Zein/phenolic antioxidant nanoparticles stabilized Pickering emulsions: Effect of antioxidant hydrophobicity on lipid oxidation

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Abstract. Due to the presence of oils and fats, emulsions undergo lipid oxidation during storage, resulting in deterioration in sensory and nutritional qualities of emulsions. Phenolic antioxidants are widely used in inhibiting lipid oxidation. We found that phenolic antioxidants could form composite nanoparticles with zein and could be utilized to prepare Pickering emulsions with strong oxidative stability. Phenolic antioxidants with different hydrophobicity (octyl gallate, Propyl gallate and gallic acid) were fabricated into nanoparticles with zein (namely, Z/OG, Z/PG, Z/GA), and the oxidative stability of the as-prepared emulsions were much stronger than Tween 20 stabilized emulsions. And the Z/OG stabilized emulsions showed stronger oxidative stability than those stabilized by Z/PG and Z/GA. We also found that the antioxidative efficiency of octyl gallate was significantly higher than propyl gallate and gallic acid in Tween 20 stabilized emulsions. Compared with the Tween 20 stabilized emulsions, the difference in antioxidation efficiency between octyl gallate, propyl gallate and gallic acid was more obvious in the Pickering emulsions due to the combined effect of physical barrier and phenolic antioxidants. The results of this study help us to improve the antioxidation efficiency of phenolic antioxidants via the rational selection of antioxidant and a well-designed Pickering stabilization strategy.

1. Introduction
Spencer Umfreville Pickering was the scientist who first systematically studied the solid particles stabilized emulsions. Then, this kind of emulsion was named as Pickering emulsions [1]. Compared with emulsions stabilized with synthetic emulsifiers, such green-label emulsion products are attracting more popularity. Moreover, a major advantage of the Pickering emulsions over the surfactant stabilized ones lies in its high resistance against coalescence. Therefore, the Pickering emulsion stabilized by food grade solid particles is emerging as a research hotspot in the food industry.

As a natural, edible, safe and non-toxic plant macromolecule, zein has good biodegradability and renewable biocompatibility. Zein can be self-assembled into nanoparticles via anti-solvent precipitation,
which have been selected as an effective Pickering stabilizer [2]. However, Pickering emulsions stabilized solely by zein nanoparticles are easily subjected to creaming and oiling [3], and instead of forming fluid-like emulsions, gelled-like emulsions are usually obtained, and the physical barrier function of zein nanoparticles to retard emulsion oxidation is limited. In order to solve this issue, a potential strategy is to add Tween 20 into the emulsion [4, 5]. Tween 20, a nonionic water-soluble surfactant, is widely used in the food industry, and it has been reported that Tween 20 could improve the surface activity of zein nanoparticles [6]. Polyphenols, the natural source of phenolic antioxidants, were widely used as effective antioxidants in emulsion systems, among which, gallic acid and its ester derivatives are the most commonly utilized ones. The oil-water interface of the emulsion is the starting point and the diffusion point of the lipid oxidation [7, 8]. Therefore, polyphenols distributed at the interface inhibit the decomposition and improve the oxidation stability of emulsions in a more efficient manner. Food grade colloidal structures, such as nanoparticles, microcapsules and complexes, can be formed by the interaction between macromolecules and micro-molecules. It has been reported that phenolic hydroxyl groups of phenolic antioxidants can bind with amide groups of proteins through hydrogen bond and hydrophobic force [9]. However, some phenolic antioxidants with low polarity are insoluble in water and are difficult to bind with water-soluble proteins. But both phenolic antioxidants and zein can dissolve in the alcohol solution. Based on this, we can expect that composite nanoparticles with phenolic antioxidants loaded in alcohol soluble proteins can be formed by the anti-solvent precipitation method. The combination of zein particles and polyphenols are expected to regulate the self-assembly behavior of zein particles, improve the stability of zein nanoparticles, and then improve the antioxidant performance of the emulsion.

In order to study the effect of hydrophobicity of phenolic antioxidants on the oxidative stability of Pickering emulsions, nanoparticles were prepared by mixing zein with hydrophilic gallic acid (GA), amphiphilic propyl gallate (PG) and lipophilic octyl gallate (OG), respectively. The Pickering emulsion was prepared by the as-prepared composite nanoparticles, named as ZPE, and the emulsion oxidative stability was evaluated by determining its primary oxidation products.

