Emulsion stabilized with phytoglycogen octenyl succinate prolongs the antimicrobial efficacy of ε-poly-L-lysine against *ESCHERICHIA coli* O157:H7

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Abstract

ε-Poly-L-lysine (EPL) is a food-grade cationic antimicrobial compound with a wide antimicrobial spectrum against bacteria, yeasts, and molds. However, EPL can be subject to rapid depletion after initial application and lose activity quickly. To address this problem, this study used *E. coli* O157:H7 and tryptic soy agar (TSA) deep-well depletion model to evaluate the prolonged antibacterial efficacy of EPL stabilized with emulsions formed by three types of emulsifier: (1) phytoglycogen octenyl succinate (PG-OS), an amphiphilic carbohydrate particulate; (2) waxy corn starch octenyl succinate (WCS-OS), an amphiphilic hyperbranched polysaccharide; and (3) Tween 20, a neutral small-molecule surfactant. During 20 days of storage at 4°C, the residual antibacterial efficacy of EPL in PG-OS emulsion was the greatest. In contrast, Tween-20 and WCS-OS emulsions were not as effective as PG-OS emulsion to retain antibacterial activity. Meanwhile, equilibrium dialysis showed the greatest EPL retention with PG-OS emulsion, suggesting the impact of electrostatic and structural properties of emulsifiers at the oil-water interface on prolonged EPL efficacy against *E. coli* O157:H7.

Keywords: ε-poly-L-lysine, phytoglycogen octenyl succinate, emulsion, prolonged efficacy, *E. coli* O157:H7

1. NTRODUCTION

Successful control and regulation of food-borne pathogenic microorganisms have a tremendous impact on social and economic wellness. While conventional food preservation technologies have dominated, novel techniques are emerging and providing new opportunities for safer foods. In this study, we explored carbohydrate-based emulsion systems to prolong the efficacy of antimicrobial peptide against food pathogens. ε-Poly-L-lysine (EPL) was utilized as the model antimicrobial peptide, and *E. coli* O157:H7 was used as the model bacterial pathogen.

As a food-grade peptide produced by *Streptomyces albulu* (Shima and Sakai, 1977), EPL is a homopolymer consisting of 25-35 L-lysine monomers and has a molecular weight of around 5,000 Da. The peptide bonds in EPL are links between the α-carbonyl groups and the ε-amino groups of adjacent lysine monomers (Chang et al., 2010 and Berg et al., 2002). EPL has a broad spectrum of inhibitory activities against Gram-positive and negative bacteria, fungi, yeasts, and bacteriophages due to its electrostatic and amphiphilic interaction with the surface constitutes of microorganisms (Chang et al., 2010, Geornaras and Sofos, 2005, and Yoshida and Nagasawa, 2003). Electrostatic interactions between cationic EPL molecules and negative components of cell membrane, as well as the hydrophobic effect between the carbon chain of EPL and phospholipid membrane are believed to destabilize the outer membrane, cause cellular disruption, and thus kill the organisms (Yoshida and Nagasawa, 2003 and Shima, et al., 1984). Due to its efficacy and safety, EPL has been used as an antimicrobial preservative (Hiraki, 2000).

E. coli O157:H7 belongs to the family of Shiga toxin-producing *Escherichia coli* (STEC) that synthesizes Shiga toxins and inhibits protein synthesis in cells and causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome at a small infectious dose of 50-100 cells (Bhunia, 2008, Thorpe, 2004, Mohsin et al, 2003, and Mufti et al., 1999). Immunocompromised

population and young children or elderly people with weakened immune system are exposed to high risk of fatal infection (Bhunia, 2008 and Philpott and Ebel, 2003). In addition, food recalls due to *E. coli* O157:H7 contamination may result in enormous economic losses (Nucci, Cuite, and Hallman, 2009). The Centers for Disease Control and Prevention (CDC) estimates that 265 000 STEC infections occur each year in the United States, and STEC O157 accounts for about 36% of these infections (CDC, 2015). As reported by the European Centre for Disease Prevention and Control (ECDC, 2014), shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) infection increased in the European Union (EU) and European Economic Area (EEA) over the period of 2008–2012, and in 2012 the overall notification rate was 1.5 cases per 100,000 population. In Japan, the annual incidence of verocytotoxin-producing *Escherichia coli* cases was 2.74 per 100 000 population (CDC, 2006).

While EPL can be used to suppress the bacterial growth, it is susceptible to multiple depletion factors that may lead to a rapid loss of activity. For example, due to its interactions with proteins and acidic polysaccharides, EPL may be rapidly depleted when applied in processed foods (Ho et al., 2000 and Hiraki, 1995). Thus, there is a strong need in the food industry to protect EPL efficacy. Studies have been carried out to improve the effectiveness of EPL, such as through its combination with organic acids (Geornaras and Sofos, 2005) and its covalent immobilization to multi-walled carbon nanotubes for constructing nanocomposite with enhanced antibacterial activity (Zhou and Qi, 2011).

In the present study, a Pickering emulsion stabilized using phytoglycogen octenyl succinate (PG-OS) was used to protect EPL and achieve prolonged antibacterial efficacy. PG-OS is a group of phytoglycogen (PG) derivatives that are substituted with octenyl succinate (OS) groups. Our previous studies have shown that PG-OS emulsion can drastically reduce the

88 oxidation of fish oil (Scheffler, Wang, Huang, Gonzalez, and Yao, 2010b) and prolong the
89 antibacterial activity of nisin against *Listeria monocytogenes* (Bi, Yang, Narsimhan, Bhunia, and
90 Yao, 2011b).

