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ARTICLE

Synthesis and Characterization of Novel Antimicrobial Emulsifiers from ε-Polylysine

Hailong Yu, Yuping Huang, and Qingrong Huang*

Department of Food Science, Rutgers, the State University of New Jersey, 65 Dudley Road, New Brunswick, New Jersey 08901

ε-Polylysine (EPL) has been used in the food industry as an antimicrobial additive and also a dietary agent. To generate amphiphilic molecules from EPL, hydrophobically modified ε-polylysine graft copolymers, which were denoted as OSA-*g*-EPLs, were synthesized by reacting EPL with octenyl succinic anhydride (OSA). The success of synthesis was confirmed by ^{1}H NMR and FT-IR spectroscopy. It was found that OSA-*g*-EPLs had glass transition temperatures lower than EPL. Furthermore, they were able to form polymer micelles in water and to lower the surface tension of water, confirming their amphiphilic properties. The antimicrobial activities of OSA-*g*-EPLs were also examined, and the minimum inhibitory concentrations of OSA-*g*-EPLs against *Escherichia coli* O157:H7 remained the same as that of EPL. Therefore, OSA-*g*-EPLs have the potential of becoming bifunctional molecules, which can be used either as surfactants or emulsifiers in the encapsulation of nutraceuticals or drugs or as antimicrobial agents.

KEYWORDS: ε-Polylysine; octenyl succinic anhydride; amphiphile; polymer micelles; antimicrobial agent; *E. coli* O157:H7

INTRODUCTION

ε-Polylysine (EPL) is a homopolymer of L-lysine through the isopeptide bond between ε-amino and α-carboxyl groups (**Scheme 1**). In contrast to poly(L-lysine), which is synthesized by chemists, EPL is generated by the bacterium *Streptomyces albulus* (*1*, *2*). The biogenic EPL usually has 25−35 lysine monomers. The polydispersity is dependent on specific bacteria strains and culture conditions (*3*).

Because of its primary amine groups, EPL is cationic in neutral aqueous solutions. Like chitosan, another cationic biopolymer, EPL also has antimicrobial activities. It has been shown that EPL is able to be absorbed onto the bacterial surface, causing stripping of the outer membrane, abnormal distribution of the cytoplasm, and finally death of the bacteria (*4*).

The antimicrobial spectrum of EPL is wide. EPL is able to inhibit the growth of both Gram-positive (such as *Listeria monocytogenes*) and Gram-negative bacteria (such as *Escherichia coli*). The minimum inhibitory concentrations of EPL against common food pathogens are in the range of 5−100 μg/mL (*4*, *5*). Based on absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies, EPL has been proven to be safe for consumption (*6*, *7*). It has been approved as a natural food preservative used in rice and in traditional Japanese food in Japan and the U.S.A. (GRAS No. 000135) (*8*).

EPL could also be used as a dietary agent. It has been shown that EPL is able to inhibit the enzymatic activity of pancreatic lipase in vitro (*9*). It has also been shown that oral consumption of EPL lowered the triglyceride level in the blood plasma of rats, which further suggested that EPL could function to suppress dietary fat intake (*9*).

Chemical modification is an effective way to impart novel properties and functions to existing materials. Hydrophobically modified starch, for instance, was synthesized from waxy maize and octenyl succinic anhydride (OSA) (*10*). The modified starch has a hydrophobic domain and thus is able to stabilize emulsions and has been widely used as wall material in flavor and bioactive encapsulation (*11*−*13*).

EPL has also been modified chemically in previous studies. Shima et al. found that conjugation of derivatives of benzoic acids to EPL caused a significant decrease in its antimicrobial activity (*4*). EPL was also modified by conjugating with glucose and dextran through the Maillard reaction (*14*, *15*). It was found that the antimicrobial activity of EPL was almost retained. Furthermore, the dextran−EPL conjugate was able to stabilize emulsions. The emulsifying capacity of synthesized conjugates was not affected by high ionic strength but was sensitive to extreme acidic conditions (*15*). The modified EPL was hydrophilic instead of amphiphilic, and the mechanism of stabilizing emulsions was thought to be the increase of viscosity caused by the high-molecular-weight dextran−EPL conjugate.

