**Resveratrol encapsulation in core-shell biopolymer nanoparticles:
Impact on antioxidant and anticancer activities**

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Abstract: Zein/pectin core-shell nanoparticles with average diameter of about 235 nm and polydispersity index (PDI) of 0.24 were developed to encapsulate, protect and deliver resveratrol. Biopolymer nanoparticles could be formed containing up to 10.2% (w/w) resveratrol, with a particle yield of 91.7% and a resveratrol loading efficiency of 77.9%. The nanoparticles formed were spherical and had good redispersibility in aqueous solutions. The encapsulated resveratrol had a higher *in vitro* antioxidant activity than free resveratrol as determined by DPPH $\cdot$, ABTS $\cdot^+$ scavenging ability, and iron (III) reducing power. The encapsulated resveratrol also exhibited higher antiproliferative activity when tested using human hepatocarcinoma Bel-7402 cells than free resveratrol, with IC₅₀ values of 17.6 μ g/mL (77.2 μ M) and 25.6 μ g/mL (112.0 μ M) respectively. The colloidal form of resveratrol developed in this study may be particularly useful for application in functional foods and beverages, as well as in dietary supplements and pharmaceutical products.

Keywords: resveratrol; zein; pectin; nanoparticles; antioxidant activity; anticancer activity.

1. Introduction

Resveratrol is a naturally occurring polyphenolic phytoalexin produced by a range of different plants, including grapes, blueberries, mulberries, cranberries, and peanuts in response to physical damage, UV irradiation, and fungal attack (Gambini, Ingles, Olaso, Lopez-Grueso, Bonet-Costa, Gimeno-Mallench, et al., 2015; Neves, Lucio, Lima, & Reis, 2012). Recently, considerable attention has been paid to the potential beneficial effects of resveratrol on human health, such as antioxidant activity (Neves, Lucio, Lima, & Reis, 2012), anti-inflammatory activity (Svajger & Jeras, 2012), hepatoprotection (Lee, Yen, Huang, Wu, Ko, Tzeng, et al., 2012), cardio- and neuro-protection (Lu, Xu, Sun, Zhu, Zheng, & Li, 2013; Nakata & Inoue, 2014), anti-carcinogenic activity (Guo, Peng, Li, Yao, Zhang, Chen, et al., 2015), anti-aging effects (Duntas, 2011), obesity and diabetes prevention (Hung, 2016; Hussein & Mahfouz, 2016). Despite the potential health benefits that may be gained from ingestion of this natural molecule, it is often difficult to incorporate resveratrol into commercial pharmaceutical, supplement, and functional food products because of its poor water solubility, chemical instability, and low bioavailability (Summerlin, Soo, Thakur, Qu, Jambhrunkar, & Popat, 2015).

A variety of nanoparticle-based systems have been investigated for their potential to encapsulate, protect, and deliver resveratrol. Resveratrol, which is primarily a hydrophobic molecule ($\text{LogP} = 3.1$), has been incorporated into lipid nanoparticles, such as liposomes (Isailović, Kostić, Zvonar, Đorđević, Gašperlin, Nedović, et al., 2013), solid lipid nanoparticles (Pandita, Kumar, Poonia, & Lather, 2014), nanoemulsions (Davidov-Pardo & McClements, 2015). Resveratrol has also been incorporated into various types of biopolymer nanoparticles fabricated from proteins that have some non-polar domains, such as gelatin (Karthikeyan, Hoti, & Prasad, 2015), bovine serum albumin (Guo, et al., 2015), silk protein (Lozano-Perez, Rodriguez-Nogales, Ortiz-Cullera, Algieri, Garrido-Mesa, Zorrilla, et al., 2014), gliadin (Joye, Davidov-Pardo, Ludescher, & McClements, 2015), and zein (Davidov-Pardo, Perez-Ciordia, Marin-Arroyo, & McClements, 2015; Penalva,

Esparza, Larraneta, Gonzalez-Navarro, Gamazo, & Irache, 2015). Zein is a attractive protein source because it is widely available and inexpensive byproduct of corn production.

Zein is a hydrophobic protein that can be solubilized in concentrated aqueous ethanol (60–90%) solutions (Dong, Padua, & Wang, 2013). This property makes zein suitable material for development of nanoparticle delivery systems to encapsulate resveratrol, because resveratrol is also readily soluble in ethanol solutions (Davidov-Pardo, Joye, & McClements, 2015; Patel & Velikov, 2014; Penalva, Larraneta, Gonzalez-Navarro, Gamazo, & Irache, 2015). However, zein nanoparticles have poor aggregation stability when exposed to certain environmental conditions, such as pH values around the isoelectric point, high salt levels, and elevated temperatures. This problem can be partially overcome by coating zein nanoparticles with emulsifiers, such as lecithin, small molecule surfactants, sodium caseinate, or sodium caseinate-dextran conjugates (Chen & Zhong, 2014; Chuacharoen & Sabliov, 2016b; Davidov-Pardo, Joye, Espinal-Ruiz, & McClements, 2015; Davidov-Pardo, Joye, & McClements, 2015; Patel, Hu, Tiwari, & Velikov, 2010). Zein nanoparticle encapsulation protected resveratrol from isomerization by UV light (Davidov-Pardo, Joye, Espinal-Ruiz, & McClements, 2015), and increased its oral bioavailability (Penalva, Esparza, Larraneta, Gonzalez-Navarro, Gamazo, & Irache, 2015). Zein nanoparticles have also been shown to be useful for encapsulating other types of hydrophobic nutraceuticals, including curcumin (Patel, Hu, Tiwari, & Velikov, 2010; Zou, Zheng, Zhang, Zhang, Liu, Liu, et al., 2016), retinol (Park, Park, & Kim, 2015), peppermint oil (Chen & Zhong, 2015), quercetin (Patel, Heussen, Hazekamp, Drost, Velikov, 2012) and carotenoids (Chuacharoen & Sabliov, 2016a, 2016b).

