 2014, Liang, et al., 2015, Luo, et al., 2012, Luo, et al., 2013). The zein solution is then poured into water, which decreases the solubility of zein and triggers a phase separation in the solution (Luo, et al., 2014). This causes a precipitation and solidification of zein nanoparticles with the final solution being opaque or light blue in color (Luo, et al., 2014, Liang, et al., 2015). The particle sizes of zein nanoparticles generally range between 100 to 400 nm (Luo, et al., 2014).

2.3 Adding Polysaccharides to Nanoparticles

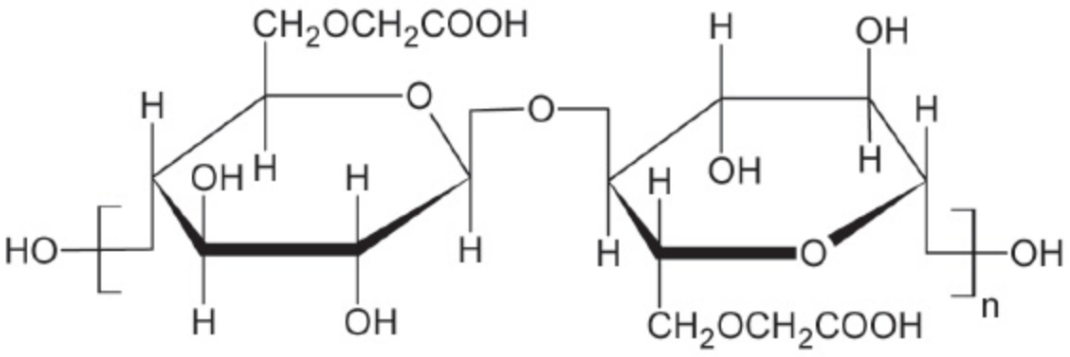
Zein nanoparticles alone may not be adequate enough to be a complete encapsulated system, as it might not provide enough desired protection and controlled release (Liang, et al., 2015). It can also cause immunogenicity effects as the zein nanoparticle can be rapidly taken up by macrophages in the body (Liu, et al., 2005, Podaralla, et al., 2012). Adding a polysaccharide layer to the zein nanoparticles provides a coating, which increases hydrophilic elements and decreases immunogenicity (Luo, et al., 2012, Luo, et al., 2013). The polysaccharide layer also provides better physiochemical properties such as improved controlled release profiles and higher encapsulation efficiency (Luo, et al., 2012). The

polysaccharides that were investigated in this research were carboxymethyl cellulose, carbodymethyl dextran, and carboxymethyl chitosan.

2.3.1 Carboxymethyl Cellulose

Carboxymethyl cellulose is a natural polysaccharide that is soluble in water. It is widely used in food and pharmaceutical industries (Liang, et al., 2015). It has been used for encapsulation and delivery of ingredients as well as the protection of coated zein nanoparticles (Liang, et al., 2015). Figure F5 shows a graphical representation of the chemical structure for carboxymethyl cellulose.

