Preparation and Characterization of Resveratrol Nanoemulsions Stabilized by Self-assembly and Complex Coacervation Consisting of Sodium Alginate, Chitosan, and β-Cyclodextrin

Ae-Jin Choi, Younghee Jo[1], Yong-Jin Cho[1], Tae-Eun Kim[1], and Chong-Tai Kim[1]*

Functional Food & Nutrition Division, National Institute of Agricultural Sciences
[1]Research Group of Bioprocess Engineering, Korea Food Research Institute

Abstract

Resveratrol was incorporated into various combinations of single- and double-layer nanoemulsions, prepared by self-assembly emulsfication and complex coacervation with chitosan, alginate, and β-cyclodextrin, respectively. Resveratrol nanoemulsions were composed of medium-chain trigacylglycerols (MCTs), Tween® 80, water, chitosan, alginate, and β-cyclodextrin. The corresponding mixtures were formulated for the purpose of being used as a nutraceutical delivery system. Resveratrol nanoemulsions were obtained with particle sizes of 10-800 nm, with the size variation dependent on the emulsification parameters including the ratio of aqueous phase and surfactant ratio. Resveratrol nanoemulsions were characterized by evaluating particle size, zeta-potential value, stability, and release rate. There were no significant changes in particle size and zeta-potential value of resveratrol nanoemulsions during storage for 28 days at 25°C. The stability of resveratrol in the double-layer nanoemulsions complexed with chitosan or β-cyclodextrin was higher, compared with the single-layer nanoemulsions.

Key words: resveratrol, nanoemulsion, self-assembly, complex coacervation, nutraceutical ingredients

Introduction

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a polyphenolic compound that is present in several plants belonging to the pineceae, mytaceae, fagaceae, moraceae, papilionaceae and vitaceae (Frémont, 2000). Resveratrol restricts platelet aggregation and coagulation, eicosanoid synthesis modification, and lipoprotein metabolism modulation. These unique properties have been shown to affect the treatment of heart disease as well as anti-cancer, anti-inflammatory, anti-oxidative, antibiotic and anti-aging activities (Frankel et al., 1993; Jang et al., 1997; Pinto et al., 1999). However, resveratrol is limited in applications that involve interaction with external environment such as air, light, and oxidative enzymes due to high hydrophobicity, poor stability and solubility. It exists in two isomeric forms *cis* and *trans* and have ability to convert into *cis* from trans isomer; unfortunately only *trans*-resveratrol has biologically activity. But *trans*-resveratrol has limited stability under the influence of light, certain pH levels and temperature, which

can cause degradation or isomerization (Zupancic et al., 2015).

At present, one promising nanotechnology is the application of a delivery system containing nutraceutical ingredients with nanometer-sized emulsion form, which offers the potential to improve the solubility and bioavailability of many bioactive ingredients such as carotenoids, polyunsaturated fatty acids, phytosterols, antioxidants, vitamins, minerals and other natural compounds (Kim et al., 2009). Nanoemulsions can be prepared by self-assembly, which can be used to introduce guest molecules having hydrophilic, lipophilic, or amphiphilic characteristics. It is thermodynamically stable and isotropic in form, having a drop size less than 100 nm. It provides ultra-low interfacial tensions and large oil-in-water (O/W) interfacial areas, so it has a higher solubilization capacity than simple micellar solutions, and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions (Lawrence & Ress, 2000). The stability of the nanoemulsion depends on the preparation method as well as the order in which its components were added in self-assembly emulsfication (Wang et al., 2008). Self-assembly emulsfication is accomplished using an isotropic mixture of oil, surfactant, and co-surfactant. Together, these form fine O/W emulsions upon introduction into aqueous media under agitation. The self-assembled structures that formed in the oil phase at a certain dilution composition can be hydrated with

water to produce bicontinuous structures inverting into an O/W structure (Garti et al., 2005).

Recently, food nanotechnology has focused on nanoencapsulation of bioactive ingredients such as capsaicin and resveratrol as well as the successful application of nanoemulsions (Cho et al., 2008; Kim et al., 2008). A bioactive ingredient can be encapsulated in nanometer-sized structures, which are transparent in form with sizes of 20-500 nm that provide solubility, stability, and bioavailability (Choi et al., 2008). Very few examples of nanoemulsion systems based on food-grade surfactants such as ethoxylated sorbitan esters (Tweens®) and sucrose esters exist (Choi et al., 2009). Medium-chain triacylglycerols (MCTs) provide different chemical and physiological properties compared to unsaturated long-chain triacylglycerols (LCTs) such as soy, corn, cotton, and sunflower oil. Compared to LCTs, MCTs such as palm and coconut oil are fully saturated. The smaller size, lower melting points, and higher water solubility allows them to be stable in aqueous phase in response to oxidative rancidity at room temperature (Jeukendrup & Aldred, 2004). As such, MCTs are now utilized in many food applications, and they have been approved as GRAS (Generally Recognized as Safe) for oral or enteral use by the U.S. Food and Drug Administration (Traul et al., 2000). A simple solution or nanoemulsion may be unstable or subject to degradation when used as a food delivery system due to the inherent structure of the emulsion as well as environmental conditions such as temperature, light, and pH. It is possible to develop nanoparticles as novel delivery systems using food-grade biopolymers such as polysaccharides (alginate, chitosan, pectin, gum arabic) and proteins (casein, gelatin, whey) by promoting self-association or aggregation of single biopolymers or by inducing phase separation in mixed biopolymer systems (Kim & Karin, 2008). Therefore, single-layer nanoemulsions were named O/W nanoemulsions layered with aqueous phase based on water, oil phase, and surfactant. Double-layered nanoemulsions were named O/W nanoemulsions layered with alginate, chitosan and β-cyclodextrin in aqueous phase.