In addition to using thickeners to increase viscosity, surfactants or emulsifiers are also commonly used to stabilize emulsions. The aim of this study is to synthesize and characterize hydrophobically modified EPL, which is expected to be a surface-active amphiphile and to be able to be used as an emulsifier. At the same time, the modified EPL is also expected to retain the antimicrobial activity of EPL. This study indeed demonstrated that hydrophobically modified EPLs are bifunctional molecules: they are amphiphilic and also antimicrobial.

*To whom correspondence should be addressed. Tel: 732-932-7193. Fax: 732-932-6776. E-mail: qhuang@aesop.rutgers.edu.

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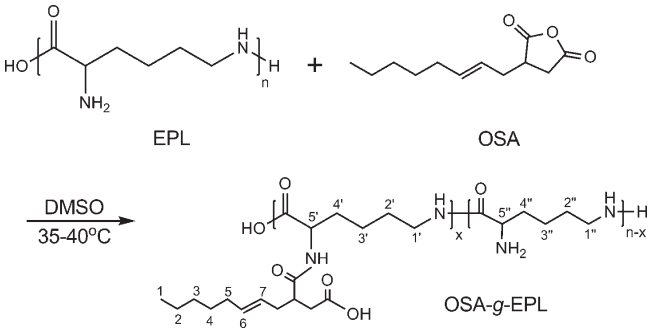
Scheme 1. Synthetic scheme of hydrophobically modified ε-polylysine (EPL) or OSA-*g*-EPLs



EXPERIMENTAL SECTION

Materials. ε-Polylyine (EPL) was purchased from Zhejiang Silver-Elephant Bioengineering Co., Ltd., China, and used without further purification, unless noted specifically. Dimethyl sulfoxide (DMSO), octenyl succinic anhydride (OSA), deuterium oxide (D_2O), sodium bicarbonate ($NaHCO_3$), methanol, and agar were purchased from Sigma-Aldrich (St. Louis, MO). Pyrene was obtained from Alfa Aesar (Ward Hill, MA). Dialysis membranes (MWCO 1000 Da) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). *E. coli* O157:H7 (ATCC 43888) was purchased from MicroBioLogics, Inc. (Saint Cloud, MN). Nutrient broth and 96-well microtiter plates were obtained from BD Biosciences (San Jose, CA).

Synthesis of Hydrophobically Modified EPL. EPL (1.5 g) was dissolved in 30 mL of DMSO, and different concentrations of OSA were added dropwise under stirring at 35−40 °C. After 18 h, the whole reaction was dialyzed in 1000 Da molecular weight cutoff (MWCO) dialysis membrane against 95% EtOH, 0.01% $NaHCO_3$, and deionized water sequentially, and then lyophilized with a Freezone4.5 freeze−dry system (Labconco, Kansas City, MO) to obtain the final products.

Infrared and ^{1}H NMR Spectroscopy. The Fourier transform infrared (FT-IR) spectra of original EPL and modified EPL were measured using Thermo Nicolet Nexus 670 FT-IR system with a Smart MIRacle horizontal attenuated total reflectance (ATR) accessory (Thermo Electron Corp., Madison, WI). Each spectrum was averaged over 512 scans with 4 cm^{-1} resolution.

^{1}H NMR spectroscopy was taken on a Varian 500 MHz NMR spectrometer (Palo Alto, CA). All of the samples were dissolved in D_2O.

Determination of the Degree of Substitution. The degree of substitution (DS) was defined as the percentage of the reacted α-amine groups on the lysine monomer in the EPL molecule. It was calculated as the peak area at 4.0 ppm divided by the total peak areas at 3.82 ppm and 4.0 ppm from the ^{1}H NMR spectra.

$$\mathrm{DS} = \frac{A_{\delta 4.0}}{A_{\delta 3.82} + A_{\delta 4.0}} \times 100\% \quad (1)$$