F5. Structure of Carboxymethyl Cellulose



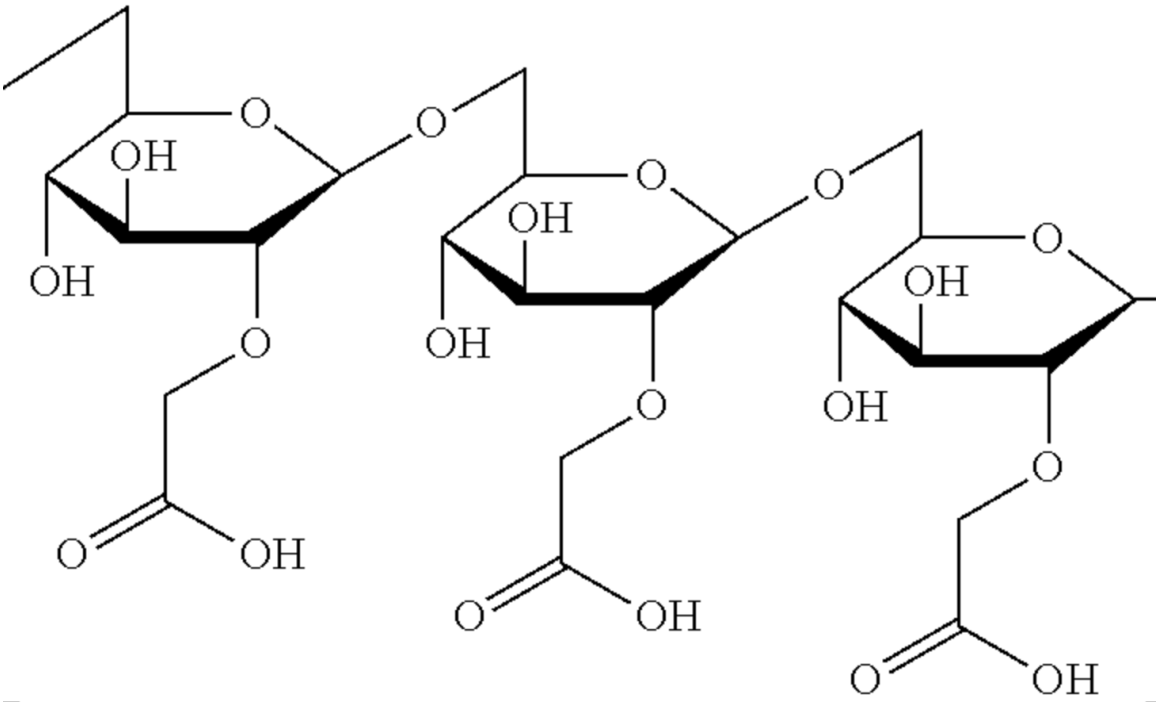
Citation: Sidley Chemical

2.3.2 Carboxymethyl Dextran

Carboxymethyl dextran is a carbohydrate polysaccharide and is soluble in water. It has been used for biomedical applications and used for encapsulation and

delivery of ingredients (Ning, et al., 2011, Zhang, et al., 2005). Figure F6 shows a graphical representation of the chemical structure for carboxymethyl dextran.

F6. Structure of Carboxymethyl Dextran

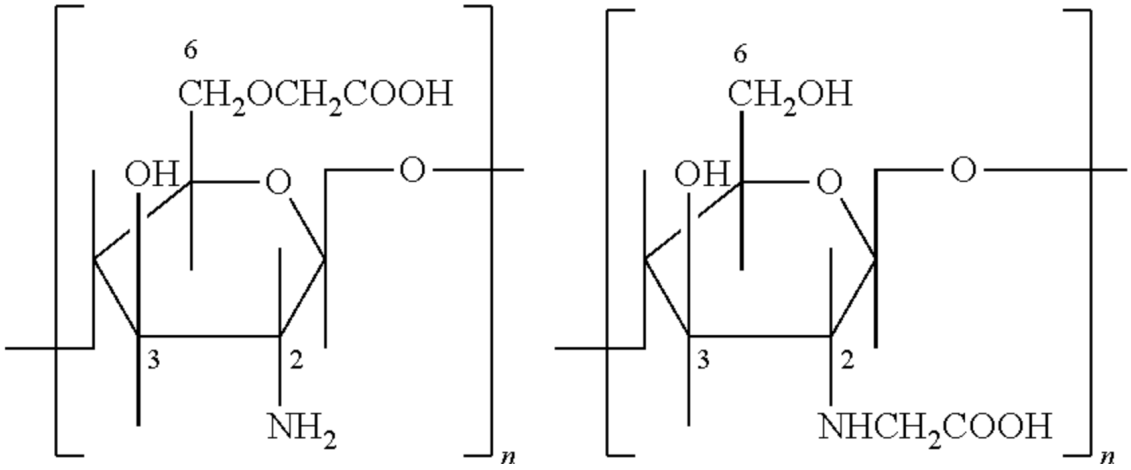


Citation: Park, J.H., 2014.

2.3.3 Carboxymethyl Chitosan

Carboxymethyl chitosan is a derivative of chitosan with improved solubility in water (Luo, et al., 2012). It is extensively used for biomedical applications as well as encapsulation and delivery of ingredients including anticancer drugs (Luo, et al., 2012, He, et al., 2010, Wang, et al., 2008, Anitha, et al., 2011, Kim, et al., 2006). Figure F7 shows a graphical representation of the chemical structure for carboxymethyl chitosan.

F7. Structure of O-Carboxymethyl Chitosan and N-Carboxymethyl Chitosan



Citation: Li, Y. and Sun, S, 2012.

2.4 Adding Calcium Chloride to the Optimized Nanoparticle Formulation

Calcium chloride is a salt and is soluble in water. It can be commonly found in stores for the use of dissolving snow and ice. It is also food-grade and GRAS and has wide applications in the food industry. Previous studies showed success with the addition of calcium chloride in nanoparticle systems. A study showed that the addition of calcium ions decreased particle sizes compared to samples without any calcium chloride (Luo, et al., 2012).

3. Materials and Methods

3.1 Materials

Naringenin of >98% purity was purchased from Quality Phytochemicals LLC (Edison, NJ). Zein was purchased from Wako Chemicals (Shanghai) Co., Ltd. (Lot#

TLH5819, Shanghai, China). Carboxymethyl cellulose (CMC) was purchased from Sigma-Aldrich Chemical Co. Ltd. (Lot# 122K1182, St. Louis, MO, USA). Carboxymethyl dextran (CMD) was acquired from the Food Science Department at Rutgers University (Piscataway, NJ). Carboxymethyl chitosan (CMCH) was purchased from Kunpoong Bio Co., Ltd. (Seoul, South Korea). Calcium chloride was purchased from Sigma-Aldrich Cell Culture (Lot# 31H03026). Tween 80, Enzyme Grade was purchased from Fisher Scientific (Lot# 943317, Pittsburgh, PA, USA). Analytical grade ethanol was purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Deionized water was utilized in preparation of all nanoparticles.