91 To protect food products from pathogens, most studies have focused on active packaging
92 in which various synthetic or biobased polymers are used to immobilize active agents, such as
93 bacterial cellulose (Gao et al, 2014), gelatin nanofibers (Xu and Zhou, 2008), and gum arabic
94 (Chang, McLandsborough, and McClements, 2014). These studies simulated the scenario where
95 the pathogen contamination occurs before package sealing or in the early stage of storage. In the
96 present study, we simulated a scenario in which the contamination of *E. coli* O157 may occur
97 over an entire period of food storage and consumption, and the retention of sufficient amount of
98 antimicrobial compounds would be essential to reducing pathogen growth.

99

100 ## 2. MATERIALS AND METHODS

101 ### 2.1 Reagents and Chemicals

102 Sweet corn cultivar Silver Queen was purchased from Burpee Co. (Warminster, PA).
103 Waxy corn starch was obtained from National Starch Food Innovation (Now a part of Ingredion,
104 Bridgewater, NJ). Tween 20, Trypan blue, and soybean oil were purchased from Sigma-Aldrich
105 (St. Louis, MO). 1-Octenyl succinic anhydride was obtained from Dixie Chemical Co. (Houston,
106 TX). Tryptic soy broth and agar were purchased from BD (Franklin Lakes, NJ). Fast micro-
107 equilibrium dialyzer and 300 kDa molecular weight cut-off (MWCO) cellulous acetate
108 membranes were purchased from Harvard Apparatus (Holliston, MA). ε-Poly-L-lysine (EPL) (25%
109 w/w) was obtained from Purac America (Lincolnshire, IL).

2.2 Extraction of Phytoglycogen

Sweet corn kernels were ground into grits and then mixed with four weights of 4°C deionized water followed by homogenization using a high-speed blender (Waring Laboratory, Torrington, CT). The solid in the homogenate was separated from the liquid using a 270-mesh sieve and the retrieved solid materials underwent further extractions. Subsequently, all liquid collected passed through the 270-mesh sieve again prior to adjusting the pH to 4.8 to precipitate proteinaceous materials. After centrifugation at 4°C for 20 min (10 000 *g*), the collected supernatant passed through the 270-mesh sieve again and was placed at 4°C for 24 h followed by 2-cycle of centrifugation at 4°C (10 000 *g*, 20 min). The collected supernatant was incubated at 4°C for additional 24 h and then subjected to centrifugation (10 000 *g*, 20 min) until no precipitation was observed. The collected supernatant was adjusted to pH 6.9 and subjected to autoclave at 121°C for 20 min. After cooling, the liquid was centrifuged (10 000 *g*, 20 min). The supernatant was collected and added with 3 volumes of ethanol to precipitate polysaccharide, which was collected, repetitively washed with ethanol, and then dried in a fume hood to yield phytoglycogen (PG) solid.

2.3 Preparation of Non-granular Waxy Corn Starch

Twenty grams of waxy corn starch (WCS) was dispersed in 400 mL of sodium hydroxide solution (2%, w/v) and the dispersion was heated in a boiling-water bath with vigorous stirring until the dispersion was clear. After cooling, the dispersion was adjusted to pH7.0 using hydrochloric acid (10%, w/w). To the dispersion, three volumes of ethanol were added to precipitate the polysaccharide. After centrifugation (5 000 *g*, 10 min), the solid was subjected to two dispersion-filtration cycles with ethanol and dried in a fume hood to yield non-granular WCS.

2.4 Substitution with Octenyl Succinic Anhydride

To the dispersions of non-granular WCS and phytoglycogen, 1-octenyl succinate anhydride was added in 2 h at the level of 3% (w/w) of glucan. The pH was maintained between 8.5 and 9.0 using 2% NaOH. The reaction was conducted at 40°C, and terminated after 24 h by reducing the pH to 6.5 using 2% HCl, followed by adding 3 volumes of ethanol. The precipitate was washed using 3 cycles of ethanol suspension-filtration. The solid collected was dried in a fume hood to yield phytoglycogen octenyl succinate (PG-OS) and waxy corn starch octenyl succinate (WCS-OS).

To determine the degree of substitution (DS) of PG-OS and WCS-OS, 0.5 g of each material was acidified with 3 mL of HCl (2.5 M) for 30 min. Subsequently, 10 mL of 90% isopropanol (v/v) was added, followed by centrifugation at 3 000 *g* for 10 min and supernatant decanting. The precipitate was subjected to repetitive isopropanol suspension-centrifugation until the test of chloride ions using silver nitrate ($AgNO_3$) showed negative outcome. For this test, one drop of 0.1M $AgNO_3$ was added to the decanted supernatant to observe white haze of silver chloride (AgCl). Once no noticeable AgCl haze was observed, 30 mL of deionized water was added to the precipitate. The mixture was heated in a boiling water bath for 30 min, and titrated using 0.01M NaOH. The DS was calculated by: DS = (162A)/(1000-210A), where A (mmol/g) was the molar amount of octenyl succinate groups in one gram of derivative, and 162 and 210 were the molecular weights of the glucosyl unit and the octenyl succinate group, respectively. The value of A was calculated as: $A = (V-V_0)\times 0.01/0.5$, where V (mL) was the volume of NaOH consumed by PG-OS or WCS-OS sample, V_0 (mL) was the volume of NaOH consumed by native PG or WCS sample, 0.5 was the weight of material, and 0.01 was the molar concentration of NaOH.