In previous studies, our group developed a nanoparticle-based delivery system consisting of core-shell nanoparticles, which had a hydrophobic zein core and a hydrophilic pectin shell (Hu, Huang, Gao, Huang, Xiao, & McClements, 2015; Huang, Huang, Gong, Xiao, McClements, & Hu, 2016). The presence of the pectin shell improved the physical stability of the nanoparticles by generating a strong

and steric repulsion. The presence of the hydrophobic zein core facilitated the encapsulation and protection of non-polar bioactive molecules. In the current study, used a similar approach to fabricate resveratrol-loaded core-shell nanoparticles, and then compared their *in vitro* antioxidant and anticancer activity with free resveratrol. The ultimate aim was to show that these nanoparticles could be used to encapsulate resveratrol in a form that could be successfully utilized in functional food, supplement, and pharmaceutical products.

2. Materials and methods

2.1. Materials

Zein (Lot SLBL9380V), pectin from citrus peel (galacturonic acid $\geq 74.0\%$, Lot SLBH9128V), L-ascorbic acid ($>99\%$, Lot STBF1107V), 2, 2'-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS, $>98\%$, Lot SLBL5589V), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Resveratrol ($>98\%$) was obtained from Adamas-Beta (Shanghai, China). 1, 1-Diphenyl-2-picryl-hydrazyl Free Radical (DPPH $\cdot$) was supplied by Tokyo Chemical Industry (Japan). All other chemicals and solvents, such as potassium persulfate, trichloroacetic acid (TCA), ethanol alcohol and dimethyl sulfoxide (DMSO) were of analytic grade.

2.2. Solution preparation

2.2.1. Resveratrol-zein solution

2.0 g zein was added to 100.0 mL 85% (v/v) aqueous ethanol solution with magnetic stirring at 500 rpm (IKA R05, UAS) for 1 h. Resveratrol was then added and the mixture was continuously stirred in the dark for another hour, then centrifuged at 1006 g for 10 min to remove any insoluble impurities.

2.2.2. Pectin solution

Pectin powder was dissolved in double distilled water and heated at 70°C with stirring for 30 min, cooled with tap water to ambient temperature and stirred for 1 h,

adjusted to pH 4.0 with 0.1 mol/L HCl, and then filtered to remove any insoluble particles.

2.3. Preparation of resveratrol loaded zein–pectin nanoparticles

Resveratrol-loaded zein nanoparticles were produced using antisolvent precipitation and electrostatic deposition as described in a previous study (Hu, Huang, Gao, Huang, Xiao, & McClements, 2015). 5.0 mL of resveratrol-zein ethanol was rapidly dropped into 20 mL water (adjusted to pH 4.0) using a syringe with constant stirring at 900 rpm. The resulting dispersion was stirred for another 3 min, ethanol was evaporated using a rotary evaporator (RE-2000, Shanghai Ya Rong biochemical instrument, China). Appropriate amounts of water (adjusted to pH 4.0) were added to compensate for any decrease in volume due to ethanol evaporation. The dispersion was poured into a 31.25 mL pectin solution (adjusted to pH 4.0) with continuous stirring at 700 rpm for 20 min.

2.4. Nanoparticle characterization

2.4.1. Particle size and ζ -potential measurements

The average diameter, polydispersity index (PDI), and electrical characteristics (ζ -potential) of freshly prepared colloidal dispersions were measured at 25°C using dynamic light scattering (DLS, Nano-ZS, Malvern Instruments, UK). Samples were diluted with pH 4.0 water before analysis to avoid multiple scattering effects.

2.4.2 . Field emission scanning electron microscopy

The morphology of resveratrol nanoparticles was characterized by field emission scanning electron microscopy (Nova NanoSEM 430; FEI, Netherlands). A thin layer of the freeze-dried sample was placed on an aluminum stub and sputtered with 3-6 nm of gold. Samples were then analyzed at 10 kV acceleration voltage under a high vacuum mode.

2.4.3 X-ray diffraction (XRD)

The X-ray diffraction patterns of powdered encapsulated resveratrol, resveratrol/zein/pectin physical mixture, resveratrol, zein, and pectin were determined

(X'Pert-Pro MRD, PANalytical, The Netherlands) with Cu K α radiation and current 40 mA. The 2 θ scan range was fixed at 10°-40°.

2.4.4. Resveratrol determination

The amount of resveratrol encapsulated in the core-shell nanoparticles was determined using a modified colorimetric method (Joye, Davidov-Pardo, & McClements, 2015). An aliquot of freeze-dried nanoparticles (10 mg) was dissolved in 10 mL DMSO, and stirred for 1h in the dark. Then the solution was centrifuged at 16099 g for 50 min. The supernatant was diluted with DMSO and analyzed by UV-visible spectroscopy (TU1901, Beijing Purkinje General Instrument Co., Ltd., China) at 327 nm. The resveratrol concentration was calculated using a calibration curve established using standard solutions in the range of 0–10 μ g/mL ($R^2 = 0.9995$).