Determination of the Critical Aggregation Concentration (CAC). The critical aggregation concentrations (CACs) of modified EPL were determined by measuring the fluorescence excitation spectra of pyrene in modified EPL solutions (*16*−*19*). Modified EPL was dissolved in deionized water at different concentrations (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/mL). Pyrene stock solution (1.0 mM) was prepared in methanol. Four microliters of this solution was added to glass tubes, and methanol was removed by purging with nitrogen. Subsequently, 4.0 mL of modified EPL solutions of different concentrations was transferred to each tube. All tubes were sonicated for 30 min to facilitate pyrene dissolution. Subsequently, the fluorescence excitation spectra of pyrene from 300 to 350 nm were obtained using Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA). The emission wavelength was set at 390 nm, and the excitation and emission slit openings were both set at 5 nm. The ratio of the intensity at 337 nm (I_{337}) to that at 334 nm (I_{334}) was calculated and plotted against the common logarithm of the concentrations of modified EPL. A major change in the slope indicated the onset of micelle formation, and the corresponding concentrations of modified EPLs were determined as the CACs of modified EPLs.

Dynamic Light Scattering (DLS). The mean hydrodynamic diameters and the corresponding polydispersity of the polymer micelles were determined using DLS-based BIC 90plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument, New York, NY) at a fixed scattering angle of 90° at 25 °C. The light source of the particle size analyzer is a solid state laser operating at 658 nm with 30 mW power, and the signals were detected by a high-sensitivity avalanche photodiode detector. The normalized field−field autocorrelation function $g(q,t)$ is obtained from the intensity−intensity autocorrelation function, $G(q,t)$, via the Sigert relation:

$$\alpha g(q,t) = [G(q,t)/A - 1]^{1/2} \quad (2)$$

where A is the experimentally determined baseline and α is the contrast factor, which is less than 1, because only a fraction of dynamic scattering intensity falls within the correlator window and also because a finite size pinhole is used in the experiment. For all micelle size measurements, the measured baseline A is in agreement with the theoretically calculated baseline to 0.01%.

The diffusion coefficient D was calculated according to $D = \tau^{-1}q^{-2}$, where q is the amplitude of the scattering vector defined as $q = (4\pi n/\lambda)\sin(\theta/2)$, n is the solution refractive index, λ is the laser wavelength, and θ is the scattering angle. The diffusion coefficient D can be converted into mean emulsion droplet diameter d using the Stokes−Einstein equation:

$$d = \frac{kT}{3\pi\eta D} \quad (3)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity.

Cumulant analysis method was used in our size measurements, where $g(q,t)$ was decomposed into a distribution of decay rate Γ ($= 1/\tau$) given by

$$g(q,t) = \int G(\Gamma)\, e^{-\Gamma t}\, d\Gamma \quad (4)$$

The first two moments of the distribution $G(\Gamma)$ are as follows:

$$\Gamma = Dq^2 \quad (5)$$

$$\mu_2 = (D^2 - D*^2)q^4 \quad (6)$$

where D* is the average diffusion coefficient. The polydispersity term defined in the cumulant analysis is

$$\text{polydispersity} = \mu_2/\Gamma^2 \quad (7)$$

Here polydispersity has no unit. It is close to zero for monodisperse or nearly monodisperse samples and larger for broader distributions.

Surface Tension Measurement. Modified EPL solutions (5 mg/mL) were prepared in deionized water. The surface tension was measured using the pendant drop method with VCA optima surface analysis system (AST Products, Billerica, MA). The surface tension values were expressed as mean ± standard deviation, with 16 repeats.

Differential Scanning Calorimetry (DSC). The thermal properties of modified EPLs were analyzed with a DSC823e thermal analyzer (Mettler Toledo, Columbus, OH). Modified EPL samples (5.5−6.0 mg) were sealed in aluminum crucibles, and the lid of each crucible was penetrated to form a small hole. The samples were first heated from 25 to 200 °C with a heating rate of 10 °C/min, subsequently cooled to −120 °C with a cooling rate of −30 °C/min, and finally heated back to 210 °C with a heating rate of 10 °C/min. Glass transition temperatures (T_g) were determined by the STARe software associated with the thermal analyzer. Before DSC analysis, EPL was first dialyzed against deionized water using a dialysis membrane with molecular weight cutoff (MWCO) of 1000 Da and then freeze-dried.