Currently, there are many different types of delivery systems for resveratrol. Such systems involve the incorporation of resveratrol into yeast cells, liposomes, calcium-pectinate or chitosan for controlled delivery as microspheres or nanospheres (Caddeo et al., 2008; Shi et al., 2008; Das & Ng, 2010; Peng et al., 2010). Although many researchers have different technologies about preparing resveratrol nanoemulsions, most of them used a high energy emulsification method such as high speed homogenization and ultra sound sonication. So we have proposed a low-energy emulsification method using ethanol in oil phase and a method of preparing nanoemulsions containing more than 100 μg of resveratrol with stable system. The objective of this study was to investigate the formulating conditions of single- and double-layers nanoemulsions containing resveratrol that are produced by self-assembly and complex coacervation using biopolymers such as chitosan, alginate and β-cyclodextrin. Furthermore, the characteristics and stabilities of the resveratrol nanoemulsions were evaluated to demonstrate the potential of resveratrol as a nutraceutical delivery system.

Materials and Methods

Chitosan was prepared by demineralization, deproteinization, and deacetylation from crab shell. Next, the chitosan (degree of deacetylation 93%, molecular weight 330,000) was dissolved in a mixed aqueous solution of 20% hydrochloride solution and distilled water, after which the mixture was adjusted to pH 5.6 and stirred for 3 h. The reaction mixture was then subjected to a continuous membrane filtration process, after which a permeate was freeze-dried (Seo & Toshio, 2002). Resveratrol, alginate sodium salt, β-cyclodextrin, and polyoxyethylene sorbitan monooleate (Tween® 80) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MCTs (medium-chain triacylglycerols) were purchased from Wellga Co. (Seoul, Korea). Deionized distilled water was purchased from Waters (Milford, MA, USA). The R-(+)-limonene was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Soybean oil, corn oil and canola oil were obtained from Baeksul Co. (Seoul, Korea). Olive oil was purchased from Sajoheapyo Co. (Seoul, Korea).

Preparation of chitosan
Chitin was prepared by treating *Chitinonecetes opilio* with 15% hydrochloride solution at 50°C for 24 h (demineralization) and, subsequently, with 5% sodium hydroxide at 90°C for 12 h (deproteinization). Then chitin was treated with 60% sodium hydroxide at 140°C for 24 h to remove the acetyl residues (deacetylation). Next, the chitosan (degree of deacetylation 93%, molecular weight 330,000) was dissolved in a mixed aqueous solution of 20% hydrochloride solution and distilled water, after which the mixture was adjusted to pH 5.6 and stirred for 3 h. The reaction mixture was then subjected to a continuous membrane filtration process, after which permeate was freeze-dried.

Screening of oil phase and surfactant concentration for solubilizing resveratrol

To measure resveratrol solubility, various oil phases were selected (MCTs, limonene, corn oil, soybean oil, olive oil, and canola oil). The selection of oil phase was evaluated with analysis of particle size, degree of turbidity, and separation of oil phase through visual observation on the preparation of the nanoemulsion. Therefore, the optimum oil phase was selected with MCTs that could manufacture particle sizes of less than 100 nm and transparent in form. After ternary mixtures consisting of various types and compositions of oil phases were pre-tested.

Resveratrol (10 mg) was added to 1 mL of ethanol, followed by the addition of the oil phases at a 1:1 ratio, respectively. The mixture was stirred for 5 min and then subjected to vacuum evaporation at 50°C to remove the ethanol. The oil phases and Tween[®] 80 were then mixed together at a weight ratio of 1:3, followed by stirring for 5 min. To prepare the nanoemulsion, the mixture was accurately weighed (0.5 g) and diluted with deionized water (100 mL) as the aqueous phase, followed by magnetic stirring for 2 h and stabilization at 25°C for 24 h. Only with MCTs, measurement of the surfactant concentration was pre-tested using Tween[®] 80 at different ratios (resveratrol +MCTs:Tween[®] 80=1:1, 1:1.5, 1:2 and 1:3), based upon the results of our prior research involving the preparation of nanoemulsions using oleoresin capsicum (Choi et al., 2009). Tweens are widely used in food emulsion systems, primarily due to their relatively low toxicity, irritation potential and the ease with which they form micro- or nanoemulsions.

Preparation of single-layer resveratrol nanoemulsions (SLRNs)

SLRNs consisted of oil droplets in an aqueous phase with the oil droplets being surrounded by a thin interfacial layer consisting of emulsifier molecules. SLRNs were prepared by self-assembly emulsification according to the following procedure. One of the six different oil phases used, MCTs produced highly dispersive emulsions consisting at all composition ratios of ethyl alcohol and resveratrol. Therefore, pre-mixture containing MCTs as the oil phase and Tween[®] 80 as surfactant was selected. Resveratrol (10 mg) was added to 1 mL of ethanol, followed by the addition of MCTs at a 1:1 (v/w) ratio. The mixture was stirred for 5 min and then subjected to vacuum evaporation at 50°C to remove the ethanol. The oil phase and Tween[®] 80 were then mixed together at weight ratios of 1:1, 1:2, and 1:3 followed by stirring for 5 min (Table

1). To prepare the nanoemulsion, the mixtures were accurately weighed (0.5 g) and diluted with deionized water (100 mL). There is magnetically stirred for 2 h and stabilized at 25°C for 24 h. This primary emulsion was used for the preparation of SLRNs by dilution with deionized water.

Preparation of double-layers resveratrol nanoemulsions (DLRNs)

Charged polyelectrolyte biopolymers are then added to the system of single layer nanoemulsions so that it absorbs to the droplet surface and produces DLRNs consisting of oil droplets coated by a biopolymer polyelectrolyte interface. DLRNs were prepared by complex coacervation with three different biopolymers: alginate (AN), chitosan (CN) and β-cyclodextrin (CYN). Alginate, chitosan and β-cyclodextrin solutions were prepared at concentrations of 0.05% in aqueous phase. Resveratrol (5 mg) was added to 1 mL of ethanol followed by the addition of MCTs at a 1:1 (v/w) ratio. The mixture was stirred for 5 min and then subjected to vacuum evaporation at 50°C to remove the ethanol. The oil phase and Tween[®] 80 were then mixed together at a weight ratio of 1:2, followed by stirring for 5 min. This mixture was accurately weighed (0.5 g), diluted in each of the above biopolymer solutions instead of deionized water, and then stirred for 2 h at 25°C. The nanoemulsions were then filtered with a 0.45 μm PVDF membrane filter (Whatman, Maidstone, Kent, UK) and stabilized at 25°C for 24 h before analysis.