2.5 Preparation of Emulsions Added with EPL

PG-OS, WCS-OS, and Tween-20 were used to prepare emulsions to which EPL was added. For PG-OS or WCS-OS, 1.0 g solid was dispersed in 100 mL sodium acetate buffer (50 mM, pH5.5, 22°C) to form a dispersion of 10 mg/mL. For Tween 20, 0.10 g was dissolved in 100 mL buffer to form a solution of 1.0 mg/mL. To each solution of emulsifier, 1.0 g of soybean oil was added. The mixtures were first treated with a high-speed homogenizer (18 000 rpm for 1 min, T25 ULTRA-TURRAX, IKA) and then with a high-pressure homogenizer (103 MPa, two cycles, Nano DeBee, BEE International). To 27 mL of each emulsion, 3.0 mL EPL solution (10 mg/mL) was added to make a final EPL concentration of 1.0 mg/mL. Each mixture was sterilized in a boiling-water bath for 3 min. EPL 1.0 mg/mL in buffer was used as the reference.

2.6 TSB Deep-well EPL Depletion Model

Thirty grams of tryptic soy broth (TSB) powder was dispersed in 1,000 mL of deionized water followed by adjusting pH to 5.5 using HCl and then adding 10 g agar. After autoclave (30 min, 121°C), the dispersion was cooled to 70°C and 500 mL of it was poured in a 600-mL beaker and allowed to solidify at room temperature. After two days, two wells were made in each beaker using a 10-mm cork borer. To each well, EPL preparation was loaded **(Figure 1)** and at each time interval over 20 days of deep-well depletion storage, 500 µL, 800 µL, and 100 µL aliquots were withdrawn for equilibrium dialysis, antimicrobial tests against *E. coli*, and total protein analysis. For each preparation, triplicate deep wells were used for evaluations. Before the deep-well loading, EPL preparations were aliquoted as the samples for “day 0”.

2.7 Equilibrium Dialysis to Evaluate EPL Retention and Availability

For the equilibrium dialysis tests, the fast micro-equilibrium dialyzer (FMED) was used

178 to evaluate the amount of available EPL after a certain period of depletion. In this study, we use
179 the amount of EPL collected in the permeate chamber of FMED to assess EPL availability. This
180 portion of EPL originated from two resources: (1) non-adsorbed EPL in the aliquoted preparation,
181 and (2) released EPL from the originally adsorbed portion. Such a procedure partly simulated the
182 scenario in which a portion of retained EPL molecules, regardless adsorbed or non-adsorbed,
183 was available to interact with bacterial cells once a pathogen contamination occurs.

184 Our early tests using EPL solution (1.0 mg/mL) showed that 30 h of dialysis at 22°C was
185 sufficient for reaching the equilibrium. Therefore, all preparations were subjected to 30 h dialysis
186 before quantifying EPL in the permeate chamber. For the aliquot withdrawn from the permeate
187 chamber, the EPL concentration was determined using the Trypan blue (TB) assay (Grotzky et al,
188 2010). For TB assay, 156 µL of diluted solution was mixed with 6 µL of TB dye (1.0 mg/mL in
189 50 mM, pH5.5 sodium acetate buffer). The mixture was incubated at 37°C for 1 h, and after
190 cooling, centrifuged (5 000 *g* for 20 min) at 15°C. The supernatant was measured for absorbance
191 at 580 nm. EPL solutions ranging 1.0 to 10 µg/mL were used as standards.

192 **2.8 Zeta-potential Measurement**

193 Emulsions (with and without EPL) formed by PG-OS, WCS-OS or Tween-20 were
194 stored in the TSB-agar deep-well model. On day 0 and day 20, aliquots were withdrawn from
195 individual wells and measured for their zeta-potentials using Zetasizer Nano (ZS90, Malvern
196 Instruments) (Bi et al., 2011a,b).

197 **2.9 Transmission Electron Microscope (TEM) Imaging of PG-OS and WCS-OS**

198 TEM imaging of PG-OS and WCS-OS was conducted as described by Putaux et al. (1999)
199 with modifications. Carbon-coated 400-mesh grid was glow discharged before use. Droplets of

0.01% (w/v) PG-OS or WCS-OS in 0.02 M NaAc buffer (pH5.5) were dried on grid and stained by 2% aqueous uranyl acetate. Specimens were imaged using a Philips CM-100 transmission electron microscope (FEI Company, Hillsboro OR) operated at 100 kV, 200 μm condenser aperture, and 70 μm objective aperture. Images were captured on a Kodak SO-163 film.

2.10 Monitoring the Retention of EPL Activity against *E. coli* O157:H7 Growth

Eight hundred microliters of liquid withdrawn from each TSA deep-well model were inoculated with 8 μL TSB broth of *E. coli* O157:H7 strain SEA13A45 (6.8 to 7.1 log CFU/mL). After incubation at 22°C for 24 h, each mixture was serially diluted using sodium acetate buffer (50 mM, pH 5.5) and spread-plated on TSA plates. Colonies formed after 24 h incubation at 37°C were enumerated.

2.11 Quantification of Soluble Proteins

The amounts of soluble protein materials in EPL preparations that were subjected to various storage stages in the TSA deep-well model were determined using Bradford assay kit (Bio-Rad).

2.12 Statistical Analysis

The data were analyzed using PROC ANOVA in SAS version 9.2. Tukey's test was utilized with a significant F test ($P < 0.05$). Each sample had 3 replicates. When monitoring the growth of pathogens, duplicates were prepared for each dilution.

3. RESULTS AND DISCUSSION

3.1 Octenyl Succinate Substitution of Phytoglycogen (PG) and Waxy Corn Starch (WCS)

The grafting of octenyl succinate (OS) groups to PG and WCS brings both hydrophobic groups and negative charges, and thus produces amphiphilic materials that can be used as emulsifiers. With 3% (w/w) OSA, the theoretical degree of substitution (DS) for PG-OS and WCS-OS is 0.0231 whereas the actual DS was 0.0128 and 0.0129, respectively. Therefore, the substitution efficiency of PG-OS and WCS-OS was 55% and 56%, respectively. The DS values of PG-OS and WCS-OS were comparable, which allowed direct comparisons in this study.