2. Materials and methods

2.1. Materials
Zein (98%) was purchased from Wako Pure Chemical Industries Co., Ltd. (Wako Inc., Osaka, Honshu, Japan). Stripped corn oil and antioxidant was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Aladdin Inc., Shanghai, China). Gallic acid (Aladdin Inc., Shanghai, China), Propyl gallate (Aladdin Inc., Shanghai, China), Octyl gallate (Aladdin Inc., Shanghai, China) and Tween 20 (Kermel Inc., Tianjin, China) were of the highest purity available and used as received. Double-distilled water was used throughout experiments, and other reagents used were of analytical grade.

2.2. Fabrication of zein/phenolic antioxidant composite nanoparticles
Synthesis of zein/phenolic antioxidant nanoparticles (Z/AO): 1 g zein powder and 0.05 mmol antioxidant (gallic acid, propyl gallate, octyl gallate, respectively) were dissolved in 40 mL ethanol solution (70%) under ultrasonic and stirring conditions. After that, the mixed ethanol solution was added to 120 mL of tertiary water under the magnetic stirring condition of 200 rpm drop by drop to obtain the zein/antioxidant composite nanoparticles ethanol solution. After that, the mixed solution was evaporated and concentrated to 50 mL, and ethanol was removed to obtain Z/GA, Z/PG and Z/OG nanoparticle solution, respectively. Finally, each group of composite nanoparticle solution was centrifuged at 4000 rpm for 10 minutes to remove large aggregates.

2.3. Particle size, polydispersity index (PDI), and zeta potential measurement
Particle size, PDI, and zeta potential were measured, using a Nanosizer ZS 90 instrument (Malvern Inc., Worcestershire, UK). All samples were diluted 20 times, using 0.04 M acetic acid solution to pH = 3.0, before measuring. All measurements were conducted in duplicate, at 25 °C.
2.4. Evaluation of antioxidant capacity

The evaluation of antioxidant capacity was carried out by determining the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. The Z/GA, Z/PG, Z/OG nanoparticle solutions with or without centrifugation (8800 g, 10 min) were used to react with 1.0 mL DPPH methanol solution (0.33 mM), and they were allowed to react for 30 min at ambient temperature. An aliquot of 200 µL of the resultant mixture was then transferred to a 96-well microplate, and the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated as follows:

\[
\text{DPPH radical scavenging activity (\%) = } \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

where \( A_{\text{blank}} \) is the control reaction without GA and zein particles, and \( A_{\text{sample}} \) is the absorbance of the test sample.

2.5. Formation of Tween 20 stabilized emulsion and ZPEs

The Tween 20 emulsions stabilized by 0.5% Tween 20 at a constant oil-water rate of 5:5 with or without the addition of antioxidant (GA, PG, OG, respectively) were set as the control. The zein nanoparticle-based Pickering emulsions (ZPE) were prepared with the Z/AO (Z/GA, Z/PG, Z/OG, respectively) solution along with 0.5% Tween 20 at a constant oil fraction of 50%. Both emulsions were prepared by IKA T18 homogenizer (IKA Inc., Staufen Im Breisgau, Baden-Württemberg, Germany) under 10,000 rpm shearing for 2 min at room temperature.

2.6. Microstructure of ZPEs revealed by fluorescence microscopy

Inverted fluorescence microscope (Axio Observer A1, Carl Zeiss, USA) was used to confirm the formation of the zein nanoparticle interfacial layer. Zein nanoparticles were pre-stained with Rhodamine B during the emulsion fabrication process. Fifty µL of the sample was then cast on a single concave glass slide, the fluorescent dyes were excited at 561 nm and examined with a 10 × objective.

2.7. Determination of surface-loading content of Z/AO nanoparticles in ZPE (Γ)

The loading content of Z/AO nanoparticles on the surface of droplets in ZPEs was quantified according to a previous study, with modification [10]. The freshly prepared ZPEs were then centrifuged at 100 g for 20 min. Then the serum phase was filtered with a 0.45 µm membrane filter. The protein concentration in the serum phase was determined by the Lowry method, and the surface protein loading (Γ) (mg/m²) was calculated by the following equation [10]:

\[
\Gamma (\text{mg/m}^2) = \frac{(C_i - C_s) \times D_{3,2}}{6\Phi}
\]

where \( C_i \) (mg/mL) is the initial protein concentration in the aqueous phase, and \( C_s \) (mg/mL) is the concentration of protein in the serum phase. \( D_{3,2} \) (µm) is the surface average diameter of emulsion droplets determined by using a Malvern MasterSizer 3000 (Malvern Inc., Worcestershire, UK), and \( \Phi \) represents the volume fraction of oil.