3.2 Preparation of Initial Nanoparticles

Methodology was based on the study performed by Liang, et al. and Luo, et al. with some modifications (Liang, et al., 2015, Luo, et al., 2012, Luo, et al., 2013). Initially stock solutions were created in preparation for the creation of nanoparticles. A zein stock of 5 mg/ml was created by dissolving zein in 75% aqueous ethanol solution. The polysaccharide stocks consisted of 5 mg / ml of polysaccharide (CMC, CMD, or CMCH) dissolved in DI water. Naringenin was dissolved in 95% aqueous ethanol solution to create a stock concentration of 5 mg/ml. The empty nanoparticles were created by mixing 1:3 ratio of zein to polysaccharide (CMC, CMCH, or CMD) and vigorously stirring for 30 minutes (Liang, et al., 2015). Naringenin filled nanoparticles were created by adding different volumes of naringenin stock drop wise into the zein stock under mild stirring for 60 minutes which was then poured into one of the polysaccharide solutions all

containing 0.2% Tween 80. The solutions were vigorously stirred for 30 minutes. Different weight ratios of naringenin (N) were investigated with the zein (Z) and polysaccharides (PLS) as follows 1Z : 3PLS, 0.1N : 5Z : 15PLS, 1N : 5Z : 15PLS, 3N : 5Z : 15PLS.

The ratios in Table T1 were used to gauge particle sizes and subsequently used for optimized formulations.

T1. Ratios of Screening Formulations

CMC	CMCH	CMD
1Z : 3CMC	1Z : 3CMCH	1Z : 3CMD
0.1N : 5Z : 15CMC	0.1N : 5Z : 15CMCH	0.1N : 5Z : 15CMD
1N : 5Z : 15CMC	1N : 5Z : 15CMCH	1N : 5Z : 15CMD
3N : 5Z : 15CMC	3N : 5Z : 15CMCH	3N : 5Z : 15CMD

Tables T2, T3, and T4 show all the ratios and formulations tested for carboxymethyl cellulose, carboxymethyl dextran, and carboxymethyl chitosan respectively.

T2. Formulations of Carboxymethyl Cellulose (CMC) Nanoparticles

Ratio	Naringenin (mg)	Zein (mg)	CMC (mg)
1Z : 3 CMC (Empty NP)	0	25	75
0.1N : 5Z : 15 CMC	0.5	25	75
0.2N : 5Z : 15 CMC	1	25	75
0.3N : 5Z : 15 CMC	1.5	25	75
0.5N : 5Z : 15 CMC	2.5	25	75
1N : 5Z : 15 CMC	5	25	75
3N : 5Z : 15 CMC	15	25	75

T3. Formulations of Carboxymethyl Dextran (CMD) Nanoparticles

Ratio	Naringenin (mg)	Zein (mg)	CMD (mg)
1Z : 3 CMD (Empty NP)	0	25	75
0.1N : 5Z : 15 CMD	0.5	25	75
1N : 5Z : 15 CMD	5	25	75
2N : 5Z : 15 CMD	10	25	75
3N : 5Z : 15 CMD	15	25	75
4N : 5Z : 15 CMD	20	25	75
5N : 5Z : 15 CMD	25	25	75

T4. Formulations of Carboxymethyl Chitosan (CMCH) Nanoparticles

Ratio	Naringenin (mg)	Zein (mg)	CMCH (mg)
1Z : 3 CMCH (Empty NP)	0	25	75
0.1N : 5Z : 15 CMCH	0.5	25	75
0.2N : 5Z : 15 CMCH	1	25	75
0.5N : 5Z : 15 CMCH	2.5	25	75
1N : 5Z : 15 CMCH	5	25	75
3N : 5Z : 15 CMCH	15	25	75
3.5N : 5Z : 15 CMCH	17.5	25	75
4N : 5Z : 15 CMCH	20	25	75
4.5N : 5Z : 15 CMCH	22.5	25	75
5N : 5Z : 15 CMCH	25	25	75

3.3 Preparation of Carboxymethyl Chitosan Nanoparticles Using Calcium Chloride

The optimized carboxymethyl chitosan nanoparticle methodology was based on the previous initial nanoparticle methodology done by Luo, et al. but with some modifications (Luo, et al., 2012, Luo, et al., 2013). The zein stock was created by dissolving zein in 70% aqueous ethanol solution with a final concentration of 2 mg/ml. The carboxymethyl chitosan stock consisted of 1 mg / ml of carboxymethyl chitosan dissolved in DI water. Naringenin was dissolved in 95% aqueous ethanol solution to create a stock concentration of 5 mg/ml. Calcium chloride was dissolved in DI water for a final concentration of 0.1 mg/ml (Luo, et al., 2012).

The empty nanoparticles were created by mixing a 2:1 ratio of zein to carboxymethyl chitosan and vigorously stirred for 30 minutes (Luo, et al., 2012, Luo, et al., 2013). Afterwards, 1 mL of calcium chloride solution was added to the mixture and was stirred for another 30 minutes.