2.4.5. Particle yield and resveratrol loading efficiency

Freshly prepared colloid dispersions were centrifuged at 1006 g for 10 min to remove large particles and free resveratrol aggregates, and then freeze dried. The particle yield and the resveratrol loading efficiency were determined using equation (1) and (2):

$$\text{Particle yield(\%)} = \frac{\text{weight of the freeze dried nanoparticles}}{\text{total weight of resveratrol, zein and pectin}} \times 100\% \quad (1)$$

$$\text{Loading efficiency(\%)} = \frac{\text{resveratrol in nanoparticles}}{\text{total resveratrol input}} \times 100\% \quad (2)$$

2.5 Antioxidant activity measurements

2.5.1 DPPH radical scavenging activity assay

The DPPH $\cdot$ scavenging capacity of the samples was determined using a method described previously (Brand-Williams, Cuvelier, & Berset, 1995; Joraholmen, Skalko-Basnet, Acharya, & Basnet, 2015) with some modification. Encapsulated resveratrol and ethanol-dissolved resveratrol were prepared at multiple concentrations. Briefly, 2 mL of a 100 μ M DPPH $\cdot$ ethanol solution was mixed with equivalent of encapsulated resveratrol and ethanol-dissolved resveratrol and subsequently

incubated at room temperature in the dark. After 30 min, the absorbance of the solution was measured at 517 nm. The anti-oxidative activity of L-ascorbic acid, and empty nanoparticles were also determined for comparison. The DPPH• scavenging percentage was calculated using the following equation:

$$\text{DPPH} \cdot \text{ scavenging percentage (\%)} = \left(\frac{A_c - A_s}{A_c} \right) \times 100\% \quad (3)$$

Where A_c is the absorbance of the control reaction, and A_s is the absorbance of the test sample. The concentrations of antioxidants (test sample) required for a 50% DPPH• radicals scavenging rate (SC_{50}) were calculated from standard curves established by plotting the antioxidant concentrations against DPPH• scavenging percentage.

2.5.2 . ABTS•⁺ scavenging activity assay

The ABTS•⁺ scavenging capacity of the samples was determined using a method described previously (Joraholmen, Skalko-Basnet, Acharya, & Basnet, 2015; Rice-Evans, 1999). An ABTS stock solution was prepared by dissolving 7 mM ABTS in water. An ABTS radical cation solution was then prepared by mixing the ABTS stock solution and a 4.9 mM potassium persulfate solution in equal quantities and allowing them to stand at room temperature in the dark for 12-16 h. The ABTS•⁺ solution was diluted with PBS (10 mM, pH 7.4) to an absorbance of 0.70 (± 0.02) cm^{-1} at 734 nm and stabilized at 30 °C. Resveratrol was dissolved in absolute ethanol, and encapsulated resveratrol was dispersed in deionized water. Test samples (140 μL) at different concentrations (10-50 μg /mL) were reacted with 4 mL of the ABTS•⁺ solution at 30 °C in the dark. After 6 min of reaction, the absorbance was taken at 734 nm using a UV-visible spectrophotometer. Phosphate buffer solution and absolute ethanol were used as blanks respectively. ABTS•⁺ scavenging percentage was calculated from the following equation:

$$\text{ABTS} \cdot^+ \text{ scavenging percentage (\%)} = \left(\frac{A_c - A_s}{A_c} \right) \times 100\% \quad (4)$$

Where A_c is the absorbance of the control reaction, and A_s is the absorbance of the test sample. The SC_{50} values were calculated from standard curves.

2.5.3 Ferric cyanide (Fe^{3+}) reducing antioxidant power assay

The reducing power of resveratrol nanoparticles was determined by a method reported previously (Gulcin, 2006) with some modification. Briefly, different concentrations of resveratrol nanoparticles in distilled water or pure resveratrol dissolved in ethanol (0-200 $\mu\text{g}/\text{mL}$) were added to a mixture of sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%, w/v). The solution was incubated at 50°C for 20 min, cooled to room temperature, and then acidified with trichloroacetic acid (2.5 mL, 10%, w/v). 2.5 mL of the resulting was mixed with distilled water (2.5 mL), reacted with FeCl_3 solution (0.5 mL, 0.1%, w/v) for 10 min, and then measured at 700 nm in a spectrophotometer. All samples carefully protected from light throughout the experimental procedure.

2.6 Cell culture and cytotoxicity assay

Human hepatocarcinoma Bel-7402 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 medium (Hyclone), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified 5% carbon dioxide incubator. Cells at 80–90% confluency were used for treatments.

Cell viability was measured using the MTT assay method (Sahu, Kasoju, & Bora, 2008). Briefly, cells (5×10^3) were seeded in 96-well microtiter plates. After 24 h the cells were treated with fresh medium containing different concentrations of dissolved in DMSO (the final concentration of DMSO in the solutions was < 0.1%), encapsulated resveratrol, and empty nanoparticles. 24 h later, MTT (5 mg/mL in PBS) was added to each well and incubated for 4 h. After incubation, the medium was removed and 150 μL of DMSO was added to each well and incubated for 15 min to solubilize the formazan crystals. The absorbance at 490 nm was measured with a microplate reader (BioTek Instruments, USA). Measurements were also carried out in the absence of treatments (control groups) in the same manner. Each concentration of sample was carried out in 5 different wells and the experiment was repeated three times. The cell viability was calculated according to the equation (5):

$$\text{Cell viability (\%)} = (A_t/A_c) \times 100\% \quad (5)$$

Where A_t and A_c are the absorbance of the treated and untreated cells, respectively. The inhibitory concentration for killing 50% of cells (IC_{50}) was determined from the resulting curves.

2.7 Statistical analysis

All experiments were performed in triplicate at least. The data were recorded as mean $\pm$ standard deviation and analyzed by statistical software (SPSS, version 13.0 for Windows, SPSS Inc.). Mean values were compared by one-way ANOVA with the Fisher's least significant difference (LSD) test or Dunnett's T3 test. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1 Effect of pectin concentration on colloidal stability

Zein contains a relatively high proportion of non-polar and cationic amino acid groups, which means that it is a strongly hydrophobic protein with a relatively high isoelectric point ($pI \approx 6.2$) (Shukla & Cheryan, 2001). These physicochemical characteristics were utilized in the fabrication of the core-shell biopolymer nanoparticles developed in the current study. The mixture of resveratrol and zein initially dissolved in an acidified aqueous ethanol solution (pH 4.0) that was injected into an acidified aqueous solution (pH 4.0) so as to generate a strong electrostatic repulsion between the zein nanoparticles when they were formed by antisolvent precipitation. This strong repulsion is associated with the high positive charge on zein molecules at pH values well below their isoelectric point. In the absence of a strong electrostatic repulsion the bare zein nanoparticles would aggregate due to hydrophobic attraction. After antisolvent precipitation, the colloid dispersion had a transparent appearance, which was attributed to the fact that the mean particle diameter (78 nm) was well below the wavelength of visible light (Fig. 1).

