Bioactive Constituents, Metabolites, and Functions

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Development of Gemini Interfacial Antioxidant for O/W Emulsion with Gallic Acid and Dodecyl Gemini Chain

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ABSTRACT: In this study, development of the gemini gallate interfacial antioxidant for O/W emulsion was performed employing Steglich esterification with gallic acid and dodecyl gemini chain through a prepacked column and peristaltic pump based purification system. The structural identity and purity of the prepared gemini and mono gallate were confirmed by NMR, FT-IR, and HPLC-MS. Further evaluation revealed that the gemini gallate possessed excellent radical scavenging activity and superior antioxidant activity in O/W emulsion relative to the mono gallate especially under the condition of reduced amount of emulsifier. Microscopic investigation by fluorescent probe and transmission electron microscopy (TEM) unveiled that the extraordinary antioxidant performance in O/W emulsion was presumably attributed to preferable interfacial location due to the unique gemini molecular architecture. The superior antioxidant activity of the gemini antioxidant hold a great promise for antioxidation in O/W emulsion.

KEYWORDS: gallic acid, gemini interfacial antioxidant, gemini antioxidant, antioxidant activity, O/W emulsion, Steglich esterification

INTRODUCTION

In recent years, functional lipids such as ω-3 fatty acid[1,2], vitamin D[3,4] and carotenoids[5,6] have received much attention for their health beneficial effects. However, the functional lipids are highly sensitive to oxidation during processing and storage especially when they are prepared as O/W emulsion due to increased exposure to oxidation.[7] As a result, great efforts have been made to develop good antioxidants for O/W emulsion. It is revealed that the oil-water interface plays an important role in oxidation where the oxidation reaction takes place and propagates, and the interfacial antioxidants which prefer to stay at the interface have superior activities in O/W emulsion.[8] A variety of examples of interfacial antioxidants have been developed including emulsifier-antioxidant bioconjugates, grafted ovalbumin-catechin conjugates and the conjugates of epigallocatechin and phytosterols which demonstrated excellent antioxidant performance in O/W emulsion.[9-11] The exceptional antioxidant performance of interfacial antioxidants in O/W emulsion has been attributed to their amphiphilic nature leading to increased effective interfacial concentration of antioxidants and so more efficient inhibition of lipid oxidation.[12-15]

It is well documented that a new class of amphiphilic molecules, gemini surfactants, containing two hydrophilic groups and two hydrophobic chains, are substantially more active relative to the conventional monomeric compounds.[16,17] Gemini's meaning in astronomy is the gemini constellation, which vividly expresses the molecular structure characteristics of such surfactants as conjoined twin babies. The unique molecular architectures of gemini surfactants result in their remarkable

activities that hold great promise in food industry, material and biological applications as emulsifiers, gene and drug delivery agents.[18-20] In previous work, our laboratory developed a novel type of highly effective nonionic gemini alkyl *O*-glucoside surfactants with intriguing self-assembly behavior.[21] Gallic acid is abundant natural antioxidant and its derivatives have been proven excellent antioxidants in lipophilic settings.[22-25] Therefore, we reasoned that incorporation of gallic acid in the architecture of gemini alkyl chain would facilitate location of gallic acid at interfacial layer and improve antioxidant performance in O/W emulsion accordingly. The concept of gemini interfacial antioxidant would be useful for design of antioxidants in complicated settings.

In this work, development of gemini interfacial antioxidant for O/W emulsion was exercised with gallic acid and dodecyl gemini chain. The gemini gallate was designed and successfully prepared. A prepacked column and pump system was built for purification. Then DPPH radical scavenging activity, oxygen radical absorbance capacity (ORAC), and the antioxidant performance in O/W emulsion were evaluated. Superior antioxidant activity of the gemini antioxidant in O/W emulsion was observed relative to the corresponding mono antioxidant. The microscopic structure of the emulsion was further investigated by fluorescent probe and transmission electron microscopy (TEM). The details of this work were disclosed as follows.