3.2 Retention of EPL in Emulsions

Table 1 shows the concentration of EPL in the permeate chamber of the FMED unit. For EPL preparations withdrawn at day 0, the EPL concentration detected in the permeate chamber was 499.7, 267.2, 307.3, and 409.9 μg/mL for EPL-alone solution and emulsions formed with PG-OS, WCS-OS, and Tween-20 respectively, suggesting the strongest adsorption of EPL molecules in the PG-OS emulsion. As shown in **Table 1**, the quickest depletion of EPL occurred with EPL-alone preparation. After 5 days of storage in the depletion model, 19.3 μg/mL of EPL was detected in the permeate chamber. Meanwhile, EPL depletion was also shown in PG-OS, WCS-OS, and Tween-20 emulsions, yet at different levels. Among them, PG-OS emulsion displayed a lowest level of EPL depletion, with 92.8 μg/mL detected in the permeate chamber at Day 5. In contrast, WCS-OS and Tween-20 emulsions resulted in 75.8 and 21.4 μg/mL EPL respectively in the permeate chamber. With continued depletion in the deep-well model, after 10 days, the EPL concentration in the permeate chamber were 11.3, 37.9, 37.7, and 20.6 μg/mL, respectively for EPL-alone and PG-OS, WCS-OS, and Tween-20 emulsion. After 20 days, only 3.8 μg/mL of EPL was detected for EPL-alone, whereas 19.8 μg/mL of EPL was detected for PG-OS emulsion. Overall, the retention of EPL with PG-OS emulsion was much greater than that with EPL-alone and Tween-20 emulsion. EPL retention with WCS-OS emulsion was less

than PG-OS emulsion but greater than EPL-alone and Tween-20 emulsion. These results were in agreement with our previous study (Bi, Yang, Bhunia, and Yao, 2011a), in which PG-OS emulsion exhibited greater capability to retain nisin than WCS-OS and Tween-20 emulsions.

3.3 Proposed Interaction between EPL and Emulsion Droplets

Zeta-potential is a technique that can be used to describe the electrostatic interactions among particles with opposite charges. In general, the addition of positively charged EPL to emulsions shifts the value of zeta-potential to the positive direction. As shown in **Figure 2**, the addition of EPL led to an increase of zeta-potential values of PG-OS, WCS-OS, and Tween-20 stabilized emulsions. The depletion (and retention) of EPL can also be reflected by the change of zeta-potential. A greater depletion usually corresponds to a greater change of zeta-potential. As shown in **Figure 2**, the zeta-potential value of EPL-alone preparation was initially +18.8 mV and reduced to +1.6 mV after 20 days in the depletion model, showing a nearly full loss of EPL. In contrast, the zeta-potential of EPL-containing PG-OS emulsion was initially -5.5 mV and slightly changed to -7.7 mV at day 20, indicating a much less EPL depletion. For EPL-containing WCS-OS emulsion, zeta-potential changed from initial -4.9 to -9.5 mV after 20 days, correlating with a greater EPL loss than PG-OS emulsion. For Tween-20 emulsion with EPL, zeta-potential changed from initial +8.6 to -0.9 mV at day 20, which showed a substantial EPL loss in the depletion model. **Figure 2** also shows that the emulsions without EPL maintained their zeta-potential in the depletion model (PG-OS, -10.0 to -10.0 mV; WCS-OS: -9.6 to -9.5 mV: and Tween-20: -0.7 to -0.8 mV). Conceivably, the changes of zeta-potential of EPL-containing emulsions in the depletion model were attributed to the loss of EPL.

Figure 3 conceptually depicts the adsorption of EPL at the interface of the PG-OS stabilized oil droplets. The adsorption of EPL molecules at the interface, which was due to the

electrostatic and hydrophobic interactions, reduced the number of free EPL molecules that were susceptible to depletion factors such as chemical degradation or physical diffusion into the bulk of gel matrix. In contrast, Tween-20 and WCS-OS based emulsions were able to adsorb EPL, however, with lower efficiencies. It appears that the performance of EPL was similar to that of nisin (Bi, Yang, Bhunia, and Yao, 2011a), and both EPL and nisin showed a much-enhanced activity retention with PG-OS stabilized emulsion. In that work (Bi, Yang, Bhunia, and Yao, 2011a), PG-OS emulsion showed the greatest ability to preserve nisin during the extended storage of 40 days, and Tween-20 emulsion and nisin-alone lost nisin activity within 10 days, and nisin activity in WCS-OS emulsion decreased rapidly after 10 days.

As shown in **Figure 4**, PG-OS was a dense nanoparticle with particle size around 50 nm, whereas WCS-OS was a highly dispersed, worm-like macromolecule ranging from 150 -200 nm. Conceivably, the structure of interfacial layers formed by PG-OS nanoparticles and WCS-OS linear segments would perform differently in adsorbing and releasing EPL molecules.

3.4 PG-OS Emulsion Prolonged EPL Activity against *E. coli* O157:H7

Table 2 shows the growth of *E. coli* O157:H7 treated with various EPL preparations that were subjected to the TSA deep-well model storage for 0, 5, 10, 15, and 20 days. Our preliminary test showed that during storage for up to 20 days no antimicrobial activity was found for PG-OS, WCS-OS, and Tween 20 emulsions without EPL. In contrast, each EPL-containing preparation showed antibacterial activity for a certain period of time during the storage. The antimicrobial effect of EPL has been attributed to its ability to interact with and disrupt anionic cell membranes, thereby leading to cell leakage (Goy et al, 2009 and Shima et al., 1984).