In the presence of pectin, the dimensions and stability of the biopolymer nanoparticles were highly dependent on pectin concentration (Fig. 1). The zein

nanoparticles strongly aggregated and precipitated at pectin concentrations of 0.005% and 0.01% (w/v), leading to the formation of white sediment at the bottom of the test tubes. At a pectin concentration of 0.033% the aggregation of the particles steeply ($d = 319$ nm), and at higher levels the particles formed had a relatively low constant diameter ($d = 217$ nm). The fact that the particles in the presence of pectin were larger than those in its absence may have been due to two reasons: (i) particle aggregation; (ii) shell formation. At intermediate pectin concentrations, it is likely that particle aggregation is more important, but at high pectin concentrations it is that shell formation is more important. An estimation of the thickness of the adsorbed pectin layer (δ) can be obtained from the difference in mean particle diameter between the coated and non-coated particles: $\delta = (d_c - d_u)/2$. This calculation suggested that pectin layer was about 70 nm thick, which is on the order of the diameter of pectin molecules in solution (Fishman, Chau, Qi, Hotchkiss, Garcia, & Cooke, 2015).

The ζ -potential of the biopolymer nanoparticles measured at pH 4 decreased around +41 mV to -9 mV when the pectin concentration was increased from 0% to 0.011% (Fig. 2), and reached a fairly constant level (-33 mV) when the pectin concentration exceeded 0.055%. This latter value corresponded to the minimum pectin concentration required to stabilize the zein nanoparticles against aggregation (Fig. 1). Interestingly, the electrical potential on the pectin-coated particles (-33 mV) was fairly similar to pectin molecules in solution (-34 mV), which can be attributed to the fact that the electrical characteristics of particles are dominated by their outer Pectin is an anionic polysaccharide that has carboxyl groups with a dissociation constant (pK_a) around pH 3.5, and would therefore be expected to be strongly negatively charged at pH 4.0 (Jones, Lesme, Dubin, & McClements, 2010). The adsorption of the anionic pectin molecules to the surfaces of the cationic zein nanoparticles would therefore account for the observed change in the particle from positive to negative.

The extensive particle aggregation observed at intermediate pectin (0.005% to 0.01%) can be attributed to two main reasons: (ii) the low net electrical potential on the particles only generates a weak electrostatic repulsion between them;

(ii) a single pectin molecule adsorbs to positive patches on the surfaces of two or zein nanoparticles, thereby inducing bridging flocculation (Hu & McClements, 2015). At high pectin concentrations, the particle surfaces are completely covered with pectin molecules, which leads to a high net electrical potential (-33.0 mV) and therefore strong electrostatic repulsion, as well as the lack of available exposed cationic sites for bridging flocculation to occur. In summary, the core-shell particles are stabilized against aggregation by a combination of electrostatic and steric repulsion due to their polysaccharide coating.

3.2 Particle characteristics and resveratrol encapsulation properties

For commercial applications it is important to fabricate delivery systems that have a high particle yield, high loading capacity, and high encapsulation efficiency. For this reason, a series of core-shell nanoparticles were prepared with different initial zein-to-resveratrol weight ratios (10:0.8, 10:1.0, 10:1.2, and 10:1.5), corresponding to zein-pectin nanoparticles theoretically containing 7.08%, 8.38%, 10.26%, and 12.06% resveratrol, respectively. The influence of initial resveratrol concentration on the nanoparticle characteristics, yield, and loading efficiency was then measured (**Table 1**). The theoretical resveratrol level used (7.08 to 12.06%) caused no significant difference in the mean particle diameter ($d \approx 230$ nm), ζ -potential ($\zeta \approx -31$ mV), or polydispersity ($PDI \approx 0.24$) of the nanoparticles ($p > 0.05$). However, both the particle yield and loading efficiency significantly decreased with increasing initial resveratrol concentration (**Table 1**).

After preparation, the initial suspension of biopolymer nanoparticles was dehydrated by freeze-drying to form a powder. SEM was used to determine the morphology of the freeze-dried biopolymer nanoparticles, which indicated that most of them were spheres with diameters around 200 nm and smooth surfaces (Fig. 3). The freeze-dried nanoparticles readily dispersed into distilled water by gentle stirring, and dynamic light scattering analysis indicated that they formed small particles ($d \approx 257$ nm) with a narrow distribution ($PDI \approx 0.12$). For many commercial applications it may be advantageous to convert the resveratrol delivery systems into a powdered

form to reduce transport and storage costs, and to facilitate their handling and utilization.

3.3 X-ray diffraction analysis

The XRD patterns of resveratrol, resveratrol in nanoparticles, and resveratrol in physical mixtures of zein and pectin were measured (Fig. 4a). In addition, the XRD patterns of pure resveratrol, pure zein, and pure pectin were also measured for comparison purposes (Fig. 4b). Diffraction peaks were observed for pure resveratrol powdered at angles of 16.31, 19.14, 22.39, 23.56, 25.16, 28.26 on a 2θ scale (Fig. 4a), which confirmed the crystalline nature of resveratrol under these conditions (Sun, Zhao, Ni, & Xia, 2014). These characteristic peaks were also observed in the diffraction pattern of the physical mixture of resveratrol, zein, and pectin, although their intensities were weaker because of the relatively low resveratrol content in the mixture. Conversely, the diffraction pattern of resveratrol encapsulated in the biopolymer nanoparticles did not show any characteristic intense sharp peaks, which indicated that it was in an amorphous form.