MATERIALS AND METHODS

Chemicals. Potassium hydroxide, hydrochloric acid (HCl), sodium dihydrogen

phosphate dihydrate, sodium phosphate dibasic dodecahydrate, anhydrous magnesium sulfate ($MgSO_4$), ethyl acetate, petroleum ether, toluene, and ethanol were purchased from Sinopharm Chemical Reagent (Shanghai, China). Ethylene glycol diglycidyl ether was obtained from Energy Chemical Reagent (Shanghai, China). Pyrene was obtained from Macklin Reagent (Shanghai, China). Gallic acid (GA), isobutyric anhydride, triethylamine, *N,N*-dimethylformamide (DMF), 4-dimethyaminopyridine (DMAP), dodecanol, *N,N*-dicyclohexylcarbodiimide (DCC), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), hydrazinium hydrate solution (80%), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone (TBHQ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5-aminofluorescein, dodecanoyl chloride, 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), ethyl butyrate, and Tween-80 were purchased from Aladdin Reagent (Shanghai, China). All chemicals were of analytical grade.

Preparation of Mono Gallate (MG) and Gemini Gallate (GG). *Preparation of Triisobutyryl Gallic Acid.* Triisobutyryl gallic acid was prepared according to the previously reported method with minor modifications.[25] Briefly, to gallic acid (6 mmol, 1020.7 mg) and DMAP (0.6 mmol, 73.3 mg) in DMF (3 mL) was added sequentially isobutyric anhydride (27 mmol, 4.477 mL), triethylamine (27 mmol, 3.763 mL). The reaction mixture was stirred at ambient temperature for 2 h and quenched by HCl (1 *N*), followed by aqueous washing to afford the pure triisobutyryl gallic acid as a white solid.

Preparation of the Mono Gallate. To a solution of triisobutyryl gallic acid (0.838

104 mmol, 318.7 mg), dodecanol (0.559 mmol, 127.1 μL), DMAP (0.056 mmol, 6.8 mg)
105 in toluene (3 mL) was added a solution of DCC (0.838 mmol, 172.9 mg in 1 mL
106 toluene). The reaction mixture was stirred at ambient temperature for 3 h. Then
107 ethanol (95%, 4 mL) was added, followed by addition of hydrazinium hydrate
108 solution (80%, 203 μL). The reaction mixture was stirred further at ambient
109 temperature for 1.5 h before quenching with HCl (1 *N*). After extracted with ethyl
110 acetate, the organic phase was dried over $MgSO_4$ and evaporated under reduced
111 pressure. Purification by a silica gel chromatography with petroleum ether/ethyl
112 acetate (2/1 to 1/1, v/v) afforded the mono gallate as a white solid (182.5 mg, 64.5%).
113 *Preparation of the Gemini Gallate.* Dodecanol and ethylene glycol diglycidyl ether
114 were applied to prepare the dodecyl gemini chain based on the previous method with
115 improvement of purification protocol.[21] To a solution of ethylene glycol diglycidyl
116 ether (10 mmol, 1558 μL) and dodecanol (30 mmol, 6816 μL) in DMSO (5 mL) was
117 added KOH (20 mmol, 1120 mg). The reaction mixture was stirred at 60 ^{o}C for 6 h
118 and then extracted with ethyl acetate and washed by water. The organic phase was
119 dried over $MgSO_4$ and evaporated under reduced pressure. The purification was
120 performed on a prepacked column system equipped with a prepacked silica gel
121 column (SepaFlash 120 g, Lisure Science Inc., Suzhou, China) and two peristaltic
122 pumps (RZ1030, Runze Fluid Inc., Nanjing, China) pumping petroleum ether (13.2
123 mL/min) and ethyl acetate (4.4 mL/min) respectively, and produced the dodecyl
124 gemini chain as a yellow oil (360 mg).
125 Then the dodecyl gemini chain and triisobutyryl gallic acid were employed to

prepare the gemini gallate. To a solution of triisobutyryl gallic acid (1.98 mmol, 753.2 mg), dodecyl gemini chain (0.66 mmol, 360.0 mg), DMAP (0.066 mmol, 8.1 mg) in toluene (3.6 mL) was added a solution of DCC (1.98 mmol, 408.5 mg in 0.8 mL toluene). The reaction mixture was stirred at ambient temperature for 4 h. After extracted with ethyl acetate and washed by water, the organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. Purification by a prepacked silica gel column (SepaFlash 25 g, Lisure Science Inc., Suzhou, China) with two peristaltic pumps (RZ1030, Runze Fluid Inc., Nanjing, China) pumping petroleum ether (16.5 mL/min) and ethyl acetate (3 mL/min) respectively, and afforded the product as a yellow oil (263 mg). After that, the product was dissolved in ethanol (95%, 3 mL), followed by addition of hydrazinium hydrate solution (80%, 121 μL). The reaction mixture was stirred further at ambient temperature for 1.5 h before quenching with HCl (1 *N*). After extracted with ethyl acetate, the organic phase was dried over $MgSO_4$ and evaporated under reduced pressure and produced the gemini gallate as a colorless oil (130 mg).