Naringenin filled nanoparticles were created by adding different volumes of naringenin stock drop wise into the zein stock under mild stirring for 30 minutes which was then poured into the carboxymethyl chitosan solution containing 200 µL of 0.5% Tween 20. The solutions were vigorously stirred for 30 minutes. Subsequently, 1 mL of calcium chloride solution was added to the mixture and was stirred for another 30 minutes (Luo, et al., 2012). Different weight ratios of naringenin (N) were investigated with zein (Z) and carboxymethyl chitosan (CMCH) and calcium chloride ($CaCl_2$) as follows 2Z : 1 CMCH : 1/20 $CaCl_2$, 1N: 2Z : 1 CMCH : 1/20 $CaCl_2$, 1.5N: 2Z : 1 CMCH : 1/20 $CaCl_2$, 2N : 2Z : 1 CMCH : 1/20 $CaCl_2$, 2.5N: 2Z : 1 CMCH : 1/20 $CaCl_2$. Table T5 includes the amounts of each material for each ratio.

T5. Formulations of Carboxymethyl Chitosan (CMCH) Nanoparticles with Calcium Chloride

Ratio	Naringenin (mg)	Zein (mg)	CMCH (mg)	$CaCl_2$ (mg)
2Z : 1 CMCH : 1/20 $CaCl_2$ (Empty NP)	0	4	2	0.1
1N: 2Z : 1 CMCH : 1/20 $CaCl_2$	2	4	2	0.1
1.5N: 2Z : 1 CMCH : 1/20 $CaCl_2$	3	4	2	0.1
2N : 2Z : 1 CMCH : 1/20 $CaCl_2$	4	4	2	0.1

3.4 Characterization of Nanoparticles

Dynamic laser scattering (DLS) of samples were performed on a commercial laser light scattering instrument. Zeta potential of samples were performed on Zetasizer Nano ZS.

3.5 Cytotoxicity

Cytotoxicity was measured by using Methyl Thiazol Tetrazolium Bromide (MTT) Assay. The MTT methodology was based on a previous study done by Yu & Huang. HepG2 cells were seeded in 96-well plates at density of 10,000 cells per well with a 100 μL final volume (Yu, et al., 2010). After 24 hours, the cells were treated with a medium containing DMSO-dissolved naringenin or loaded naringenin nanoparticles (Yu, et al., 2010). Other cells were treated with DMSO or HMS with sufficient concentration to dissolve the naringenin filled nanoparticle, or the cells were left untreated as negative controls (Yu, et al., 2010). After another 24 hours, the media was aspirated and the cells were incubated with 100 μL of MTT solution (Yu, et al., 2010). After 2 hours at 37 °C, the MTT solution was aspirated and the formed formazan crystals were dissolved in 100 μL of DMSO (Yu, et al., 2010). Light absorbance was recorded at 560 and 670 nm (Yu, et al., 2010). The relative cell viability was expressed as A560-A670 and normalized to that of the untreated wells (Yu, et al., 2010). Eight-well repeats were performed and data was presented with the average mean of repeats with standard deviations.

4. Results and Discussion

4.1 Particle Size

Particle size results for all nanoparticles are listed in Tables T6-T9. The smallest particle size for each polysaccharide was the empty nanoparticle system. Optimal ratios of naringenin were identified for each polysaccharide. Statistical differences between particle sizes were analyzed using one-way Analysis of Variance (ANOVA) at the 95% confidence level.

For the carboxymethyl cellulose system, the optimal naringenin ratio was identified to be 0.1N : 5Z : 15CMC. No significant difference was found between this ratio and the empty CMC nanoparticle system. No statistical difference was found up to the level of the 1N : 5Z : 15CMC ratio when compared to the empty CMC system. However, the particle sizes for those formulations were much larger and might not be as effective as the 0.1N : 5Z : 15CMC formulations with a particle size in the nanometer level.

For carboxymethyl dextran, statistical differences were found for all naringenin formulations when compared to the empty nanoparticle system. The optimal naringenin ratio was identified to be 3N : 5Z : 15CMD. It had the highest naringenin load and was not significantly different in size from 0.1N : 5Z : 15 CMD and 1N : 5Z : 15 CMD ratios.

In the carboxymethyl chitosan system, the optimal naringenin ratio was identified as 3N : 5Z : 15 CMCH. This ratio had the highest naringenin load without being significantly larger in size compared to the empty CMCH nanoparticle system.

Ratios with higher concentrations of naringenin showed significantly larger particle sizes. Across the three polysaccharides, carboxymethyl chitosan showed to be the most optimal polysaccharide with the lowest particle size and the highest naringenin load.