3.4 Antioxidant activities of encapsulated resveratrol

Resveratrol is an excellent scavenger of hydroxyl, superoxide, and other radicals (Neves, Lucio, Lima, & Reis, 2012), and is known to have a high solubility in ethanol (50 g/L) (Summerlin, Soo, Thakur, Qu, Jambhrunkar, & Popat, 2015). For these reasons, we compared the DPPH $\cdot$ and ABTS $\cdot^+$ scavenging capacity, and the Fe $^{3+}$ reducing power, of encapsulated resveratrol (in biopolymer nanoparticles) and free resveratrol (in ethanol solution).

The DPPH $\cdot$ scavenging capacity increased linearly with increasing ascorbic acid concentration from 0 to 10 $\mu\text{g/mL}$ (data not shown), and reached to about 95% when the ascorbic acid concentration equaled or exceeded 10 $\mu\text{g/mL}$ (Fig. 5a). The DPPH $\cdot$ scavenging activity of both encapsulated and free resveratrol increased with increasing resveratrol concentration from 10 to 50 $\mu\text{g/mL}$, but the encapsulated resveratrol had a significantly higher activity ($p < 0.05$). The SC_{50} values of encapsulated and free resveratrol were 10.5 $\mu\text{g/mL}$ and 35.5 $\mu\text{g/mL}$, respectively (Fig.

5b), indicating that the encapsulated resveratrol was about three times more efficient at scavenging DPPH $\cdot$ than the free resveratrol. The ABTS $\cdot^+$ scavenging activity of encapsulated resveratrol was significantly higher than that of both free resveratrol and ascorbic acid (Fig. 6a). Indeed, the SC₅₀ values of ascorbic acid, encapsulated resveratrol, and free resveratrol were around 61, 19, and 28 μ g/mL, respectively (Fig. 6b). The resveratrol-free biopolymer particles had little antioxidant activity in both assays used.

The reducing power of the different systems was determined using a modified iron (III) to iron (II) reduction assay method. The presence of reductants in the solution causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. Therefore, the Fe²⁺ can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm (Zou, Lu, & Wei, 2004). The reducing power of encapsulated and free resveratrol was compared with that of ascorbic acid, which is known to be a strong reducing agent (Fig. 7). In addition, the reducing power of the empty nanoparticles was also tested as a negative control. The empty nanoparticles had little reducing power, whereas the resveratrol-loaded nanoparticles had a reducing power that was just lower than that of ascorbic acid. In addition, the reducing power of resveratrol encapsulated in nanoparticles was appreciably higher than that of free resveratrol at the same resveratrol concentration.

3.5 Hepatocarcinoma cancer cell cytotoxicity

Hepatocarcinoma is the sixth most common cancer in the world, and the third leading global cause of cancer-related deaths. Hepatocarcinoma is generally insensitive to anticancer drugs, and roughly 67% hepatoma patients treated with chemotherapeutic agents develop rapidly acquired resistance (Shi, Ma, Lu, Xu, Zhu, Wang, et al., 2008). Previous studies have reported that resveratrol may have anti-proliferative activity against hepatocarcinoma cells (Bu, Gan, Guo, Chen, Song, Qi, et al., 2013). Consequently, we used human hepatocarcinoma Bel-7402 cells to evaluate the anti-proliferative activity of encapsulated resveratrol (in biopolymer nanoparticles) and free resveratrol (solubilized in DMSO). The results from the MTT

assay suggested a strong cytotoxic effect of both encapsulated and free resveratrol against the model cancer cells (Fig. 8a). There was dose-dependent cytotoxic effect of resveratrol on the cancer cells after 24 h treatment, with the cell viability being around 24.7% and 19.3% for free and encapsulated resveratrol at a concentration of 197.2 μ M, respectively. As anticipated, the empty biopolymer nanoparticles had no cancer cell cytotoxicity. The encapsulated resveratrol had a significantly higher cytotoxicity than the free resveratrol ($p < 0.05$), with IC_{50} values of 17.61 μ g/mL (77.2 μ M) for encapsulated resveratrol and 25.57 μ g/mL (112.0 μ M) for free resveratrol (Fig 8b). These results suggest that encapsulated resveratrol may inhibit the proliferation of hepatocarcinoma cells.

4. Conclusion

Resveratrol is poorly soluble in water and only slightly soluble in oil, and so colloidal delivery systems that utilize oil as a carrier phase, such as nanoemulsions or solid lipid nanoparticles, are limited by a low loading efficiency. We successfully developed core-shell biopolymer nanoparticles that consisted of a hydrophobic zein core and a hydrophilic pectin shell. These nanoparticles could be produced at a high yield (91.7%) using an antisolvent precipitation/electrostatic deposition method, and they had a relatively high resveratrol content of 10.2% (w/w). The biopolymer nanoparticles formed were relatively small ($d < 260$ nm) and uniform ($PDI < 0.25$) spheres that had good re-dispersibility in water after they had been dehydrated. The encapsulated resveratrol had stronger antioxidant and anticancer activity than free resveratrol. In future work, we intend to evaluate the bioavailability and *in vivo* bioactivities of encapsulated resveratrol. The resveratrol-loaded biopolymer nanoparticles developed in this work may be effective delivery systems for application in functional foods and beverages, as well as in dietary supplements and pharmaceutical products.

Acknowledgements

This work was funded by International Cooperation Research Program of

Guangdong Provincial Department of Science and Technology, China (No. 2015A050502051). This material was partly based upon work supported by the Cooperative State Research, Extension, Education Service, USDA, Massachusetts Agricultural Experiment Station (MAS00491) and USDA, NRI Grants (2013-03795 and 2014-67021).