2.8. Oxidative stability of ZPEs

To study the effect of AO hydrophobicity on the ZPE oxidative stability, ZPEs stabilized by zein/antioxidant nanoparticles (Z/AO) were prepared and allowed to spontaneously oxidize at 55 °C, in the dark, for further measurement. Then, the primary oxidation products of ZPEs were determined by the ferric thiocyanate method every other day, to evaluate their oxidative stability [11]. Emulsion with or without AO addition, stabilized only by 0.5% Tween 20 (T20), was selected as a control. All measurements were taken three times.

3. Results and discussions

3.1. Characterization of composite nanoparticles
Table 1. The average particle size, polydispersity index (PDI), ζ-potential of composite Z/AO nanoparticle.

|        | Size (nm)          | PDI    | ζ-potential (mV) |
|--------|--------------------|--------|-----------------|
| Z/GA   | 139.13±1.04        | 0.1±0.02| +28.53±0.64     |
| Z/PG   | 142.53±1.1         | 0.15±0.01| +27.5±1.06     |
| Z/OG   | 173.53±1.37        | 0.12±0.02| +24.43±1.25     |

As showed in Table 1, the prepared Z/AO nanoparticles were in nano scale. With the increase in hydrophobicity of phenolic antioxidants, particles with larger averaged particle size was obtained, which indicated that the hydrophobicity of phenolic antioxidants affected the self-assembly process of zein in the anti-solvent precipitation. The phenolic hydroxyl group of phenolic antioxidants bond with the amide group of zein through hydrogen bond and hydrophobic force [5]. After crosslinking with zein in ethanol solution, the more hydrophobic antioxidant is, the more hydrophobicity of nanoparticles increased. Therefore, in the process of antisolvent precipitation, larger composite nanoparticles are more likely to be formed with the addition of lipophilic OG. The PDI values of the three groups of nanoparticles were within 0.15, which indicated that the size distributions were within a narrow range as showed in Figure 1. It meant that these nanoparticles solutions are homogenous monodisperse dispersion, without significant aggregation. The strength of the repulsive force between oil droplets usually depends on the surface charge of the droplet itself. The charge density on the droplet surface can generally be reflected by the Zeta potential, and electrostatic exclusion plays a key role in stabilizing emulsion droplets and preventing coalescence of emulsion droplets. The zeta potential of these composite nanoparticles is close to +30 mV, which presents an electrostatic repellent effect, which is conducive to the long-term stability of the emulsion.

![Figure 1. The size distribution of Z/AO nanoparticles.](image)

Table 2. DPPH radical scavenging capacities of composite nanoparticles.

|        | DPPH radical scavenging rate before centrifugation | DPPH radical scavenging rate after centrifugation | Difference in DPPH radical scavenging rate before and after centrifugation |
|--------|--------------------------------------------------|--------------------------------------------------|------------------------------------------------------------------------|
| Z/GA   | 73.56±0.4%                                       | 31.34±0.2%                                       | 42.22±0.2%                                                            |
The DPPH radical scavenging rate before centrifugation represented the overall DPPH free radical scavenging rate of the composite nanoparticles solution, while after centrifugation, it represented the DPPH free radical scavenging rate of the phenolic antioxidants that are not combined with the zein nanoparticles, that is, the DPPH radical scavenging rate of the free phenolic antioxidants, and their difference indicated the DPPH free radical scavenging rate of the composite nanoparticles. As presented in Table 2, before centrifugation, Z/GA (73.56 ± 0.4%) showed the highest radical scavenging rate, and Z/OG (58.61 ± 0.5%) performed the lowest scavenging rate. However, according to the difference between before centrifugation and after centrifugation, the highest DPPH radical scavenging rate of composite nanoparticles was Z/OG (50.97 ± 0.1%), which indicated that only a small amount of OG distributed in water and the bonding of lipophilic OG and zein was the strongest. On the contrary, the free hydrophilic GA which distributed in the water phase has a DPPH radical scavenging rate above 30%, suggesting the weakest bonding between GA and zein.