The addition of calcium chloride to the carboxymethyl chitosan system further decreased the particle sizes. Within the CMCH and $CaCl_2$ system set, no significant differences were found between the formulations. However, the 1.5N: 2Z : 1 CMCH : 1/20 $CaCl_2$ ratio was numerically the smallest. It was also the smallest across all the formulations tested. This could be attributed to the cross-link between the calcium and carboxymethyl chitosan within the nanoparticle

T6. Particle Sizes of Carboxymethyl Cellulose (CMC) Nanoparticles

Ratio	Effective Diameter Mean (nm)	ANOVA Group	Polydispersity
1Z : 3 CMC (Empty NP)	222.6 ± 6.3	B	0.208 ± 0.003
0.1N : 5Z : 15 CMC	320.1± 2.4	B	0.246 ± 0.012
0.2N : 5Z : 15 CMC	3235.9 ± 759.7	AB	0.375 ± 0.058
0.3N : 5Z : 15 CMC	6358.2 ± 3450.4	AB	0.473 ± 0.049
0.5N : 5Z : 15 CMC	4898.3 ± 3559.5	AB	0.475 ± 0.044
1N : 5Z : 15 CMC	3124.3 ± 469.7	AB	0.243 ± 0.123
3N : 5Z : 15 CMC	11509.1 ± 652.7	A	0.526 ± 0.067

T7. Particle Sizes of Carboxymethyl Dextran (CMD) Nanoparticles

Ratio	Effective Diameter Mean (nm)	ANOVA Group	Polydispersity
1Z : 3 CMD (Empty NP)	283.8 ± 26.8	D	0.216 ± 0.013
0.1N : 5Z : 15 CMD	476.2 ± 11.0	BC	0.168 ± 0.081
1N : 5Z : 15 CMD	409.6 ± 4.9	C	0.077 ± 0.072
2N : 5Z : 15 CMD	557.7 ± 24.6	A	0.005 ± 0.000
3N : 5Z : 15 CMD	441.5 ± 6.8	C	0.008 ± 0.003
4N : 5Z : 15 CMD	527.9 ± 9.8	AB	0.035 ± 0.018
5N : 5Z : 15 CMD	574.6 ± 1.6	A	0.021 ± 0.014

T8. Particle Sizes of Carboxymethyl Chitosan (CMCH) Nanoparticles

Ratio	Effective Diameter Mean (nm)	ANOVA Group	Polydispersity
1Z : 3CH (Empty NP)	192.7 ± 7.8	C	0.142 ± 0.016
0.1N : 5Z : 15 CMCH	375.2 ± 2.9	ABC	0.188 ± 0.026
0.2N : 5Z : 15 CMCH	335.0 ± 6.0	BC	0.231 ± 0.034
0.5N : 5Z : 15 CMCH	344.9 ± 8.5	BC	0.239 ± 0.005
1N : 5Z : 15 CMCH	445.8 ± 9.9	ABC	0.235 ± 0.025
3N : 5Z : 15 CMCH	364.0 ± 74.0	ABC	0.005 ± 0.000
3.5N : 5Z : 15 CMCH	643.4 ± 85.4	A	0.005 ± 0.000
4N : 5Z : 15 CMCH	522.9 ± 56.5	AB	0.005 ± 0.000
4.5N : 5Z : 15 CMCH	650.0 ± 120.5	A	0.005 ± 0.000
5N : 5Z : 15 CMCH	2109.5 ± 409.7	*	0.082 ± 0.051

*** Excluded in analysis due to the large particle size.**

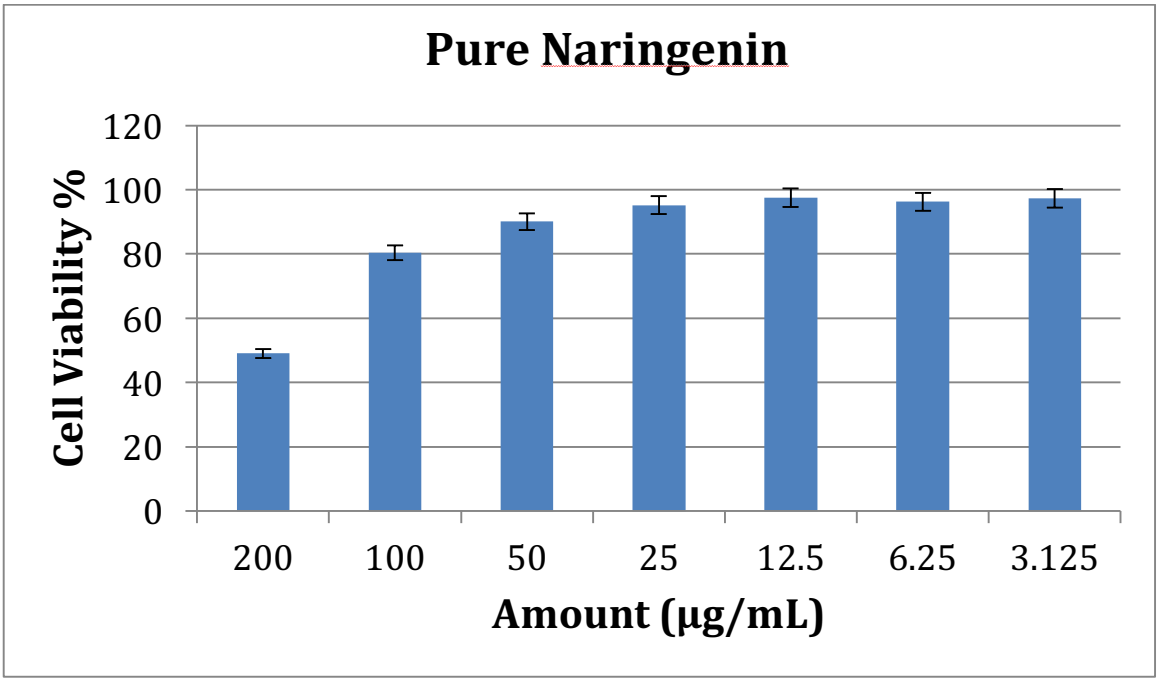
T9. Particle Sizes of Optimized Carboxymethyl Chitosan (CMCH) Nanoparticles