References

- Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. u.-Technol.*, 28(1), 25-30.
- Bu, L., Gan, L. C., Guo, X. Q., Chen, F. Z., Song, Q., Qi, Z., Gou, X. J., Hou, S. X., & Yao, Q. (2013). Trans-resveratrol loaded chitosan nanoparticles modified with biotin and avidin to target hepatic carcinoma. *Int J Pharm*, 452(1-2), 355-362.
- Chen, H. Q., & Zhong, Q. X. (2014). Processes improving the dispersibility of spray-dried zein nanoparticles using sodium caseinate. *Food Hydrocolloids*, 35, 358-366.
- Chen, H. Q., & Zhong, Q. X. (2015). A novel method of preparing stable zein nanoparticle dispersions for encapsulation of peppermint oil. *Food Hydrocolloids*, 43, 593-602.
- Chuacharoen, T., & Sabliov, C. M. (2016a). The potential of zein nanoparticles to protect entrapped beta-carotene in the presence of milk under simulated gastrointestinal (GI) conditions. *Lwt-Food Science and Technology*, 72, 302-309.
- Chuacharoen, T., & Sabliov, C. M. (2016b). Stability and controlled release of lutein loaded in zein nanoparticles with and without lecithin and pluronic F127 surfactants. *Colloids and Surfaces a-Physicochemical and Engineering Aspects*, 503, 11-18.
- Davidov-Pardo, G., Joye, I. J., Espinal-Ruiz, M., & McClements, D. J. (2015). Effect of Maillard Conjugates on the Physical Stability of Zein Nanoparticles Prepared by Liquid Antisolvent Coprecipitation. *J Agric Food Chem*, 63(38), 8510-8518.
- Davidov-Pardo, G., Joye, I. J., & McClements, D. J. (2015). Encapsulation of resveratrol in biopolymer particles produced using liquid antisolvent precipitation. Part 1: Preparation and characterization. *Food Hydrocolloids*, 45, 309-316.
- Davidov-Pardo, G., & McClements, D. J. (2015). Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification. *Food Chem*, 167, 205-212.
- Davidov-Pardo, G., Perez-Ciordia, S., Marin-Arroyo, M. R., & McClements, D. J. (2015). Improving resveratrol bioaccessibility using biopolymer nanoparticles and complexes: impact of protein-carbohydrate maillard conjugation. *J Agric Food Chem*, 63(15), 3915-3923.
- Dong, F., Padua, G. W., & Wang, Y. (2013). Controlled formation of hydrophobic surfaces by self-assembly of an amphiphilic natural protein from aqueous solutions. *Soft Matter*, 9(25), 5933.
- Duntas, L. H. (2011). Resveratrol and its impact on aging and thyroid function. *J Endocrinol Invest*, 34(10), 788-792.

472 Fishman, M. L., Chau, H. K., Qi, P. X., Hotchkiss, A. T., Garcia, R. A., & Cooke, P. H. (2015).
 473 Characterization of the global structure of low methoxyl pectin in solution. *Food*
 474 *Hydrocolloids*, 46, 153-159.

475 Gambini, J., Ingles, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L.,
 476 Mas-Bargues, C., Abdelaziz, K. M., Gomez-Cabrera, M. C., Vina, J., & Borrás, C. (2015).
 477 Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and
 478 Biological Effects in Animal Models and Humans. *Oxid Med Cell Longev*, 2015, 837042.

479 Gulcin, I. (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology*, 217(2-3),
 480 213-220.

481 Guo, L., Peng, Y., Li, Y., Yao, J., Zhang, G., Chen, J., Wang, J., & Sui, L. (2015). Cell death pathway
 482 induced by resveratrol-bovine serum albumin nanoparticles in a human ovarian cell line.
 483 *Oncol Lett*, 9(3), 1359-1363.

484 Hu, K., Huang, X., Gao, Y., Huang, X., Xiao, H., & McClements, D. J. (2015). Core-shell biopolymer
 485 nanoparticle delivery systems: synthesis and characterization of curcumin fortified
 486 zein-pectin nanoparticles. *Food Chem*, 182, 275-281.

487 Hu, K., & McClements, D. J. (2015). Fabrication of biopolymer nanoparticles by antisolvent
 488 precipitation and electrostatic deposition: Zein-alginate core/shell nanoparticles. *Food*
 489 *Hydrocolloids*, 44, 101-108.

490 Huang, X., Huang, X., Gong, Y., Xiao, H., McClements, D. J., & Hu, K. (2016). Enhancement of curcumin
 491 water dispersibility and antioxidant activity using core-shell protein-polysaccharide
 492 nanoparticles. *Food Research International*, 87, 1-9.

493 Hung, C.-C. C. K.-Y. L. K.-Y. P. Y.-J. D. L.-M. (2016). Resveratrol exerts anti-obesity effects in high-fat diet
 494 obese mice and displays differential dosage effects on cytotoxicity, differentiation, and
 495 lipolysis in 3T3-L1 cells. *Endocrine Journal*, 63(2), 169-178.

496 Hussein, M. M. A., & Mahfouz, M. K. (2016). Effect of resveratrol and rosuvastatin on experimental
 497 diabetic nephropathy in rats. *Biomedicine & Pharmacotherapy*, 82, 685-692.

498 Isailović, B. D., Kostić, I. T., Zvonar, A., Đorđević, V. B., Gašperlin, M., Nedović, V. A., & Bugarski, B. M.
 499 (2013). Resveratrol loaded liposomes produced by different techniques. *Innovative Food*
 500 *Science & Emerging Technologies*, 19, 181-189.

501 Jones, O. G., Lesme, U., Dubin, P., & McClements, D. J. (2010). Effect of polysaccharide charge on
 502 formation and properties of biopolymer nanoparticles created by heat treatment of
 503 β -lactoglobulin-pectin complexes. *Food Hydrocolloids*, 24, 374-383.