Structural Determination. The ^{1}H and ^{13}C NMR spectra of the prepared mono gallate and gemini gallate were carried out on a 400 MHz NMR spectrometer (Bruker Corporation, Fällanden, Zürich, Switzerland) at room temperature applying DMSO-d_6 (^{1}H, 2.50 ppm; ^{13}C, 39.52 ppm) as solvent.[26] FT-IR spectra were performed on an AVA TAR370 spectrophotometer (Thermo Nicolet Corporation, Madison, WI, USA) by attenuated total reflectance method within 400-4000 cm^{-1}. Mass spectra were collected on a Thermo Finnigan LCQ Deca XP Max system (Thermo Fisher

148 Scientific, Waltham, MA, USA) with application of negative ion electron spray
149 ionization (ESI) mode within 300−2000 (m/z).

150 **HPLC-MS Analysis.** The HPLC-MS analysis was performed by an Agilent 1200
151 system (Agilent, Santa Clara, CA, USA) employing the Agilent SB-C18 (2.1×150
152 mm, 3.5 μm) column. The mobile phase A was formic acid solution (0.1%, v/v) and B
153 was methanol. The flow rate was 0.5 mL/min. 5 μL of sample was injected and
154 detected at 277 nm. The gradient was starting from 80% B to 100% within 20 min,
155 followed by 30 min with 100% B. Mass spectra were collected on a Thermo Finnigan
156 LCQ Deca XP Max system (Thermo Fisher Scientific, Waltham, MA, USA) with
157 application of negative ion electron spray ionization (ESI) mode within 300−2000
158 (m/z).

159 **Antioxidant Activity Evaluated by the DPPH Scavenging Activity Assay.** The
160 method described by Wang et al. was applied to evaluate the DPPH radical
161 scavenging activity with slight modification.[11] Briefly, 0.1 mL of the antioxidant
162 ethanol solution of specified concentration was added to 3.9 mL of DPPH ethanol
163 solution (0.025 g/L). The mixture was placed in the dark for 30 min at ambient
164 temperature. Then the absorbance was measured at 515 nm by a UV−vis
165 spectrophotometer (SP-756, Shanghai Spectrum Corporation, Shanghai, China). The
166 assay was carried out in triplicate. The DPPH scavenging activity ($DPPH_{SCA}$ %) was
167 calculated as follows.

168 $$DPPH_{SCA}\% = \left(1 - \frac{As - Ab}{Ac}\right) \times 100\%$$

169 where A_s represents the absorbance of sample mixture (addition of 0.1 mL of

antioxidant ethanol solution to 3.9 mL of DPPH ethanol solution)，A_b the absorbance of the blank mixture (addition of 0.1 mL of antioxidant ethanol solution to 3.9 mL ethanol), and A_c the absorbance of the control mixture (addition of 0.1 mL ethanol to 3.9 mL of DPPH ethanol solution).

Antioxidant Activity Evaluated by the Oxygen Radical Absorbance Capacity (ORAC) Assay. *Preparation of Dodecanoyl 5-Aminofluorescein (DAF).* According to Chaiyasit et al.[27], 5-aminofluorescein (AF) was applied to synthesize fat-soluble fluorescent probe DAF. Briefly, to a solution of AF (0.58 mmol, 200 mg), dodecanoyl chloride (1.16 mmol, 266.6 μL) in DMF (10 mL) was added triethylamine (1.73 mmol, 239.2 μL) dropwise. The reaction mixture was stirred at ambient temperature for 2.5 h, and then extracted with ethyl acetate and washed by HCl (1 *N*). Purification by a silica gel chromatography with petroleum ether/ethyl acetate (2/1 to 1.5/1, v/v) produced DAF as a yellow oil (30 mg).