Determination of particle size distribution and zeta-potential value

The particle size distributions of the nanoemulsions were determined by photon correlation spectroscopy which analyzes the fluctuations in light scattering due to the Brownian motion of the particle using a Nanotrac NPA 250 (Microtrac Inc., Montgomeryville, PA, USA). Light scattering was monitored at 25°C at a 90° angle. The zeta-potentials of the nanoemulsions were measured by a ZetaPlus (Brookhaven Instruments Corp., Holtsville, NY, USA), which measures the distribution of the electrophoretric mobility of the particles using laser light scattering. 1.6 mL of each sample was poured into the electrophoretic cell. The particle size distribution and zeta-potential value was analyzed at the optimum concentration range of Nanotrac and ZetaPlus through diluting between 10 and 100 times with deionized water. Each measurement was repeated 5 times on three different samples of the same composition.

Table 1. Particle sizes and resveratrol contents of resveratrol nanoemulsions

Composition ratio		Composition contents (%)			Resveratrol (μg)	Particle size (nm)
Res+MCTs[1]	Tween® 80	Res+MCTs	Tween® 80	Water		
		0.25	0.25	99.5	12.5	136±10.78[2)d4)]
		0.50	0.50	99.0	25.0	155±22.94[d]
1	1	1.50	1.50	97.0	75.0	176±64.12[d]
		2.50	2.50	95.0	125	251±9.26[c]
		5.00	5.00	90.0	250	342±47.49[b]
		10.0	10.00	80.0	500	655±36.76[a]
F-value						81.94[***3)]
		0.17	0.33	99.5	8.33	27±1.11[c]
		0.33	0.67	99.0	16.7	25±2.15[c]
1	2	1.00	2.00	97.0	50.0	24±0.99[c]
		1.67	3.33	95.0	83.3	30±4.65[c]
		3.33	6.67	90.0	167	170±42.64[b]
		6.67	13.33	80.0	333	700±34.70[a]
F-value						428.4[***]
		0.13	0.38	99.5	6.25	9±0.18[c]
		0.25	0.75	99.0	12.5	14±0.14[a]
1	3	0.75	2.25	97.0	37.5	14±0.07[a]
		1.25	3.75	95.0	62.5	14±0.12[a]
		2.50	7.50	90.0	125	12±0.66[b]
		5.00	15.00	80.0	250	9±0.57[c]
F-value						142.09[***]

[1] Res+MCTs: resveratrol+medium-chain triacylglycerols
[2] Mean±SD
[3] ***: $p<0.001$
[4] a-d: Means with different superscripts in the same column differ significantly at $p<0.05$ by Duncan's multiple range test

Storage stability of SLRNs and DLRNs

The storage stability of SLRNs and DLRNs were evaluated by measuring the particle size and zeta-potential value during storage at 25°C for 4 weeks. The determination methods of particle size and zeta-potential value were same as above described method.

Stability of resveratrol

The stability of resveratrol from the SLRNs and DLRNs was determined by measuring the amount of resveratrol using liquid-liquid extraction. In this study, only CYN was used in the release test of resveratrol from DLRNs. The initial resveratrol content of the nanoemulsions was designated as 100%, whereas the decreasing resveratrol content was calculated via HLPC for 7 storage days at 25°C. The liquid-liquid extraction of resveratrol was performed as follows. Nanoemulsions (1 mL) were mixed with 100% MeOH (2.5 mL) for 1 min, followed by the addition of hexane (10 mL) with stirring for 3 min. This mixture was maintained for 10 min at room temperature prior to the extraction of resveratrol. The methanol layer was recovered using a micro-syringe and then filtered with a 0.45 μm syringe filter. The extracted resveratrol was then analyzed by HPLC using a gradient controller unit 601-602 (FLOM, Tokyo, Japan) with an injection volume of 20 μL. Separation was performed on a C_{18} column (Zorbax Eclipse XDB-C18, 4.6×250 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase used was 100% acetonitrile, and the flow rate was 0.6 mL/min for 10 min. The UV/VIS detector S-3740 (Soma, Tokyo, Japan) was set at 308 nm. Standard curves were made using resveratrol dissolved in methanol (10, 30, 50 and 100 ppm).

Statistical analysis

All results are expressed as the mean±standard deviation. Statistical analyses of the data were carried out using SAS software (SAS Institute, Cary, NC, USA). Significant differences among groups of nanoemulsions were determined by Duncan's multiple range test with $p<0.05$, $p<0.01$ and $p<0.001$, were considered statistically significant.

Results and Discussion

Effect of oil phase on resveratrol nanoemulsions

Resveratrol at a concentration of 5 mg/mL was solubilized in ethanol and then mixed with MCTs, limonene, corn oil, soybean oil, olive oil and canola oil at ratios of 1:1. These resulting mixtures were then added to Tween[®] 80 at ratios of 1:3 (w/w). The emulsion was prepared by dissolving the mixtures with water at concentrations of 0.5%. When the ethanol-resveratrol solution was mixed with corn oil, soybean oil, olive oil, and canola oil, it became cloudy and separated from the oil phase. Additionally, the particle size was found to be within 191-936 nm (Table 2). In contrast, MCTs and limonene showed particle sizes of about 16.15 and 12.11 nm, respec- tively, which implies they can serve as the oil phase for the production of resveratrol-containing nanoemulsions. Resveratrol does not completely dissolve in buffering solutions with pHs of 4-5, but it does show relatively high solubility in coconut oil as well as glycerol (Hung et al., 2006). Additionally, the use of limonene is limited due to its characteristic flavor. Therefore, MCTs are considered to be the appropriate oil phase for the production of nanoemulsions containing resveratrol. In addition, due to its high solubility in ethanol, resveratrol could be used for the manufacture of nanoemulsions containing high concentrations of resveratrol.