504 Joraholmen, M. W., Skalko-Basnet, N., Acharya, G., & Basnet, P. (2015). Resveratrol-loaded liposomes
 505 for topical treatment of the vaginal inflammation and infections. *Eur J Pharm Sci*, 79,
 506 112-121.

507 Joye, I. J., Davidov-Pardo, G., Ludescher, R. D., & McClements, D. J. (2015). Fluorescence quenching
 508 study of resveratrol binding to zein and gliadin: Towards a more rational approach to
 509 resveratrol encapsulation using water-insoluble proteins. *Food Chem*, 185, 261-267.

510 Joye, I. J., Davidov-Pardo, G., & McClements, D. J. (2015). Encapsulation of resveratrol in biopolymer
 511 particles produced using liquid antisolvent precipitation. Part 2: Stability and functionality.
 512 *Food Hydrocolloids*, 49, 127-134.

513 Karthikeyan, S., Hoti, S. L., & Prasad, N. R. (2015). Resveratrol loaded gelatin nanoparticles
 514 synergistically inhibits cell cycle progression and constitutive NF-kappaB activation, and
 515 induces apoptosis in non-small cell lung cancer cells. *Biomed Pharmacother*, 70, 274-282.

516 Lee, C. W., Yen, F. L., Huang, H. W., Wu, T. H., Ko, H. H., Tzeng, W. S., & Lin, C. C. (2012). Resveratrol
517 nanoparticle system improves dissolution properties and enhances the hepatoprotective
518 effect of resveratrol through antioxidant and anti-inflammatory pathways. *J Agric Food Chem*,
519 60(18), 4662-4671.

520 Lozano-Perez, A. A., Rodriguez-Nogales, A., Ortiz-Cullera, V., Algieri, F., Garrido-Mesa, J., Zorrilla, P.,
521 Rodriguez-Cabezas, M. E., Garrido-Mesa, N., Utrilla, M. P., De Matteis, L., de la Fuente, J. M.,
522 Cenis, J. L., & Galvez, J. (2014). Silk fibroin nanoparticles constitute a vector for controlled
523 release of resveratrol in an experimental model of inflammatory bowel disease in rats. *Int J*
524 *Nanomedicine*, 9, 4507-4520.

525 Lu, X., Xu, H., Sun, B., Zhu, Z., Zheng, D., & Li, X. (2013). Enhanced neuroprotective effects of
526 resveratrol delivered by nanoparticles on hydrogen peroxide-induced oxidative stress in rat
527 cortical cell culture. *Mol Pharm*, 10(5), 2045-2053.

528 Nakata, R., & Inoue, H. (2014). Resveratrol and Cardiovascular Disease. *Current Nutrition Reports*, 3(3),
529 163-169.

530 Neves, A. R., Lucio, M., Lima, J. L. C., & Reis, S. (2012). Resveratrol in Medicinal Chemistry: A Critical
531 Review of its Pharmacokinetics, Drug-Delivery, and Membrane Interactions. *Current*
532 *Medicinal Chemistry*, 19(11), 1663-1681.

533 Pandita, D., Kumar, S., Poonia, N., & Lather, V. (2014). Solid lipid nanoparticles enhance oral
534 bioavailability of resveratrol, a natural polyphenol. *Food Research International*, 62,
535 1165-1174.

536 Park, C. E., Park, D. J., & Kim, B. K. (2015). Effects of a chitosan coating on properties of
537 retinol-encapsulated zein nanoparticles. *Food Science and Biotechnology*, 24(5), 1725-1733.

538 Patel, A., Hu, Y., Tiwari, J. K., & Velikov, K. P. (2010). Synthesis and characterisation of zein-curcumin
539 colloidal particles. *Soft Matter*, 6(24), 6192.

540 Patel, A. R., Heussen, P. C. M., Hazekamp, J., Drost, E., & Velikov, K. P. (2012). Quercetin loaded
541 biopolymeric colloidal particles prepared by simultaneous precipitation of quercetin with
542 hydrophobic protein in aqueous medium. *Food Chem*, 133(2), 423-429.

543 Patel, A. R., & Velikov, K. P. (2014). Zein as a source of functional colloidal nano- and microstructures.
544 *Current Opinion in Colloid & Interface Science*, 19(5), 450-458.

545 Penalva, R., Esparza, I., Larraneta, E., Gonzalez-Navarro, C. J., Gamazo, C., & Irache, J. M. (2015).
546 Zein-Based Nanoparticles Improve the Oral Bioavailability of Resveratrol and Its
547 Anti-inflammatory Effects in a Mouse Model of Endotoxic Shock. *J Agric Food Chem*, 63(23),
548 5603-5611.

549 Rice-Evans, R. R. N. P. A. P. A. P. M. Y. C. (1999). Antioxidant activity applying an improved ABTS radical
550 cation decolorization assay. *Free Radical Biology & Medicine*, 26(9-10), 1231-1237.

551 Sahu, A., Kasoju, N., & Bora, U. (2008). Fluorescence study of the curcumin-casein micelle
552 complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules*,
553 9(10), 2905-2912.

554 Shi, L. X., Ma, R., Lu, R., Xu, Q., Zhu, Z. F., Wang, L., Zhou, C. L., Li, X. L., Zhang, H. L., & Yao, Z. (2008).
555 Reversal effect of tyroservatide (YSV) tripeptide on multi-drug resistance in resistant human
556 hepatocellular carcinoma cell line BEL-7402/5-FU. *Cancer Lett*, 269(1), 101-110.

557 Shukla, R., & Cheryan, M. (2001). Zein the industrial protein from corn. *Industrial Crops and Products*,
558 13 171-192.