ORAC Assay. 1.25 mL of DAF PBS solution (pH 7.4, 0.1 μmol/L) was added to 2.5 mL of antioxidant solution of specified concentration with 1 μL of Tween-80, then treated by ultrasonication for 5 min. The microplate containing 150 μL of the prepared solution was incubated at 37 °C for 15 min. Then 50 μL of AAPH ethanol solution (640 mmol/L) was added to the prepared solution and analyzed every 5 min for 150 min with an excitation wavelength of 485 nm and an emission wavelength of 495 nm by a microplate reader (SpectraMax M5/M5e, American molecular instruments, America). The temperature was maintained at 37°C. The assay was carried out in triplicate. The fluorescence decay area under the curve (AUC), NET

AUC, and ORAC value were calculated as follows.

$$\mathrm{AUC} = 0.5 \times [2 \times (f_0 + f_1 + \ldots + f_{n-1} + f_n) - f_0 - \mathrm{fn}] \times \Delta\mathrm{t}$$

$$\mathrm{NET\ AUC} = AUC_s - AUC_b$$

$$\mathrm{ORAC} = \frac{NET\ AUC_s}{NET\ AUC_{Trolox}} \times \frac{C_{Trolox}}{C_s}$$

Where f_n represents the relative fluorescence intensity at the nth measure point, Δt the time interval, AUC_s the sample's or Trolox's AUC, AUC_b the control's AUC, NET AUC_s the sample's NET AUC, NET AUC_{Trolox} the Trolox's AUC, C_{Trolox} the Trolox's concentration, C_s the sample's concentration.

β-Carotene AAPH Bleaching Assay in O/W Emulsion. The protocol was improved on the basis of the previous study.[11] To a round-bottom flask containing 2 mL of β-carotene ethyl butyrate solution (0.2 mg/mL) were added 3.35 or 2.6 mL of Tween-80 and 2.25 mL of ethanol. The mixture was stirred forcefully at 50 °C and then added 100 mL of AAPH aqueous solution (0.3 mmol/L) to form the O/W emulsion. To 4.8 mL of the emulsion was added 0.2 mL of antioxidant ethanol solution of specified concentration. The mixture was incubated in a 50 °C water bath for 2.5 h. Then the absorbance at 470 nm was measured applying a UV−vis spectrophotometer (SP-756, Shanghai Spectrum Corporation, Shanghai, China). The assay was carried out in triplicate. The antioxidant activity (AA %) was calculated as follows.

$$\mathrm{AA\ (\%)} = \left(1 - \frac{A_0 - A_t}{AO_0 - AO_t}\right) \times 100\%$$

where A_0 represents the initial absorbance of each sample, A_t the absorbance after

incubation for 2.5 h, and AO the absorbance of the blank (addition of 0.2 mL ethanol to 4.8 mL of the emulsion).

Critical Micelle Concentration (CMC) Determined by Fluorescent Probe. The CMCs of the mono gallate and gemini gallate were measured by the steady state fluorescence method. Pyrene was applied as the fluorescent probe. 10 μL of pyrene ethanol solution (1 mmol/L) was added into a standard volumetric flask, followed by evaporation of the solvent. The sample in aqueous solution of specified concentration was added, and kept the pyrene concentration at 10^{-3} mmol/L. All the samples were treated by ultrasonication for 5 min before measured at 298.15 K. The fluorescence emission spectra of pyrene were recorded by a fluorescence spectrometer (Cary Eclipse, Varian, America). Excitation was done at 335 nm while the emission spectra were scanned over the range of 350-600 nm. The intensity ratio of the first peak (I_1 at around 371 nm) to the third peak (I_3 at around 382 nm) of the emission spectra was used as an index of the environment around pyrene molecules.[28] The CMC was determined by the intersection point of the extended two lines.

Microscope and Transmission Electron Microscopy (TEM) Analysis. The samples in β-carotene AAPH bleaching assay were analyzed. Micrographs were obtained by a microscope (CKX53, Olympus Corporation, Tokyo, Japan) and a transmission electron microscope (SU-8010, Hitachi, Japan). For microscope analysis, the samples were dropped on a glass slide before observation. For TEM analysis, the samples were dropped on a copper grid and placed on a filter paper, and then dried in air before observation.