Formulation and phase diagram of resveratrol nano-emulsions

Table 1 displays the particle sizes and resveratrol contents of nanoemulsions based on various ratios of resveratrol and MCTs with Tween[®] 80. The highest resveratrol content (250 μg/mL) was produced when the resveratrol and MCTs mixture was mixed with Tween[®] 80 at a 1:3 ratio (Resveratrol+MCTs :

Tween[®] 80 : water composition ratio of 5 : 15 : 80, w/w) (Table 1). In addition, when resveratrol and MCTs were mixed with Tween[®] 80 at a 1:2 ratio, the particle size was 30 nm and the resveratrol content was 83.3 μg/mL (Resveratrol+MCTs : Tween[®] 80 : water composition ratio of 1.67 : 3.33 : 95, w/w). The particle sizes of the nanoemulsions after mixing of resveratrol and MCTs with Tween[®] 80 at ratios of 1:1, 1:1.5, 1:2 and 1:3 are presented in Table 3. When the mixing ratio was 1:2 or 1:3, the particle sizes of the nanoemulsions were 16.3 and 16.2 nm, respectively. However, mixing ratios of 1:1 and 1:1.5 resulted in sizes of 231 and 554 nm, respectively, which indicates that the emulsions must be unstable if subjected to storage. Based on the results, the appropriate mixing ratio of Tween[®] 80 for the production of resveratrol-containing nanoemulsions is considered to be two times that of the weight of the resveratrol and MCTs mixture. A phase diagram of the three-component resveratrol+MCTs/Tween[®] 80/water system at ratios of 1:1, 1:1.5, 1:2 and 1:3 is presented in Fig. 1. This composition ratio of Tween[®] 80 could be used as a quality indicator related to the allowance limit of additives in the food industry. The phase behavior of such samples was determined only after sharp interfaces become visible. When the mixing ratio of the resveratrol and MCTs mixture with Tween[®] 80 was 1:2 or 1:3 in 60% water, nanoemulsions formed with particle sizes of less than 100 nm, which is indicated as the white region in the diagram. There were exhibited white areas as one-phase nanoemulsions region (below 100 nm) and gray areas as multiple-phase nanoemulsions (between 100 and 700 nm), respectively. Because nano-sized emulsions below 100 nm absorb light, these samples will have a transparent appearance. This differs from the micro-sized emulsions larger than 300 nm which will scatter light sufficiently to allow them to be seen by naked eye and have a cloudy white appearance.

Table 2. Effect of various oil phases on the resveratrol nanoemulsion particle sizes

Oil phase	Particle size(nm)
MCTs[1]	16.15±0.22[2) e4)]
Limonen	12.11±0.77[e]
Corn oil	190.67±6.03[d]
Soybean oil	634.67±23.59[c]
Olive oil	764.67±12.58[b]
Canola oil	935.67±31.94[a]
F-value	1,651.02[***3)]

[1] MCTs : medium-chain triacylglycerols
[2] Mean±SD
[3] [***]: $p < 0.001$
[4] [a-e]: Means with different superscripts in the same column differ significantly at $p < 0.05$ by Duncan's multiple range test

Table 3. Effect of various mixture ratios of oil phases and surfactants on the resveratrol nanoemulsion particle size

Mixture ratio Res+MCTs:T[1]	Particle size(nm)
1 : 1.0	554.00±112.94[2)a4)]
1 : 1.5	230.87±23.53[b]
1 : 2.0	16.29±0.47[c]
1 : 3.0	16.15±0.22[c]
F-value	58.23[***3)]

[1] Res+MCTs:T : resveratrol+medium-chain triaylglycerols:Tween80
[2] Mean±SD
[3] [***]: $p < 0.001$
[4] [a-c]: Means with different superscripts in the same column differ significantly at $p < 0.05$ by Duncan's multiple range test
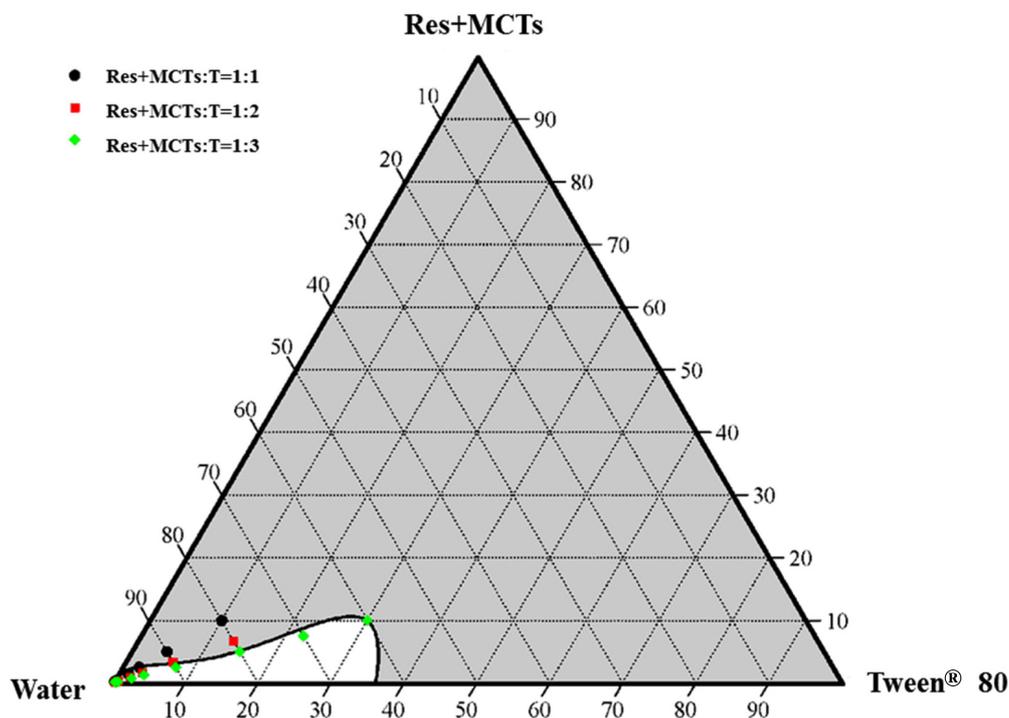
Fig. 1. Phase diagram of nanoemulsions formed by self-assembly of the water/resveratrol+MCTs/Tween® 80 system. Res+ MCTs:T: resveratrol+medium-chain riaylglycerols:Tween® 80. White area is the region of the nanoemulsions ranging below 100 nm.