Determination of the Minimum Inhibitory Concentration against *E. coli* O157:H7. Mininum inhibitory concentrations (MIC) of different modified EPLs were determined by the microtiter broth dilution method (*20*). Briefly, 10^5 CFU/mL of *E. coli* O157:H7 was cultured in nutrient broth with EPL or modified EPLs of different concentrations (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, and 0.391 μg/mL). Media inoculated with

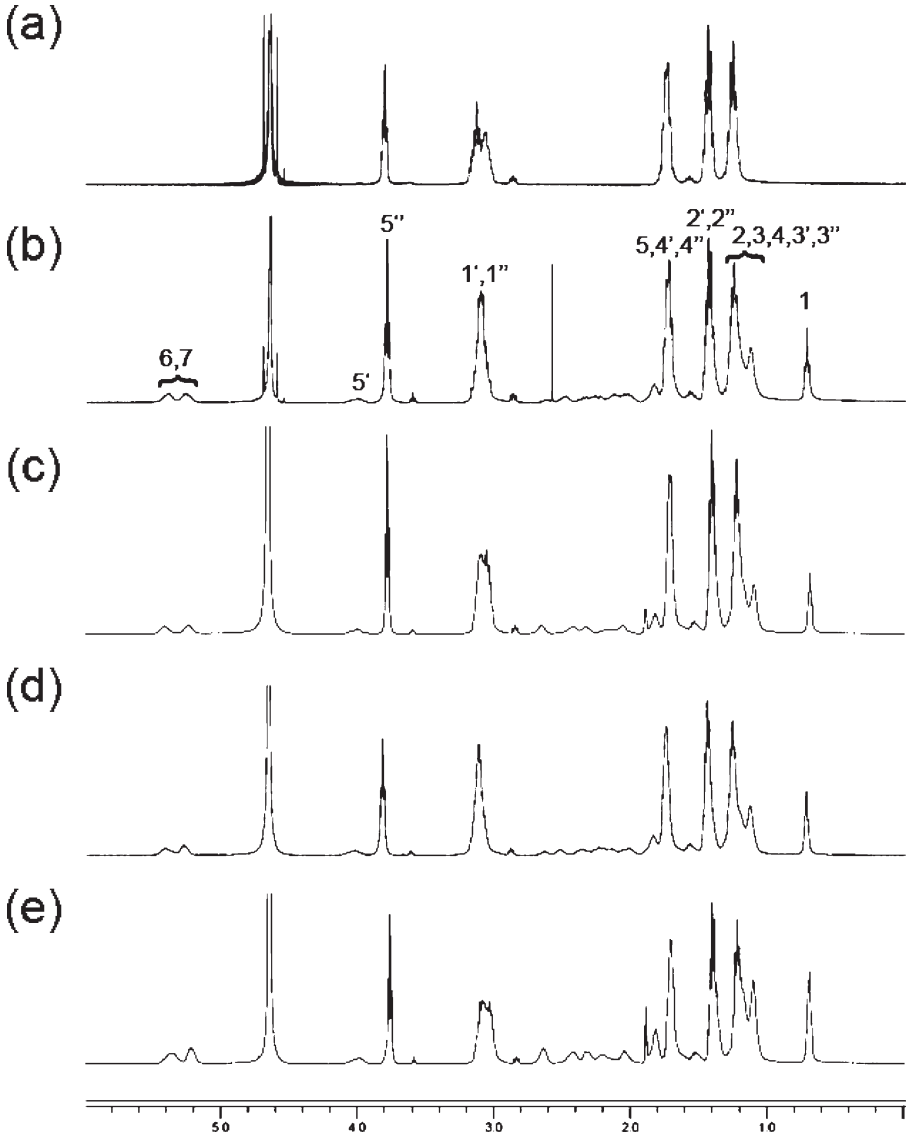


Figure 1. ^{1}H NMR spectra of (a) EPL, (b) OSA-*g*-EPL6.2, (c) OSA-*g*-EPL8.5, (d) OSA-*g*-EPL12.4, and (e) OSA-*g*-EPL20.5.