559 Summerlin, N., Soo, E., Thakur, S., Qu, Z., Jambhrunkar, S., & Popat, A. (2015). Resveratrol

560 nanoformulations: challenges and opportunities. *Int J Pharm*, 479(2), 282-290.

561 Sun, R., Zhao, G., Ni, S., & Xia, Q. (2014). Lipid based nanocarriers with different lipid compositions for
 562 topical delivery of resveratrol: comparative analysis of characteristics and performance.
 563 *Journal of Drug Delivery Science and Technology*, 24(6), 591-600.

564 Svajger, U., & Jeras, M. (2012). Anti-inflammatory effects of resveratrol and its potential use in therapy
 565 of immune-mediated diseases. *Int Rev Immunol*, 31(3), 202-222.

566 Zou, L. Q., Zheng, B. J., Zhang, R. J., Zhang, Z. P., Liu, W., Liu, C. M., Xiao, H., & McClements, D. J. (2016).
 567 Enhancing the bioaccessibility of hydrophobic bioactive agents using mixed colloidal
 568 dispersions: Curcumin-loaded zein nanoparticles plus digestible lipid nanoparticles. *Food*
 569 *Research International*, 81, 74-82.

570 Zou, Y., Lu, Y., & Wei, D. (2004). Antioxidant Activity of a Flavonoid-Rich Extract of *Hypericum*
 571 *perforatum* L. in Vitro. 52, 5032-5039.

572

CAPTIONS

Figure 1. Effect of pectin concentration on the mean particle diameter and colloidal stability of the biopolymer nanoparticles (zein: resveratrol = 10:1 w/w, pH 4.0). The photograph was taken after 24 hours storage at room temperature.

Figure 2. Effect of pectin concentration on the electrical potential of biopolymer nanoparticles (zein: resveratrol = 10:1 w/w) dispersed in aqueous solutions (pH 4.0).

Figure 3. Particle size distribution of biopolymer nanoparticles loaded with resveratrol. The pectin concentration in the nanoparticles was 0.11%, while the zein-to-resveratrol ratio was 10:1.5. The inset shows the microstructure of freeze dried nanoparticles determined by SEM.

Figure 4a. XRD spectra of crystalline resveratrol, encapsulated resveratrol (in nanoparticles), and crystalline resveratrol simply mixed with zein/pectin (physical mixture). The resveratrol content in the nanoparticles and physical mixture was 6.25%, w/w.

Figure 4b. XRD spectra of crystalline resveratrol, zein powder, and pectin powder.

Figure 5a. DPPH $\cdot$ radical scavenging activity of ascorbic acid, free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles) and empty nanoparticles. The letters above the data show significant differences between similar sample types with different concentrations (n=4, p<0.05).

Figure 5b. SC₅₀ values of free resveratrol (in ethanol) and encapsulated resveratrol (in nanoparticles) compared to ascorbic acid (n=3, p<0.05).

Figure 6a. ABTS $\cdot^+$ scavenging activity of ascorbic acid, free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles), and empty nanoparticles (no resveratrol). The letters above the data show significant differences between similar sample types with different concentrations (n=4, p<0.05).

Figure 6b. SC₅₀ values of free resveratrol (in ethanol), encapsulated resveratrol (in biopolymer nanoparticles) and ascorbic acid. The letters above the bars indicate significant differences between samples (n=3, p<0.05).

Figure 7. The reducing power capacity of ascorbic acid (control), free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles), and empty nanoparticles determined by spectrophotometry at 700 nm (n=3).

Figure 8a. Cell viability of hepatocarcinoma Bel-7402 cells treated with different concentrations of free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles) and empty nanoparticles (without resveratrol), incubated at 37°C for 24 h (n=3).

Figure 8b. IC₅₀ values of resveratrol in DMSO and resveratrol in particles (n=3, p<0.05). Cells were treated with different concentrations of free resveratrol and resveratrol nanoparticles incubated at 37 °C for 24 h.

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□ Ascorbic acid ■ Resveratrol in ethanol ■ Resveratrol in nanoparticles ■ Empty nanoparticles

Figure 5a. DPPH· radical scavenging activity of ascorbic acid, free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles) and empty nanoparticles. The letters above the data show significant differences between similar sample types with different concentrations (n=4, p<0.05).

Figure 5b. SC_{50} values of free resveratrol (in ethanol) and encapsulated resveratrol (in nanoparticles) compared to ascorbic acid ($n=3$, $p<0.05$).

Figure 6a. ABTS•⁺ scavenging activity of ascorbic acid, free resveratrol (in ethanol), encapsulated resveratrol (in nanoparticles), and empty nanoparticles (no resveratrol). The letters above the data show significant differences between similar sample types with different concentrations (n=4, p<0.05).

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Table 1. The particle yield, resveratrol loading efficiency, size and charge of resveratrol-loaded zein-pectin nanoparticles (n=3, p<0.05), the pectin concentration was 0.11% in colloid dispersions before freeze drying. The theoretical resveratrol content is the amount added originally.

Theoretical resveratrol content (%)	Determined resveratrol content (%)	Particle yield (%)	Resveratrol loading efficiency (%)	Particle diameter (nm)	ζ-potential(mV)	PDI
7.08	6.2±0.2	96.1±0.5 ^a	84.8±2.5 ^a	224.6±9.1 ^a	-31.17±1.09 ^a	0.231±0.010 ^a
8.38	7.5±0.3	97.6±0.7 ^a	86.4±3.5 ^a	229.8±9.2 ^a	-31.37±2.73 ^a	0.233±0.011 ^a
10.26	8.4±0.1	93.2±0.6 ^b	76.2±0.8 ^b	237.6±4.4 ^a	-30±2.78 ^a	0.243±0.021 ^a
12.06	10.2±0.2	91.7±1.8 ^b	77.9±1.8 ^b	235.4±4.3 ^a	-30.07±2.04 ^a	0.243±0.019 ^a