Storage stability of SLRNs and DLRNs

To confirm the stability of SLRNs, the mixing ratios of the resveratrol and MCTs mixture with Tween® 80 were varied as 1:1, 1:2 and 1:3, and the particle sizes of the resveratrol nanoemulsions during 4 weeks of storage at 25°C were measured (Table 4). When the mixing ratio was 1:1, the particle sizes of emulsions were 136-176 nm at mixture concentrations of 0.5, 1 and 3% (w/w); whereas, the particle sizes of 251-655 nm were observed at concentrations of 5, 10, and 20% (w/w) at 0 day. When the mixing ratio was 1:2, particle sizes of 27, 25, 24, and 30 nm were observed to be concentrations of 0.5, 1, 3, and 5%, respectively, showing that particle size was reduced by increasing the amount of Tween® 80 and maintaining a stable state during storage. A mixing ratio of 1:3 resulted in particle size of less than 15 nm when the Tween® 80 was added at concentrations of 0.5, 1, 3, 5, and 10% (w/w); these nanoemulsions were stable throughout storage. For the production of nanoemulsions, the amount of Tween® 80 added has large effects on particle size reduction and stability of the nanoemulsion (Choi et al., 2009). The storage stabilities of the resveratrol nanoemulsions prepared by mixing resveratrol and MCTs with Tween® 80 with a ratio of 1:2 are presented in Table 5. The DLRNs prepared with alginate and chitosan were unstable and showed slight increases in particle size during the 4 week storage period.

However, the DLRNs prepared with β-cyclodextrin were more stable with particle sizes of less than 40 nm during storage. For the SLRNs, they were relatively stable during storage, but their stability was slightly reduced after 2 weeks of storage. These differences in stability between the SLRNs and DLRNs may be due to their initial particle sizes governed by their composition that included surfactants, bio-polymers (e.g. alginate and chitosan), and lipids.

The droplets in most nanoemulsions have an appreciable electrical charge; therefore, electrostatic interactions may play an important role in determining their overall stability and physicochemical properties. The zeta potential, which represents the electric charge between the shear plane of the final external layer and the bulk solution, has been used to estimate the property of dispersions. The zeta potentials of the resveratrol nanoemulsions prepared by mixing resveratrol and MCTs with Tween® 80 at a ratio of 1:2 are indicated in Table 6. The zeta potential of the DLRN prepared with chitosan was 0-37.7 mV during storage due to the positive charge of the chitosan molecules coated on the surface of nanoemulsions. In contrast, the zeta potential of the DLRN prepared with alginate was -14.4 - -24.8 mV due to the negative charge of the alginate molecules. Meanwhile, the zeta potential of the DLRN prepared with β-cyclodextrin resulted in a stable negative value ranging from -5.6 - -14.6 mV. The droplets of most

Table 4. Changes in particle size of single-layer resveratrol nanoemulsions formed by self-assembly during storage 28 days