For each antibacterial test, the concentration of inoculated *E. coli* O157 was around 5.0 log CFU/mL, which was achieved by diluting the broth of 6.8-7.1 log CFU/mL with 100 times

290 volume of EPL preparations. As shown in Table 2, at day 0, *E. coli* population was fully
291 removed by all types of EPL preparation. However, after 5 days of deep-well depletion storage,
292 the efficacies of EPL preparation to inhibit the growth of *E. coli* varied. For PG-OS emulsion-
293 stabilized EPL preparation, 5 days of storage retained strong inhibitory activity against the
294 bacteria, leading to a reduction of bacterial count from 5.0 log CFU/mL (at inoculation) to an
295 undetectable level. After 20 days of deep-well storage, the preparation showed a reduced
296 inhibitory activity but still led to a reduction of bacterial count form 5.0 to 2.38 log CFU/mL.
297 This shows that the PG-OS emulsion was able to retain a substantial level of EPL activity for at
298 least 20 days and realized an evident antibacterial efficacy.

299 In contrast, EPL-alone and Tween-20 emulsion stabilized EPL have shown very low
300 antibacterial efficacies after prolonged storage. After 5 days of deep-well storage, both EPL-
301 alone and Tween-20 emulsion stabilized EPL were able to partially inhibit bacterial growth,
302 showing over 2 log CFU/mL *E. coli* population, in comparison with the inoculation level of 5 log
303 CFU/mL. After 10 days of deep-well storage, EPL-alone and Tween-20 emulsion stabilized EPL
304 still inhibited the bacterial growth, but with further lower capability (*E. coli* population increased
305 to 3.22 and 2.45 log CFU/mL, respectively). After 20 days, the EPL efficacy seemed mostly lost,
306 leading to an *E. coli* population of 7.15 and 6.90 log CFU/mL, respectively.

307 WCS-OS emulsion showed appreciable effect in retaining EPL activity. After 5 days,
308 EPL preparation was able to fully inhibit the bacterial growth (undetectable *E. coli* colony). After
309 20 days of deep-well storage, however, the EPL preparation was not able to inhibit bacterial
310 growth, resulting in 5.40 log CFU/mL. Apparently, WCS-OS emulsion was less efficacious than
311 PG-OS emulsion.

312 Similar observation was reported in our previous study (Bi, Yang, Bhunia and Yao,

313 2011a) showing that during 50 days of storage, the anti-listerial activity of nisin-containing PG-
314 OS emulsion was much greater than that of nisin-alone solution. In contrast, the emulsions
315 stabilized with Tween-20 or WCS-OS was either ineffective or less effective than the PG-OS-
316 stabilized emulsion to retain nisin activity. We consider that the retention of EPL activity in
317 emulsions was mainly due to the interactions between the positively charged EPL molecules
318 with the negatively charged PG-OS or WCS-OS molecules at the oil-water interface. PG-OS
319 showed a greater capacity than WCS-OS to retain EPL molecules, which could be associated
320 with its dense particulate structure (Fig. 4). In contrast, Tween-20 emulsion showed negligible
321 capability to retain EPL due to its lack of negative charges.

322 To further reveal the relationship between the antibacterial efficacy of EPL preparations
323 and their EPL retention, we plotted *E. coli* population after EPL treatments vs. EPL
324 concentration in FMED's permeate chamber. As shown in **Figure 5**, there was a strong negative
325 correlation between the bacteria survival and the EPL concentration. Undoubtedly, the EPL
326 concentration in permeate chamber, which reflects the abundance of available EPL in the
327 formulation, is an effective indicator of antibacterial efficacy.

328 To understand the potential effect of nutrient concentration on the detected antibacterial
329 efficacy, we measured the concentration of proteinaceous materials in EPL preparations. For
330 each preparation, the starting peptide concentration was 1.0 mg/mL that originated from added
331 EPL. When EPL preparations were treated in the depletion models, proteinaceous materials
332 diffused from the TSA wall of deep wells to the dispersion. **Table 3** shows that at day 5, the total
333 proteinaceous materials reached around 17-18 mg/mL, and then stabilized at around 20 mg/mL
334 after 10 days. Conceivably, the total amounts of proteinaceous materials originated from TSA
335 were essentially the same, thus their impact on the difference of *E. coli* growth should be

negligible. In general, we consider that the retention of EPL should be the prevailing factor that controlled the bacterial growth.

From an industry perspective, this study may help design highly effective, low-cost delivery systems to enhance or prolong antimicrobial efficacy against pathogenic bacteria. PG-OS is a novel, plant-based material that has shown potential applications for food, whereas WCS-OS is an established food ingredient. Both had shown, although at different levels, the property of retaining EPL in model environment. In general, this study will support EPL formulations to improve food safety.

4. CONCLUSION

In this study, we used TSA deep-well depletion model to simulate food surfaces where EPL preparations were applied and subjected to depletion. During the storage of model systems, there was a continuous depletion of various EPL preparations at different levels. The order of the retention of antibacterial efficacy was: PG-OS emulsion > WCS-OS emulsion > Tween-20 emulsion > EPL-alone. Such a difference in antibacterial efficacy correlated with EPL retention in the preparations, with greater retention leading to higher efficacy. In general, the PG-OS emulsion showed the highest capability to prolong the efficacy of EPL against *E. coli* O157:H7, which further demonstrated the performance of PG-OS as an effective delivery vehicle to protect and deliver bioactive compounds. The structure of PG-OS can be engineered chemically and enzymatically, allowing for the generation of suitable colloidal assemblies.