E. coli O157:H7 but without antimicrobial compounds served as positive controls. Medium alone was taken as negative control. After the bacteria were cultured for 24 h at 37 °C, optical absorbance at 600 nm (OD_{600}) of each well was recorded using an absorbance microplate reader (Molecular Devices). The MIC of each tested compound was defined as the lowest concentration of the compound at which bacteria had no optically prominent growth.

RESULTS AND DISCUSSION

Synthesis of Modified ε-Polylysine. To hydrophobically modify a biopolymer containing primary amine or hydroxyl groups, fatty acid with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), acid chlorides, and acid anhydrides are often used. In this study, we chose octenyl succinic anhydride (OSA) because of its long history of use in the food industry, especially in synthesizing hydrophobically modified starch (*10*, *21*−*23*). ε-Polylysine (EPL) and OSA were reacted in DMSO through nucleophilic reaction (**Scheme 1**). The products were then dialyzed to remove unreacted OSA (and its possible hydrolysates) and lyophilized to obtain the products denoted as OSA-*g*-EPLs.

The success of reaction was confirmed by ^{1}H NMR spectroscopy. As shown in **Figure 1**, the peaks specific to EPL (**Figure 1a**.) appeared between 1.2 and 4.0 ppm. Peaks at about 3.82 and 3.12 ppm corresponded to terminal methine and methylene groups, respectively. In the products (OSA-*g*-EPLs, **Figure 1b**−**d**.), peak assignments were as follows: δ 0.74 (CH_3), 1.27 [CH_2 (β, γ, δ) of OSA and CH_2 (γ) of EPL], 1.46 [CH_2 (β) of EPL], 1.76 [(CH_2 (α) of OSA and CH_2 (δ) of EPL], 3.12 [CH_2 (α) of EPL], 3.82 [unreacted methine of EPL], 4.0 [reacted methine of EPL], 5.2 and 5.4 [H on CH═CH of OSA].

The peak of the methine group of EPL shifted partially from 3.82 to 4.0 ppm when acylation reaction occurred in the amine groups. This shift indicated the presence of major functional groups linked to EPL.

The degree of substitution (DS) of EPL can be calculated as the area ratio of the reacted methine proton (δ 4.0) to the total methine proton (δ 3.82 and δ 4.0), or proton on the double bond (δ 5.2 and 5.4) to the total methine proton (δ 3.82 and δ 4.0). In this study, the former method was used. The DS of OSA-*g*-EPLs at different feed ratios of OSA to EPL are listed in **Table 1**. One notices that the DS of OSA-*g*-EPLs increased with the increase of feed ratio. Once the DS values were determined, the reaction products were further denoted as OSA-*g*-EPL*n*, with *n* indicating the DS value. For example, in OSA-*g*-EPL6.2, 6.2% of amine groups in EPL were reacted with OSA.

Table 1. Summary of Degree of Substitution (DS), Glass Transition Temperatures (T_g), Critical Aggregation Concentrations (CAC), Surface Tension, and Minimum Inhibitory Concentrations (MIC) of OSA-*g*-EPLs

feed ratio	DS (%)	T_g (°C)	CAC (mg/mL)	surface tension (mN/m)	MIC (μg/mL)
10:0	0	133.1	a	72.3 ± 1.0	12.5
10:1	6.2	126.1	1.76	40.6 ± 2.1	12.5
10:3	8.5	94.0	0.64	44.8 ± 2.8	12.5
10:6	12.4	78.9	0.32	35.6 ± 1.8	12.5
10:10	20.5	60.6	0.18	39.5 ± 2.2	12.5

[a] Not detected.