Composition ratio	Mixture:Water	Storage (day)							F-value
		0	1	3	7	14	21	28	
Res+MCTs:T =1:1[1]	0.5:99.5	$^{5)D}136.00\pm10.78^{2)}$	$^{C}149.20\pm23.87$	$^{D}155.70\pm7.50$	$^{D}113.00\pm19.14$	$^{D}134.60\pm20.30$	$^{C}147.90\pm8.32$	$^{D}148.70\pm15.42$	$1.86^{n.s.}$
	1:99	$^{D}154.90\pm22.94$	$^{C}168.60\pm34.68$	$^{D}138.80\pm20.13$	$^{D}131.40\pm10.10$	$^{D}123.40\pm3.61$	$^{C}138.80\pm26.54$	$^{D}163.30\pm7.67$	$1.94^{n.s.}$
	3:97	$^{D}176.40\pm64.12$	$^{C}177.20\pm23.82$	$^{CD}191.70\pm29.03$	$^{D}150.00\pm20.37$	$^{D}147.00\pm41.26$	$^{C}174.20\pm21.67$	$^{CD}183.00\pm9.54$	$0.63^{n.s.}$
	5:95	$^{C}250.60\pm9.26^{b}$	$^{B}323.00\pm11.36^{a}$	$^{BC}216.70\pm40.60^{b}$	$^{C}212.80\pm13.33^{b}$	$^{C}210.30\pm25.69^{b}$	$^{B}236.00\pm36.72^{b}$	$^{BC}252.20\pm36.48^{b}$	5.01^{**}
	10:90	$^{B}342.00\pm47.49$	$^{B}361.00\pm29.82$	$^{B}269.20\pm52.82$	$^{B}297.40\pm26.23$	$^{B}321.00\pm33.34$	$^{B}283.50\pm35.62$	$^{B}305.00\pm10.53$	$2.18^{n.s.}$
	20:80	$^{A}655.00\pm36.76^{ab4)}$	$^{A}696.00\pm49.00^{a}$	$^{A}568.00\pm41.07^{bc}$	$^{A}528.00\pm39.69^{c}$	$^{A}589.00\pm36.61^{bc}$	$^{A}424.00\pm37.07^{d}$	$^{A}632.00\pm93.31^{ab}$	7.07^{**}
	F-value	$81.94^{***3)}$	134.37^{***}	61.53^{***}	88.51^{***}	74.14^{***}	40.21^{***}	37.60^{***}	
Res+MCTs:T =1:2	0.5:99.5	$^{C}26.47\pm1.11^{cd}$	$^{C}40.20\pm9.31^{ab}$	$^{C}20.28\pm0.81^{d}$	$^{C}31.20\pm2.32^{bcd}$	$^{D}35.40\pm1.01^{bc}$	$^{C}50.30\pm16.45^{a}$	$^{D}31.20\pm0.37^{bcd}$	46.12^{***}
	1:99	$^{C}25.30\pm2.15^{b}$	$^{C}50.70\pm18.42^{a}$	$^{C}26.91\pm2.32^{b}$	$^{C}41.30\pm1.01^{a}$	$^{D}47.80\pm3.41^{a}$	$^{C}44.60\pm3.86^{a}$	$^{D}47.90\pm1.64^{a}$	5.46^{**}
	3:97	$^{C}24.00\pm0.99^{e}$	$^{C}26.34\pm0.34^{e}$	$^{C}32.70\pm1.10^{d}$	$^{C}39.30\pm2.19^{c}$	$^{D}45.80\pm5.03^{b}$	$^{C}51.70\pm3.78^{ab}$	$^{CD}55.60\pm2.29^{a}$	60.39^{***}
	5:95	$^{C}30.00\pm4.65^{e}$	$^{C}44.60\pm10.19^{d}$	$^{C}42.90\pm2.97^{d}$	$^{C}60.40\pm1.61^{c}$	$^{C}63.00\pm11.06^{bc}$	$^{C}72.60\pm8.73^{ab}$	$^{C}82.20\pm6.50^{a}$	19.51^{***}
	10:90	$^{B}169.90\pm42.64$	$^{B}212.80\pm63.40$	$^{B}229.90\pm29.82$	$^{B}174.20\pm39.85$	$^{B}235.50\pm4.38$	$^{B}178.90\pm32.81$	$^{B}217.20\pm17.43$	$1.46^{n.s.}$
	20:80	$^{A}700.00\pm34.70^{b}$	$^{A}799.00\pm57.30^{a}$	$^{A}661.00\pm52.05^{bc}$	$^{A}558.00\pm32.30^{d}$	$^{A}603.00\pm16.87^{cd}$	$^{A}447.00\pm36.50^{e}$	$^{A}582.00\pm27.43^{d}$	23.00^{***}
	F-value	428.40^{***}	211.86^{***}	320.23^{***}	193.98^{***}	2444.14^{***}	161.87^{***}	492.12^{***}	
Res+MCTs:T =1:3	0.5:99.5	$^{C}8.77\pm0.18^{b}$	$^{A}18.96\pm0.36^{b}$	$^{E}11.34\pm0.10^{b}$	$^{B}24.07\pm0.10^{b}$	$^{D}20.26\pm0.38^{b}$	$^{C}24.89\pm1.15^{b}$	$^{B}118.60\pm56.06^{a}$	31.44^{***}
	1:99	$^{A}13.86\pm0.14^{d}$	$^{B}17.40\pm0.10^{c}$	$^{D}13.11\pm0.12^{d}$	$^{B}22.89\pm0.03^{ab}$	$^{CD}22.72\pm0.39^{ab}$	$^{C}23.58\pm0.60^{a}$	$^{D}22.11\pm12.92^{b}$	192.79^{***}
	3:97	$^{A}13.92\pm0.07^{d}$	$^{DE}15.81\pm0.06^{c}$	$^{CD}14.26\pm0.02^{d}$	$^{B}22.94\pm0.24^{b}$	$^{CD}24.25\pm1.09^{a}$	$^{C}25.47\pm0.77^{a}$	$^{D}24.34\pm13.85^{a}$	128.90^{***}
	5:95	$^{A}14.17\pm0.12^{e}$	$^{E}15.58\pm0.11^{d}$	$^{C}14.89\pm0.12^{de}$	$^{B}23.26\pm0.24^{c}$	$^{C}25.66\pm1.38^{b}$	$^{C}28.75\pm1.17^{a}$	$^{D}26.58\pm15.26^{b}$	172.22^{***}
	10:90	$^{B}12.39\pm0.66^{f}$	$^{CD}16.52\pm0.30^{e}$	$^{B}16.54\pm0.33^{e}$	$^{B}23.82\pm0.30^{d}$	$^{B}35.70\pm1.56^{c}$	$^{B}45.20\pm3.67^{b}$	$^{C}49.70\pm25.96^{a}$	122.39^{***}
	20:80	$^{C}9.04\pm0.57^{d}$	$^{BC}16.95\pm0.93^{d}$	$^{A}17.97\pm1.93^{d}$	$^{A}91.10\pm2.34^{c}$	$^{A}127.80\pm4.71^{b}$	$^{A}120.10\pm10.03^{b}$	$^{A}151.30\pm75.21^{a}$	299.81^{***}
	F-value	142.09^{***}	24.36^{***}	25.99^{***}	1606.13^{***}	753.23^{***}	218.69^{***}	49.82^{***}	

[1] Res+MCTs:T=1:1 : resveratrol+medium-chain riaylglycerols:Tween® 80=1:1, [2] Mixture:Water : Resveratrol+MCTs+Tween® 80:Water.
[2] Mean±S.D.
[3] ***: $p<0.001$, **: $p<0.01$, n.s.: not significantly
[4] a-f: Means with different superscripts in the same row differ significantly at $p<0.05$ by Duncan's multiple range test
[5] A-E: Means with different superscripts in the same column differ significantly at $p<0.05$ by Duncan's multiple range test

Table 5. Changes in particle sizes of single- and double-layers resveratrol nanoemulsions prepared by complex coacervation during storage for 28 days

Sample	Storage (day)								F-value
	0	1	3	5	7	14	21	28	
SLRN[1]	[5)B]27.09±2.04[2)d4)]	[B]29.48±3.71[cd]	[C]29.89±1.70[cd]	[B]35.7±2.33[bc]	[B]41.30±1.40[b]	[B]42.70±3.47[b]	[B]56.60±7.90[a]	[C]51.90±7.77[a]	17.3[***]
CN	[A]45.60±11.89[d]	[B]50.87±0.40[d]	[B]70.00±19.75[cd]	[AB]53.70±26.31[d]	[A]99.80±26.36[bc]	[A]115.40±22.44[ab]	[A]144.30±19.46[a]	[B]105.00±28.20[bc]	8.56[***]
AN	[A]55.40±14.27[c]	[A]133.90±39.16[ab]	[A]150.40±26.21[a]	[A]94.00±37.17[bc]	[A]116.40±35.54[ab]	[A]115.50±20.40[ab]	[A]152.30±4.52[a]	[A]166.50±38.57[a]	4.48[**]
CYN	[B]26.85±1.99[d]	[B]33.00±3.63[cd]	[C]38.20±6.26[bc]	[B]28.39±2.97[d]	[B]38.20±5.32[bc]	[B]41.80±3.62[ab]	[B]42.70±3.25[ab]	[C]45.90±5.20[a]	7.88[**]
F-value	6.79[*3)]	18.45[***]	32.4[***]	4.96[*]	9.70[**]	22.71[***]	83.52[***]	15.96[**]	