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FIGURE CAPTIONS

Figure 1. Schematic of tryptic soy agar (TSA) deep-well depletion model. Various ε-poly-L-lysine (EPL) preparations were filled in the wells and stored for 20 days

Figure 2. Zeta-potential values of base emulsions, ε-poly-L-lysine (EPL)-alone dispersion, and EPL-containing emulsions stabilized using PG-OS, WCS-OS, and Tween-20 at storage stages of day 0 and day 20 in the deep-well depletion model. PG-OS: phytoglycogen octenyl succinate; WCS-OS: waxy corn starch octenyl succinate; PG-OS + EPL: EPL-containing PG-OS emulsion; WCS-OS + EPL: EPL-containing WCS-OS emulsion; Tween-20 + EPL: EPL-containing Tween-20 emulsion

Figure 3. Schematic of ε-poly-L-lysine (EPL) depletion with EPL-alone and EPL-containing PG-OS emulsion (PG-OS + EPL) at the initial (day 0) and final stage (day 20) of depletion storage in the agar deep-well model. The values of zeta-potential are labeled with individual scenarios, showing the change of zeta-potential as associated with the abundance of EPL molecules and their adsorption to the oil droplets of PG-OS emulsion. An oil droplet of PG-OS emulsion without EPL is shown to highlight the impact of EPL on zeta-potential value. PG-OS: phytoglycogen octenyl succinate; PG-OS + EPL: EPL-containing PG-OS emulsion

Figure 4. Transmission electron microscopy (TEM) of PG-OS (left) and WCS-OS (right), with scale bar of 100 nm

Figure 5. Relationship between the residual populations of *E. coli* O157:H7 treated with ε-poly-L-lysine (EPL) preparations and the concentrations of EPL in the fluids collected from the permeate chamber of fast micro-equilibrium dialyzer, plotted from **Table 1 and 2**

Table 1. EPL concentrations in the permeate chamber of the fast micro-equilibrium dialyzer (FMED) for EPL preparations subjected to various storage stages in the agar deep-well depletion model

EPL Delivery system	**EPL concentration, μg/mL**				
	Day 0	**Day 5**	**Day 10**	**Day 15**	**Day 20**
EPL-alone	499.7 ± 11.3a	19.3±4.3c	11.3±1.3c	5.2±0.9c	3.8±0.2c
PG-OS emulsion	267.2±10.3c	92.8±2.3a	37.9±6.1a	29.5±6.6a	19.8±3.9a
WCS-OS emulsion	307.3±9.0b	75.8±5.4b	37.7±2.7a	18.6±0.4b	7.7±1.0b
Tween-20 emulsion	409.9 ± 11.4a	21.4±1.6c	20.6±2.6b	7.9±1.3c	5.0±0.4c

Data are expressed in mean ± SD (n=3). Significant differences within each of Day 0, Day 5, Day 10, Day 15, and Day 20 groups are denoted with different letters ($p<0.05$).

EPL: ε-poly-L-lysine; PG-OS: phytoglycogen octenyl succinate; WCS-OS: waxy corn starch octenyl succinate

Table 2. Survival of *E. coli* O157:H7 (log CFU/mL) affected by EPL preparations subjected to various storage stages in the agar deep-well depletion model

EPL delivery system	***E. coli* population, log CFU/mL**				
	Day 0	**Day 5**	**Day 10**	**Day 15**	**Day 20**
EPL-alone	-*	2.53±0.06a	3.22±0.04a	5.90±0.01a	7.15±0.01a
PG-OS emulsion	-	-	1.45±0.15b	1.79±0.20c	2.38±0.03c
WCS-OS emulsion	-	-	1.58±0.21b	2.94±0.08b	5.40±0.03b
Tween-20 emulsion	-	2.27±0.12a	2.45±0.14a	5.42±0.01a	6.90±0.06a

* - Below detection limit (<1.0 log CFU/mL)

Initial inoculation level was 5 log CFU/mL.

Data are expressed in mean ± SD (n=3). Significant differences within each of Day 0, Day 5, Day 10, Day 15, and Day 20 groups are denoted with different letters ($p<0.05$).

EPL: ε-poly-L-lysine; PG-OS: phytoglycogen octenyl succinate; WCS-OS: waxy corn starch octenyl succinate

Table 3. The amount of proteinaceous materials in EPL preparations subjected to various storage stages in the agar deep-well depletion model

EPL delivery system	**Concentration of proteinaceous materials, mg/mL**				
	Day 0	**Day 5**	**Day 10**	**Day 15**	**Day 20**
EPL-alone	1.01± 0.01a	17.8±0.2a	20.5±0.1a	20.1±0.1a	19.7±0.1a
PG-OS emulsion	1.11±0.11a	17.9±0.4a	19.6±0.2a	19.8±0.3a	19.2±0.3a
WCS-OS emulsion	1.15±0.12a	18.3±0.5a	20.6±0.3a	20.2±0.2a	20.5±0.3a
Tween-20 emulsion	1.02±0.01a	17.1±0.2a	19.4±0.2a	19.7±0.3a	19.7 ±0.2a

Data are expressed in mean ± SD (n=3). Significant differences within each of Day 0, Day 5, Day 10, Day 15, and Day 20 groups are denoted with different letters ($p<0.05$)

EPL: ε-poly-L-lysine; PG-OS: phytoglycogen octenyl succinate; WCS-OS: waxy corn starch octenyl succinate