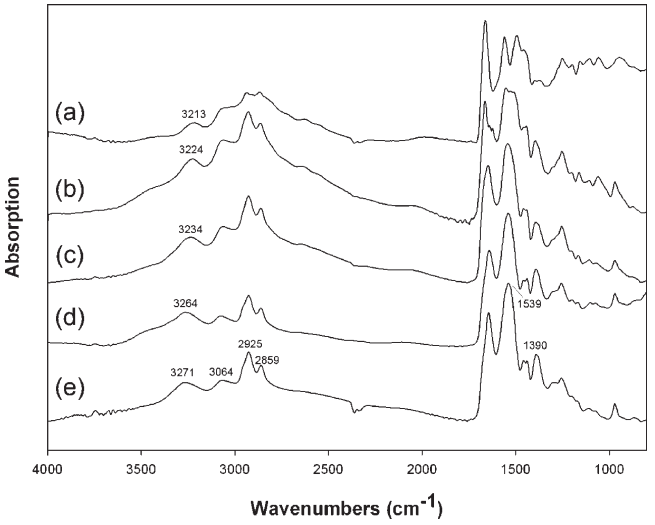


Figure 2. ATR FT-IR spectra of (a) EPL, (b) OSA-*g*-EPL6.2, (c) OSA-*g*-EPL8.5, (d) OSA-*g*-EPL12.4, and (e) OSA-*g*-EPL20.5. Numbers on the spectra indicate the wavenumbers of the corresponding peak positions.

To further confirm the synthesis, infrared spectra of OSA-*g*-EPLs were collected and compared with that of EPL, as shown in **Figure 2**. The N−H stretching bands were located between 3300−3200 cm^{-1}. With the increase of DS, the position of this band shifted to larger wavenumbers, that is, from 3213 to 3224, 3234, 3263, and 3271 cm^{-1}, suggesting the existence of hydrogen bonding between the N−H group of EPL moiety and the increasing number of carbonyl (C═O) groups from OSA moieties on the products. The weak band at approximate 3070−3060 cm^{-1} corresponded to the overtone of the N−H stretch band (amide II band) at 1560−1530 cm^{-1}. The intensity of this band was stronger for OSA-*g*-EPLs than for EPL. The two bands at 2925 and 2859 cm^{-1} were designated as C−H stretching. With hydrophobic substitution, the two bands were naturally more prominent. The band at 1539 cm^{-1} in the spectrum of EPL corresponded to the amide II band originating from N−H bending. After conjugation, it was intensified as anticipated. The band at 1390 cm^{-1} was designated as methyl C−H bending and was increasingly prominent with the increase of DS.

The results of ^{1}H NMR and ATR-FTIR spectroscopies both confirmed that OSA-*g*-EPLs were successfully synthesized by conjugating OSA onto EPL. Subsequently, thermal properties and self-assembly behavior of OSA-*g*-EPLs were analyzed and compared with those of original EPL.

Thermal Properties of OSA-g-EPLs. The thermal properties of modified EPLs were analyzed by differential scanning calorimetry (DSC). For comparison, a dialyzed EPL sample was also analyzed. From **Figure 3**, it was shown that each of the five samples gave a single glass transition temperature (T_g), and with

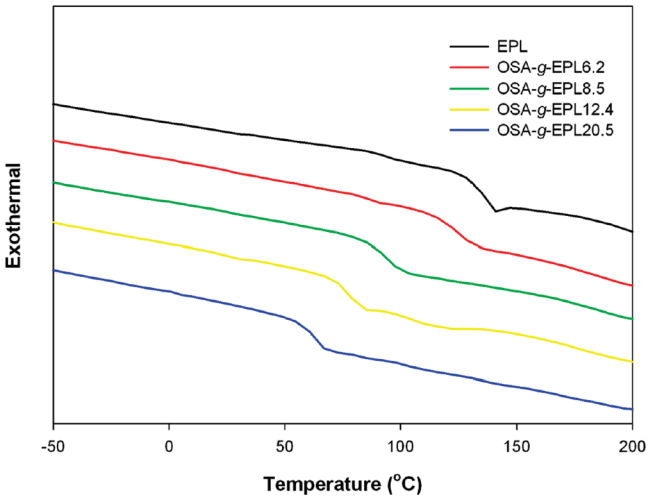


Figure 3. Differential scanning calorimetry analysis of EPL and OSA-*g*-EPLs. Only the curves from the second heating procedure are shown.

the increase of DS, T_g decreased from 133.1 °C for EPL to 60.6 °C for OSA-*g*-EPL20.5 (**Figure 3** and **Table 1**). The decrease of T_g was due to the plasticizing effect of OSA moieties, since OSA has a much lower melting point and glass transition temperature (*24*). It was noteworthy that the dialyzed EPL only showed a single T_g at 133.1 °C, which was different from what was reported (T_g at 88 °C and T_m at 172.8 °C) (*1*). This may be due to the different chemical constitutions and purification procedures from different manufacture sources. Meanwhile, it was also noted that before dialysis, the EPL sample has two T_g's at 92.6 and 165.1 °C (not shown), suggesting that the salt contents and small-molecular-weight lysine oligomers may interfere with the DSC results.