3.2. Effect of AO hydrophobicity on the oxidative stability of ZPEs

Inverted fluorescence microscope images of ZPEs stabilized by composite nanoparticles (Figure 2) showed oil droplets surrounding with fluorescence, confirming the formation of a nanoparticle interfacial layer in all formulations.

|          | D_{3,2} (μm) | C₀ (mg/mL) | Cᵣ (mg/mL) | Γ (mg/m²) |
|----------|--------------|------------|-------------|-----------|
| Z/PG     | 11.21±0.06   | 1.463±0.007| 1.138±0.017 | 1.22±0.09 |
| Z/OG     | 11.07±0.03   | 1.453±0.052| 1.138±0.017 | 1.16±0.26 |
| Z/GA     | 10.7±0.28    | 1.488±0.017| 1.33±0.035  | 0.56±0.07 |

In Table 3, the average droplet size of the ZPEs followed the order of: Z/PG > Z/OG > Z/GA, but the difference was not significant (P<0.05). And the difference is not large enough to impact the antioxidant stability of emulsion, so we excluded the effect of emulsion particle size on the antioxidant properties of emulsions.

Both ZPEs stabilized by Z/PG and Z/OG has a significant higher interfacial loading content of protein than ZPE stabilized by Z/GA. This may be due to the better combination of lipophilic OG and amphiphilic PG with zein. Therefore, OG and PG obtained a higher concentration than GA in the interfacial region.
To study the effect of hydrophobicity of antioxidants on the oxidative stability of ZPEs, ZPEs stabilized by zein composite nanoparticles were prepared and allowed to spontaneously oxidize at 55 °C in the dark. Then, the primary oxidation products of ZPEs were determined by the ferric thiocyanate method every other day, to evaluate their oxidative stability. As showed in Figure 3, the amount of primary oxidation products generated by control group stabilized by Tween 20 without adding any antioxidants increased with time and reached 375 mmol/kg·oil at the eighth day. The formation of primary oxidation products of Tween 20 stabilized emulsions with the addition of phenolic antioxidants, gallic acid, propyl gallate, octyl gallate, increased slowly within six days, and the difference occurred on the eighth day. Among these emulsions, the group added with GA produced the highest amount of primary oxidation products but did not reach to 275 mmol/kg·oil. The order of primary oxidation products followed the order of GA added emulsions > PG added emulsions > OG added emulsions. It could be concluded that OG, octyl gallate, had the strongest antioxidant activity in Tween 20 stabilized emulsions.

As for Pickering emulsions stabilized composite nanoparticles, they showed a better oxidative stability than Tween 20 stabilized emulsion with the addition of phenolic antioxidants, and the amount of primary oxidation products produced was much less, and the order of primary oxidation was Z/GA > Z/PG > Z/OG. This was the combined effect of Pickering emulsions and phenolic oxidants, which increased the interfacial concentration of antioxidants. The results are in agreement with the concentration data of Table 1. Pickering emulsions stabilized by Z/OG exhibited the strongest oxidation stability. Thus, compared with the DPPH radical scavenging data before centrifugation, the DPPH radical scavenging rate of the composite nanoparticles was consistent with the results of emulsion oxidation stability.

4. Conclusion
In this paper, gallic acid, propyl gallate and octyl gallate, were selected to form composite nanoparticles with zein respectively, and their average particle size, PDI and potential were measured. The PDI and ζ-potential values of the nanoparticles were similar. With the increase in hydrophobicity of phenolic antioxidants, we found that the averaged particle size of nanoparticles was larger, because when crosslinking with zein in ethanol solution, the more hydrophobic antioxidant is, the more hydrophobicity of nanoparticles increased, and larger particles formed during the antisolvent precipitation process of zein. According to the primary oxidation products of emulsion under 14 days, we found that the antioxidant efficiency of octyl gallate in both Pickering emulsion and traditional emulsion was the highest, followed by propyl gallate and gallic acid. Among these three phenolic antioxidants, octyl
gallate is lipophilic, gallic acid is hydrophilic, and propyl gallate is both hydrophilic and lipophilic. This indicates that the hydrophobicity of phenolic antioxidants affected the oxidative stability of emulsions. Our work also suggested that phenolic antioxidants effectively improved their antioxidant efficiency by binding with zein. It was due to the increase in the interfacial concentration of phenolic antioxidants, and the formation of nanoparticle physical barriers. The results of this study will help to rationally design the emulsion with improved antioxidant efficiency.

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