Figure 1

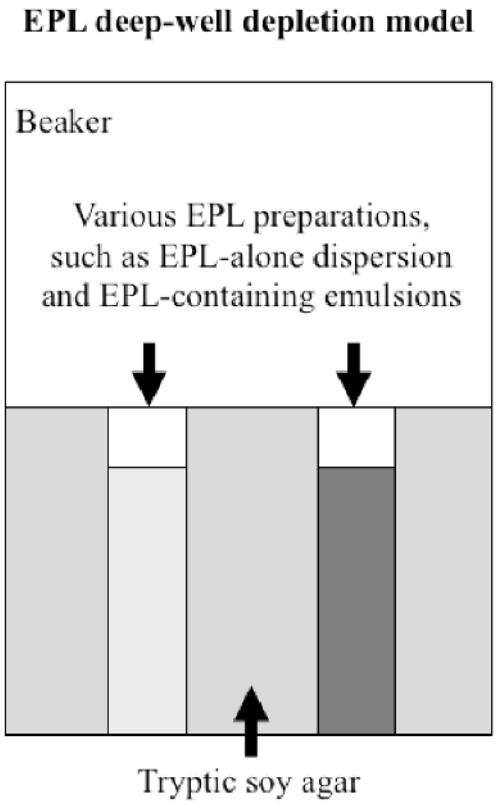

EPL deep-well depletion model
Beaker
Various EPL preparations, such as EPL-alone dispersion and EPL-containing emulsions
Tryptic soy agar

Figure 2

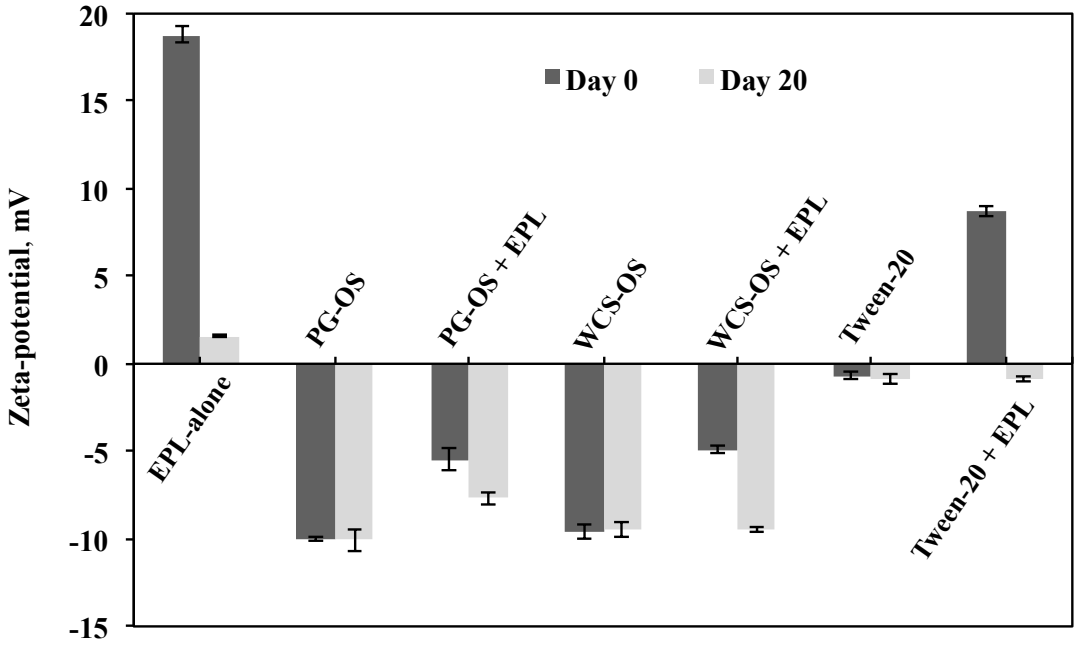

Zeta-potential, mV
20
15
10
5
0
-5
-10
-15
Day 0
Day 20
EPL-alone
PG-OS
PG-OS + EPL
WCS-OS
WCS-OS + EPL
Tween-20
Tween-20 + EPL