[1] SLRN: single-layer resveratrol nanoemulsion based on water, CN: double-layers resveratrol nanoemulsion prepared by complex coaervation with chitosan, AN: double-layers resveratrol nanoemulsion prepared by complex coaervation with alginate, CYN: double-layers resveratrol nanoemulsion prepared by complex coaervation with β-cyclodextrin
[2] Mean±S.D.
[3] [***]: $p<0.001$, [**]: $p<0.01$, [*]: $p<0.05$
[4] [a-d]: Means with different superscripts in the same row differ significantly at $p<0.05$ by Duncan's multiple range test
[5] [A-C]: Means with different superscripts in the same column differ significantly at $p<0.05$ by Duncan's multiple range test

Table 6. Zeta-potential of single- and double-layers resveratrol nanoemulsions prepared by complex coacervation during storage for 28 days

Sample	Storage (day)								F-value
	0	1	3	5	7	14	21	28	
SLRN[1]	[5)B]-8.55±1.37[2)b4)]	[C]-11.63±2.78[c]	[B]-11.25±1.24[bc]	[A]0.00±0.00[a]	[B]-7.97±1.94[b]	[B]-7.56±2.12[b]	[B]-11.32±1.62[bc]	[B]-9.27±1.03[b]	8.73[***]
CN	[A]14.17±4.12[d]	[A]14.47±1.44[d]	[A]17.85±0.60[c]	[A]0.00±0.00[e]	[A]18.62±0.69[c]	[A]17.93±0.61[c]	[A]37.72±1.03[a]	[A]31.06±0.81[b]	153.63[***]
AN	[C]-22.76±0.74[bc]	[C]-15.41±3.09[a]	[C]-22.02±1.37[bc]	[C]-20.41±0.88[bc]	[D]-21.22±1.43[bc]	[D]-24.76±2.01[c]	[C]-19.94±0.36[b]	[D]-14.36±0.68[a]	5.30[***]
CYN	[B]-7.67±1.13[ab]	[B]-5.64±1.99[a]	[B]-10.35±0.67[bc]	[B]-14.58±0.85[c]	[C]-14.64±2.02[c]	[C]-14.00±1.46[c]	[B] -12.82±2.10[c]	[C]-11.88±1.21[bc]	4.96[***]
F-value	163.49[***3)]	42.20[***]	273.57[***]	362.61[***]	117.90[***]	118.98[***]	391.96[***]	695.75[***]	

[1] SLRN: single-layer resveratrol nanoemulsion based on water, CN: double-layers resveratrol nanoemulsion prepared by complex coaervation with chitosan, AN: double-layers resveratrol nanoemulsion prepared by complex coaervation with alginate, CYN: double-layers resveratrol nanoemulsion prepared by complex coaervation with β-cyclodextrin.
[2] Mean±S.D.
[3] [***]: $p<0.001$
[4] [a-e]: Means with different superscripts in the same row differ significantly at $p<0.05$ by Duncan's multiple range test
[5] [A-D]: Means with different superscripts in the same column differ significantly at $p<0.05$ by Duncan's multiple range test

nanoemulsions have an appreciable electrical charge, suggesting electrostatic interactions may play an important role in determining overall stability and physicochemical properties (Nielloud & Marti-Mestres, 2000). The zeta potential represented by the electric charge between the shear plane of the final external layer and the bulk solution, is used to estimate the extent of dispersion. Furthermore, this factor is influenced by the electrical characteristics between nanoemulsion particles.

Stability of resveratrol

The stability of resveratrol from the DLRN prepared with β-cyclodextrin is shown in Table 7. The resveratrol content of the DLRN prepared with β-cyclodextrin was much higher than the SLRN, which it was reduced to 94.6% after 7 days. However, for the SLRN, the content of resveratrol was reduced to about 88.5% in the same storage period, demonstrating lower stability compared to the DLRN. This result indicates that delayed resveratrol release when resveratrol was present in an emulsion or aqueous micelle system. Moreover, the stability of resveratrol is related with its reduction in the order of aqueous micelle system > tocol emulsion > lipid emulsion (Hung et al., 2007). Therefore, the DLRNs prepared with β-cyclodextrin have potential as a nutraceutical delivery system for the purpose of increasing the stability and utility of physiological active compositions obtained from functional foods within the body. Controlled release of resveratrol is dependent on pH with slower release kinetics at higher pH values due to the protonated amino groups of chitosan resulting in a soluble, positively-charged polysaccharide that causes fast swelling in acidic medium (Peng et al., 2010).

The biopolymers such as chitosan and alginate were increased the stability of resveratrol via complexation with DLRNs. However, the DLRNs prepared with β-cyclodextrin resulted in the highest stability. Therefore, β-cyclodextrin was the best in maintaining the stability of the resveratrol nanoemulsions. In addition, β-cyclodextrin is available in solid and powder form, making it ideal in a nutraceutical delivery system.