Ratio	Effective Diameter Mean (nm)	ANOVA Group	Polydispersity
2Z : 1 CMCH : 1/20 $CaCl_2$	140.5 ± 0.2	A	0.201 ± 0.016
1N: 2Z : 1 CMCH : 1/20 $CaCl_2$	206.6 ± 4.7	A	0.145 ± 0.032
1.5N: 2Z : 1 CMCH : 1/20 $CaCl_2$	167.2 ± 2.0	A	0.144 ± 0.031
2N : 2Z : 1 CMCH : 1/20 $CaCl_2$	183.0 ± 0.9	A	0.141 ± 0.018

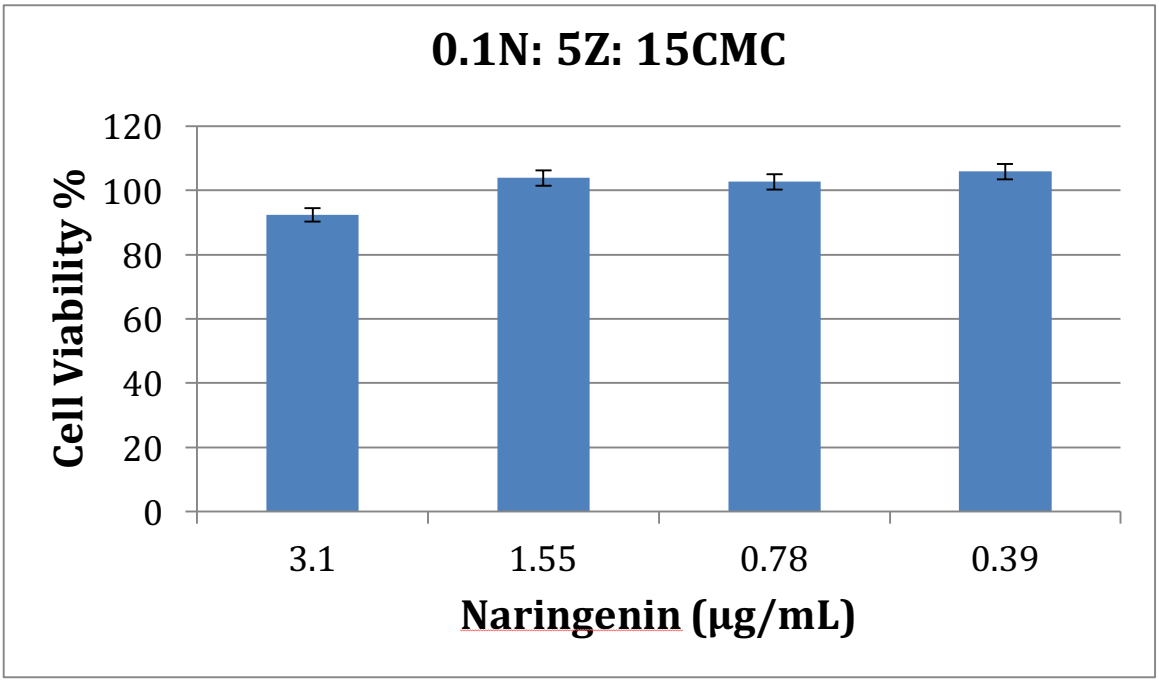
4.2 Cytotoxicity

Cytotoxicity results for are listed in Tables T10-T13. The MTT testing showed no cytotoxicity effects in the encapsulized systems. The cytotoxicity threshold for pure naringenin was 50 μl / mL. Cell viability was found to be over 90% in the polysaccharide formulations investigated including 0.1N: 5Z: 15CMC, 3N: 5Z: 15 CMD, 3N: 5Z: 15CMCH.