Figure 3

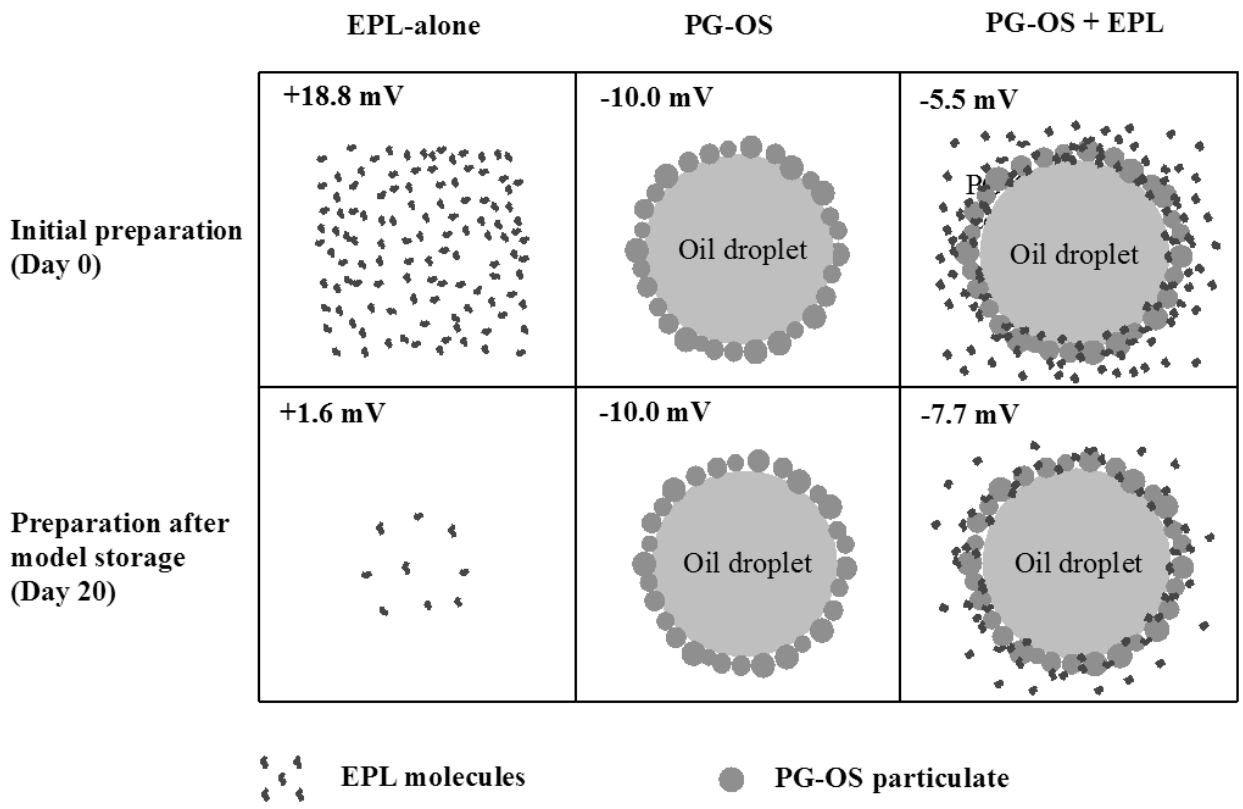

EPL-alone
PG-OS
PG-OS + EPL
Initial preparation (Day 0)
Preparation after model storage (Day 20)
+18.8 mV
-10.0 mV
-5.5 mV
+1.6 mV
-10.0 mV
-7.7 mV
Oil droplet
Oil droplet
Oil droplet
Oil droplet
EPL molecules
PG-OS particulate

Figure 4

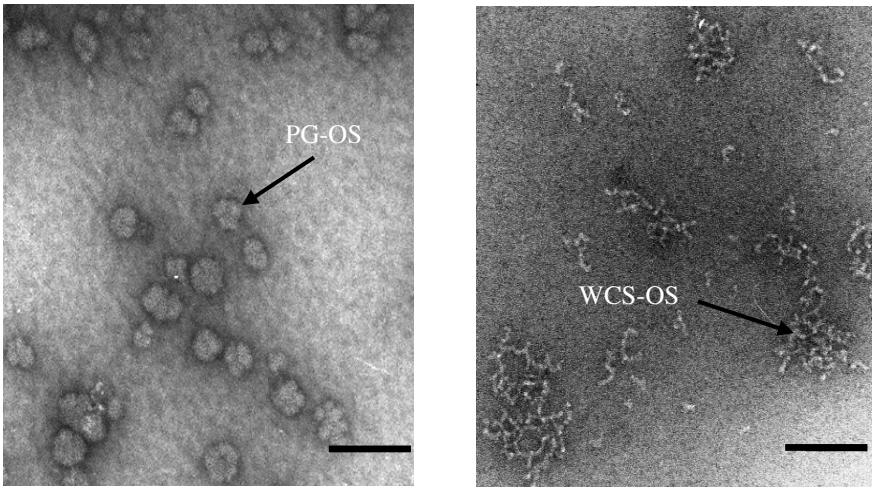
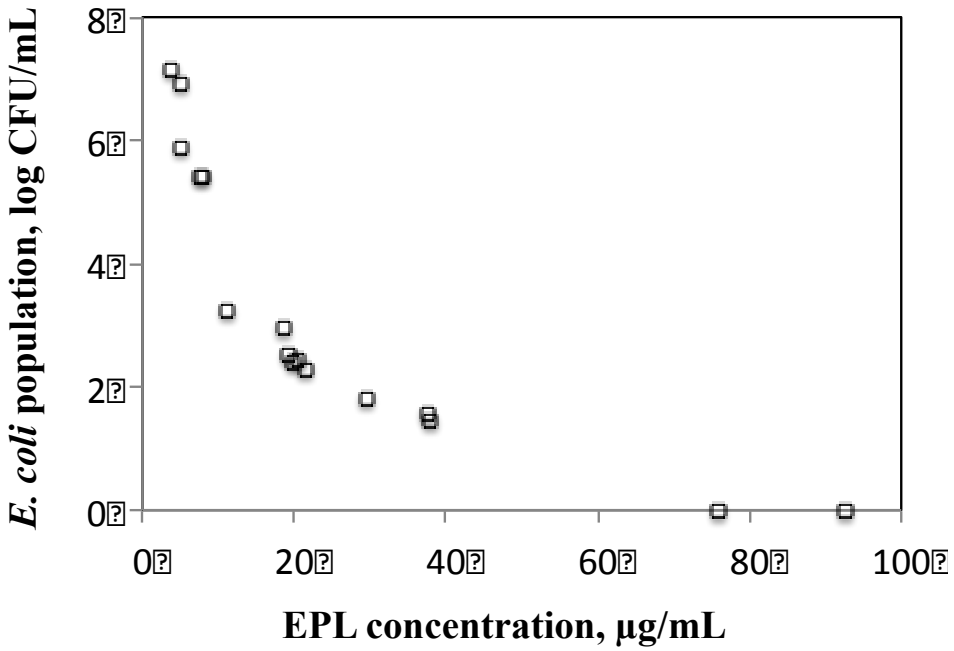


Figure 5


E. coli population, log CFU/mL
8
6
4
2
0
0
20
40
60
80
100
EPL concentration, µg/mL