Summary

Resveratrol nanoemulsions prepared with chitosan, alginate, and β-cyclodextrin were obtained with particle sizes of 10-800 nm, depending on the emulsification parameters such as the ratio of aqueous phase and surfactant. Resveratrol nanoemulsions maintained their particle size and zeta-potential value during storage for 28 days at 25°C. The double-layers resveratrol nanoemulsions prepared with alginate or chitosan showed slight increase in particle size from 45 to 166 nm during storage period. The double-layers resveratrol nanoemulsions prepared with β-cyclodextrin relatively stable with a particle size of less than 40 nm. Stability of resveratrol in the double-layers nanoemulsion prepared by complex coaervation with β-cyclodextrin was higher than single-layer nanoemulsion, maintained approximately 95% of resveratrol content until 6 days of storage. Consequently, the double-layers of resveratrol nanoemulsion prepared with β-cyclodextrin showed the best stability of the resveratrol having particle sizes below 100 nm. Further, β-cyclodextrin is available in solid and powder form, which makes its use ideal in a nutraceutical delivery system for increasing solubility, stability, and bioavailability of functional components.

Acknowledgements

References

Table 7. Changes in resveratrol contents of single- and double-layers resveratrol nanoemulsions during storage for 7 days

Sample	Storage (day)			
	0	5	7	F-value
SLRN[1]	100[a]	92.09±3.41[2)b4)]	88.48±4.10[b]	10.99[**3)]
CYN	100[a]	98.82±0.27[b]	94.58±0.56[c]	187.48[***]

[1] SLRN: single-layer resveratrol nanoemulsion based on water, CYN: double-layers reveratrol nanoemulsion prepared by complex coaervation with β-cyclodextrin
[2] Mean±S.D.
[3] **: $p<0.01$, ***: $p<0.001$
[4] a-c: Means with different superscripts in the same row differ significantly at $p<0.05$ by Duncan's multiple range test

Caddeo C, Teskac K, Sinico C, Kristl J. 2008. Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells. Int. J. Pharm. 336: 183-191.

Cho YJ, Kim CJ, Kim NS, Kim CT, Park B. 2008. Some cases in applications of nanotechnology to food and agricultural systems. Biochip. J. 2: 183-185.

Choi AJ, Kim CJ, Cho YJ, Hwang JK, Kim CT. 2008. Solubilization of capsaicin and its nanoemulsion formation in the sonication and self assembly methods. In: Proceeding of Nano Science & Technology Institute. June 1-5, Boston, USA, p. 61.

Choi AJ, Kim CJ, Cho YJ, Hwang JK, Kim CT. 2009. Effects

of surfactants on the formation and stability of capsaicin-loaded nanoemulsions. Food Sci. Biotechnol. 18: 1161-1172.

Das S, Ng KY. 2010. Colon-specific delivery of resveratrol: Optimization of multi-particulate calcium-pectinate carrier. Int. J. Pharm. 385: 20-28.

Frankel EN, Waterhouse AL, Kinsella JE. 1993. Inhibition of human DHL oxidation by resveratrol. Lancet. 341: 1103-1104.

Frémont L. 2000. Minireview: biological effects of resveratrol. Life Sci. 66: 663-673.

Garti N, Spernath A, Aserin A, Lutz R. 2005. Nano-sized self-assemblies of nonionic surfactant as solubilization reservoirs and microreactors for food system. Soft Matter. 1: 206-218.

Hung CF, Chen JK, Liao MH, Lo HM, Fang JY. 2006. Development and evaluation of emulsion-liposome blends for resveratrol delivery. J. Nanosci. Nanotechnol. 6: 2950-2958.

Hung CF, Fang CL, Liao MH, Fang JY. 2007. The effect of oil components on the physicochemical properties and drug delivery of emulsions: Tocol emulsion versus lipid emulsion. Int. J. Pharm. 335: 193-202.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275: 218-220.

Jeukendrup AE, Aldred S. 2004. Fat supplementation, health and endurance performance. Nutrition 20: 678-688.

Kim CT, Cho YJ, Kim N, Kim CJ. 2009. The use of nano-technology in functional food product development. Int. Rev. Food Sci. Technol. 1: 48-53.

Kim CT, Choi AJ, Kim CJ, Cho YJ. 2008. Preparation of food nanoemulsions containing bioactive ingredients and its application for use as nutraceutical delivery system. In: Proceeding of 6th International Nanotech Symposium & Exhibition. August 27-29, Ilsan, Korea, p.231.

Kim CT, Karin S. 2008. Nano- and microtechnology for emulsification. Food Sci. Technol. 22: 19-22.

Lawrence MJ, Ress GD. 2000. Microemulsion-based media as novel drug delivery systems. Adv. Drug Deliv. Rev. 45: 89-121.

Nielloud F, Marti-Mestres G. 2000. Suspension Formulation. In: Pharmaceutical emulsions and suspensions. Swarbrick J. (eds). AAI press, Inc., Wilmington, NC, USA, pp.156-157.

Peng H, Xiong H, Li J, Xie M, Liu Y, Bai C, Chen L. 2010. Vanillin cross-linked chitosan microspheres for controlled release resveratrol. Food Chem. 121: 23-28.

Pinto MC, García-Barrato JA, Macías P. 1999. Resveratrol is a potent inhibitor of the dioxigenase activity of lipoxygenase. J. Agric. Food Chem. 47: 4842-4846.

Seo SB, Toshio K. 2002. Preparation of water soluble chitosan blendmer and their application to removal of heavy metal ions from wastewater. Macromol. Res. 10: 103-107.

Sessa M, Balestrieri ML, Ferrari G, Servillo L, Castaldo D, D'Onofrio N, Donsi F, Tsao R. 2014. Bioavailability of encapsulated resveratrol into nanoemulsion-based delivery systems. Food Chem. 147: 42-50.

Shi G, Rao L, Yu H, Xiang H, Yang H, Ji R. 2008. Stabilization and encapsulation of photosensitive resveratrol within yeast cell. Int. J. Pharm. 349: 83-93.

Traul KA, Driedger A, Ingle DL, Nakhasi D. 2000. Review of the toxicology properties of medium-chain triglycerides. Food Chem. Toxicol. 38, 79-98.

Wang L, Mutch KJ, Eastoe J, Heenan RK, Dong J. 2008. Nanoemulsions prepared by a two-step low-energy process. Langmuir. 24: 6092-6099.

Zupancic P, Lavric Z, Kristl J. 2015. Stability and solubility of trans-resveratrol are strongly influenced by pH and temperature. Eur. J. Pharm. Biopharm. 93: 196-204.