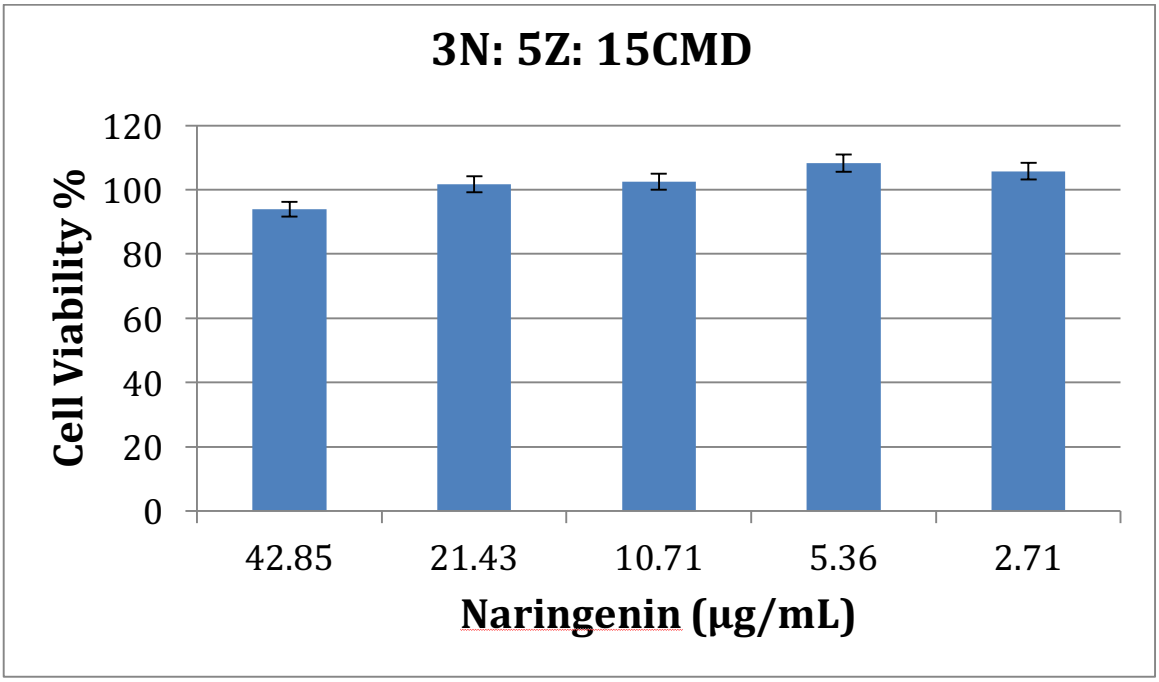
T10. Cytotoxicity Results of Pure Naringenin



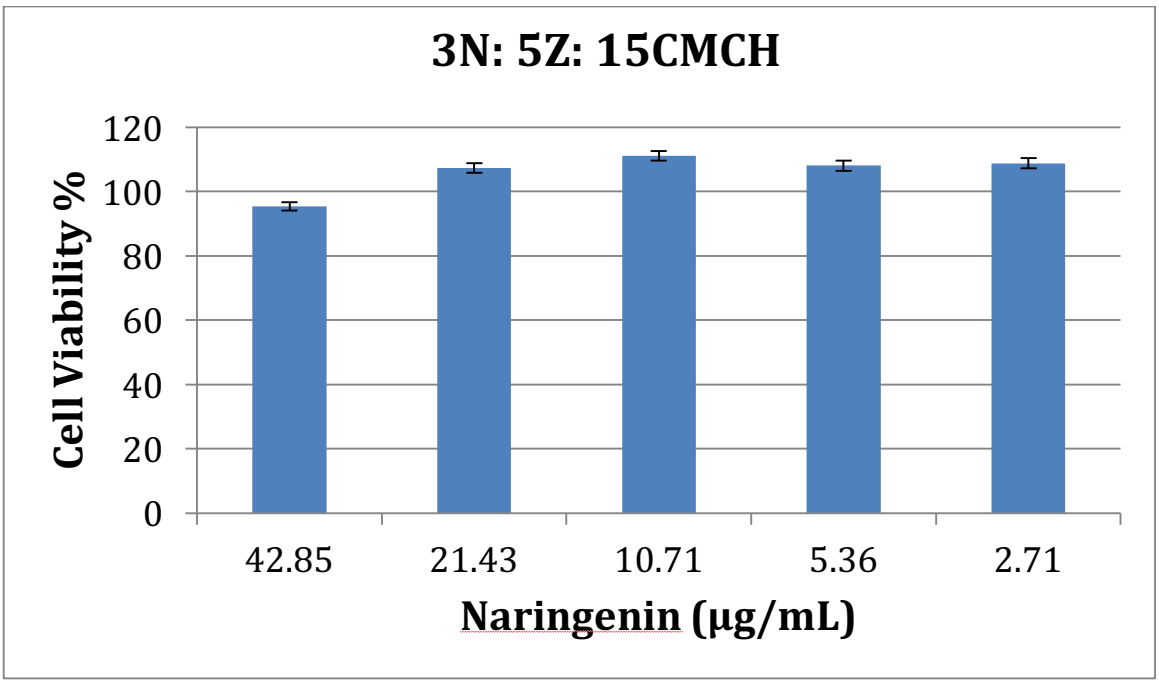
T11. Cytotoxicity Results of Optimized Carboxymethyl Cellulose (CMC)



T12. Cytotoxicity Results of Optimized Carboxymethyl Dextran (CMD)



T13. Cytotoxicity Results of Optimized Carboxymethyl Chitosan (CMCH)



4.3 Zeta Potential & Mobility

The zeta potential of the empty carboxymethyl chitosan nanoparticle with calcium chloride ranged between -37.2 and -42.6. The mobility of the nanoparticle ranged between -1.656 and -1.895.