Self-Assembly of OSA-g-EPLs in Water. It is self-evident that OSA-*g*-EPLs were amphiphilic and would aggregate and form polymer micelles in aqueous solutions above their critical aggregation concentrations (CACs). Considering that polymer micelles have been well applied in delivery systems (*25*, *26*), it is important to investigate whether and above what concentration the OSA-*g*-EPLs are able to form polymer micelles. Therefore, the CACs of OSA-*g*-EPLs were determined with the pyrene fluorescence excitation spectra method (*18*, *25*). For instance, the excitation spectra of pyrene in OSA-*g*-EPL12.4 water solution are shown in **Figure 4A**. At low polymer concentration, one peak in the spectrum was at 334 nm. With increase of the OSA-*g*-EPL12.4 concentration, pyrene migrated into the hydrophobic microdomains formed by the polymer aggregate, and the peak shifted gradually to 337 nm. By calculating the ratio of the fluorescence intensity at 337 to that at 334 (I_{337}/I_{334}), one can determine the critical aggregation concentration. As shown in **Figure 4B**, 0.32 mg/mL was the onset concentration where I_{337}/I_{334} started to increase abruptly and thus was the CAC of OSA-*g*-EPL12.4 in deionized water. As shown in **Table 1**, all of the OSA-*g*-EPLs molecules were able to form polymer micelles in water. The increase of the OSA substitution caused a decrease of the CAC value, with the lowest CAC being 0.18 mg/mL for OSA-*g*-EPL20.5.

The particle size of the OSA-*g*-EPLs was determined using dynamic light scattering at the concentration of 5 mg/mL [above the CMCs of all the OSA-*g*-EPLs]. It was found that the hydrodynamic diameters of the polymer micelles were in the range of 40−110 nm (**Table 2**). The corresponding hydrodynamic radii of hydration were from 20 to 55 nm. The smaller particle size of OSA-*g*-EPL20.5 may be due to the compact packing of the hydrophobic core of the polymer micelle.

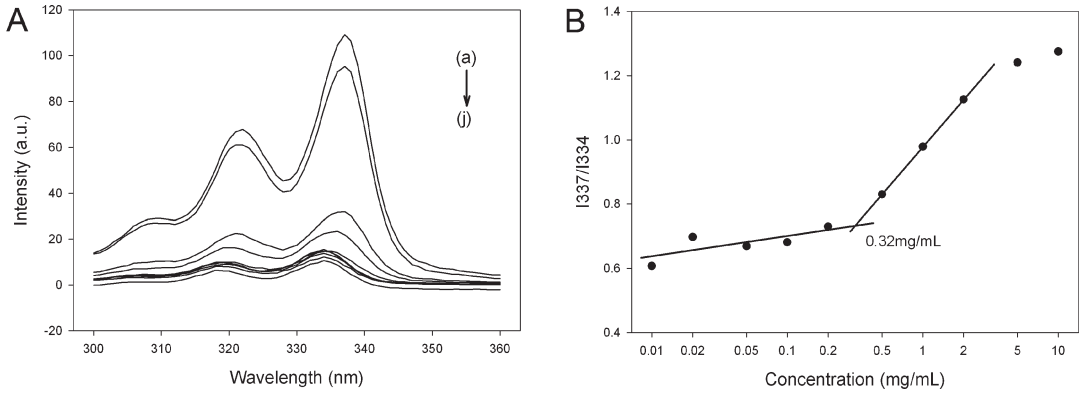


Figure 4. Determination of the critical aggregation concentrations (CACs) of OSA-*g*-EPL12.4: (**A**) Pyrene fluorescence excitation spectra of OSA-*g*-EPL12.4 solutions of different polymer concentrations (a−j) 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 mg/mL, respectively; (**B**) plot of the ratios I_{337}/I_{334} against common logarithm of concentrations of OSA-*g*-EPL12.4. The change in slope corresponded to the critical aggregation concentration (CAC) of OSA-*g*-EPL12.4.