T14. Zeta Potential & Mobility of Optimized Carboxymethyl Chitosan (CMCH) Nanoparticles with Calcium Chloride

Record	Zeta Potential	Zeta Deviation	Mobility	Mobility Deviation
1	-41.8	13.5	-1.860	0.6023
2	-42.6	14.9	-1.895	0.6631
3	-39.3	13.1	-1.748	0.5822
4	-37.4	14.1	-1.665	0.6284
5	-39.2	14.1	-1.744	0.6286
6	-37.2	11.1	-1.656	0.4960

5. Conclusion and Future Work

Naringenin is a powerful flavanone with multiple health benefits. Although naringenin is found in citrus fruits that can be obtained all over the world, the absorption of naringenin in the body is low which prevents us from maximizing these health benefits. An intravenous solution would be a quick fix to increase bioavailability. However, this approach is not convenient or consumer friendly. A more consumer focused approach using a system with natural and food grade

ingredients would provide consumers an opportunity for increased health benefits as well as provide industries a tool to create a variety of different formats and options for the consumers.

This research showed that naringenin can be encapsulized using zein nanoparticles with the use of polysaccharides. Based on these results, carboxymethyl chitosan provided better encapsulation over carboxymethyl cellulose and carboxymethyl dextran. The addition of calcium chloride to the carboxymethyl chitosan system provided further encapsulation benefits including smaller particle size. Due to the smaller particle size and low cytotoxicity, the carboxymethyl chitosan system with calcium chloride would be a great candidate to improve bioavailability of naringenin and possibly increase the health benefits of naringenin.

Possible future work of the optimized system would include further in vitro investigation to quantify the bioavailability and cellular uptake of narginenin. It would be also useful to see whether this system could encapsulate other flavanones or flavonoids. To make the system more consumer and industry friendly, it would also be beneficial to reduce or remove the alcohol in the system and only have the naringenin filled nanoparticle system remaining.

With consumers taking medication whether for the short term or long term, investigation is required to determine whether there are any increased interactions of the optimized naringenin nanoparticle system with certain drugs. Previous findings showed that there are interactions between naringenin in grapefruit juice and medications. As a result, prescription bottles for those drugs are labeled to

avoid consumption of grapefruit juice during the course of the treatment to avoid adverse effects. For this reason, further research is required to compare the optimized naringenin nanoparticle system (without grapefruit juice) with normal levels of naringenin in grapefruit juice. If increased interactions exist, maximum levels of nargingenin would be required as well as clear communication to the consumers.

Another opportunity of investigation would be to test the stability of the naringenin encapsulation system by itself and in other food matrices over time. Packaged foods have a certain amount of shelf life before it is no longer fit for consumption. A longer shelf life provides more flexibility with CPG companies and more convenience for consumers. It would be beneficial to understand the efficacy of the naringenin encapsulation system over time and investigate whether the system is prone to interaction with other ingredients in certain conditions.

Once the efficacy and safety of the nanoparticle system is fully established, further optimization for taste would be beneficial for CPG food application. As naringenin itself is bitter and provides the natural bitterness in grapefruit, sensory testing would be required to assess the flavor profile and determine if there are high levels of bitterness or off flavors that might distort the overall experience of the product (Fuhr, et al., 1993). As there are certain people in the population that are very sensitive to different types of bitterness, care should be taken to ensure that this nanoparticle system does not turn consumers away due to the taste (Tepper, et al., 2014). The encapsulation system itself may inhibit some of these undesirable taste effects. Microencapsulation systems have been used for other bioactive

ingredients. Omega-3 fatty acids, which are naturally derived from fish oils, have fish-like aromas and flavors that are typically disliked by consumers and very difficult to cover up with other flavors or ingredients. However, with the use of microencapsulation, omega-3 fatty acids are now added to breakfast cereals and dairy products without any fish-like aromas or tastes in the finished product (IFT). For the naringenin system, it is hypothesized that the encapsulation system would also minimize the unpleasant flavors of naringenin. However, formal sensory testing would be required to confirm this hypothesis.

Overall, the encapsulation system including zein, carboxymethyl chitosan, and calcium chloride shows to be a promising delivery vehicle for naringenin. With naringenin's vast health benefits and the safe and food grade properties of the ingredients of the delivery vehicle, the complete naringenin encapsulation system would be a great addition to the world of functional foods. Referring back to Figure F1, the Role of Functional Foods in Health Care Continuum, the naringenin encapsulation system has the potential to be anywhere between the fortified/enhanced foods, supplements, and medical foods depending on the concentration of naringenin and the intended health benefit of the particular food system. It has the potential to be more easily absorbed and in higher concentrations than in it's natural state in grapefruit. With it's versatility, novelty, and numerous benefits, the naringenin encapsulation system would be an opportunity for scientists, food and health industries, and most of all, the consumers that would be using the product.

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