Table 2. Summary of the Effective Hydrodynamic Diameters and Polydispersities of OSA-*g*-EPLs

samples	effective diameter (nm)	polydispersity
OSA-*g*-EPL6.2	42.0 ± 2.6	0.359
OSA-*g*-EPL8.5	110 ± 2.0	0.080
OSA-*g*-EPL12.4	102.2 ± 0.1	0.089
OSA-*g*-EPL20.5	72.2 ± 4.2	0.263

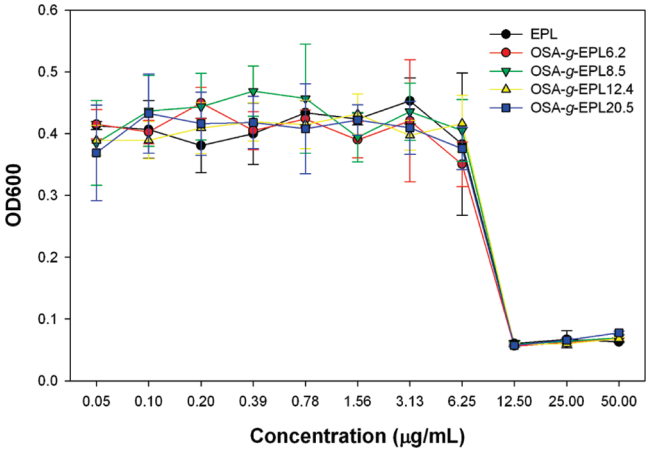


Figure 5. Optical absorption at 600 nm (OD_{600}) of *E. coli* O157:H7 versus the concentrations of EPL, OSA-*g*-EPL6.2, OSA-*g*-EPL8.5, OSA-*g*-EPL12.4, and OSA-*g*-EPL20.5. The minimum inhibitory concentrations of these polymers against *E. coli* O157:H7 were all found to be 12.5 μg/mL.

Interfacial Properties of OSA-*g*-EPLs. As amphiphiles, OSA-*g*-EPLs could also form adsorption layers on the air/water interface and lower the interfacial tension. The air/water surface tension of the OSA-*g*-EPLs at the concentration of 5 mg/mL [above CACs of all the OSA-*g*-EPLs] was measured with pendant drop method and was in the range of 35−40 mN/m, while the surface tension of original EPL remained at a similar level to pure water (**Table 1**), which was consistent with a previous report (*15*). This result further confirmed the amphiphilicity of synthesized OSA-*g*-EPLs and suggested that OSA-*g*-EPLs could be used as emulsifiers in emulsion formulation.

Antimicrobial Activity of OSA-*g*-EPLs. A high antimicrobial activity is essential for a bifunctional emulsifier. However, previous studies suggested that chemical modification to the α-amine groups of EPL would lower its antimicrobial activity (*4*, *15*). Therefore, to examine the antimicrobial activities of OSA-*g*-EPLs, the minimum inhibitory concentrations (MICs) of OSA-*g*-EPLs against *E. coli* O157:H7 were determined and compared with that of unmodified EPL. Interestingly, the MICs of all four OSA-*g*-EPLs remained the same as that of EPL, being 12.5 μg/mL (**Figure 5**). This result suggested that OSA-*g*-EPLs retained the antimicrobial activity of EPL.

CONCLUSION

In summary, OSA-*g*-EPLs with different degrees of OSA substitution were successfully synthesized by conjugating OSA onto the amino groups of EPL molecules, confirmed by ^{1}H NMR and ATR-FTIR spectroscopy. Furthermore, they were demonstrated as bifunctional materials: on one hand, they were amphiphiles and able to self-assemble to form micelles and lower the surface tension of water; on the other hand, they retained the antimicrobial activity of EPL. Our results suggested that OSA−EPLs were novel functional ingredients that could be used as antimicrobial emulsifiers in the food industry.

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