Statistical Analysis. The data of all the experiments were presented as mean ± standard deviation (SD). The results were subjected to least significant difference (LSD) in one-way analysis of variance (ANOVA) applying the SPSS 20.0 software to analyze the difference. Differences with a p value less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Design and Preparation of Gemini Gallate. To realize a gemini gallate antioxidant, the key issue was how to construct the gemini molecular architecture. Ethylene glycol and its polymeric forms have been ubiquitously employed as building blocks for molecular modification owing to biodegradable and biocompatible nature.[29,30] As a result, polymeric ethylene glycol was chosen to build the gemini architecture in this study. Owing to the complexity in selective manipulation of galloyl moieties, it was preferred to build the gemini chain first followed by introduction of galloyl moieties at final stage. In previous study, a class of gemini chain was developed based on polymeric ethylene glycol with alkyl alcohols, which was proven very effective to promote the activities of the prepared emulsifiers. During the developed gemini chains, the dodecyl gemini chain exhibited significantly higher efficiency than other alkyl gemini chains. Hence, the dodecyl gemini chain was applied in this study to construct the gemini gallate antioxidant, which could be readily prepared from ethylene glycol epoxide and dodecanol. The molecular structure of designed gemini gallate and the proposed synthetic route were

257 demonstrated in **Figure 1A**.

258 The synthesis of the gemini gallate commenced with protection of the three
259 hydroxyl groups of gallic acid. According to previous study, isobutyryl was utilized
260 for protection due to exceptional stability and operational simplicity. Following the
261 established protocol,[25] the protection was smoothly performed by the action of
262 isobutyric anhydride. Then the dodecyl gemini chain was prepared from
263 regioselective ring-opening of ethylene glycol epoxide by the dodecanol.[21] This step
264 produced a mixture of the gemini chain and other unidentified side products which
265 were not easy to separate by traditional flash chromatography. Therefore, a prepacked
266 column and peristaltic pump based purification system was developed in this study as
267 indicated in **Figure 1B** to facilitate isolation of the dodecyl gemini chain. The
268 prepacked silica gel column had better column efficiency and the system readily
269 provided analytically pure dodecyl gemini chain for further synthesis. Then the
270 protected gallic acid was coupled with the dodecyl gemini chain through Steglich
271 esterification applying the optimized condition to minimize side reactions.[25] Finally,
272 deprotection of the protected gemini gallate by aqueous hydrazine successfully
273 provided the gemini gallate antioxidant. After preparation of the gemini gallate, the
274 mono gallate was also synthesized for comparison from the protected gallic acid and
275 dodecanol through Steglich esterification and deprotection with the same conditions.
276
277 **Structural Analysis of the Mono Gallate and the Gemini Gallate.** The molecular
278 structures of the mono gallate (MG) and the gemini gallate (GG) were confirmed by

NMR, FT-IR and HPLC-MS.

^{1}H NMR of MG (400 MHz，DMSO-d6) δ 9.17 (br, 2 H; phenolic proton), 8.88 (br, 1 H; phenolic protons), 6.93 (s, 2 H; aromatic protons of the galloyl group), 4.14 (t, *J* = 6.5 Hz, 2 H; oxygen adjacent protons of dodecanol), 1.82-0.72 (m, 23 H; protons of dodecanol).

^{13}C NMR of MG (100 MHz，DMSO-d6) δ 165.80, 145.47, 138.26, 119.57, 108.44, 63.87, 31.28, 29.03, 29.00, 28.97, 28.70, 28.28, 25.51, 22.08, 13.91. The ^{13}C chemical shifts of 165.80 ppm corresponded to the signal of the galloyl ester carbon, 145.47 to 108.44 ppm corresponded to the signals of the aromatic carbons of gallic acid, and 63.87 to 13.91 ppm corresponded to the signals of dodecyl carbons.

^{1}H NMR of GG (400 MHz, DMSO-d6) δ 9.23 (s, 4 H; phenolic protons), 8.93 (m, 2 H; phenolic protons), 6.94 (s, 4 H; aromatic protons of the galloyl group), 5.12 (t, *J* = 5.2 Hz, 2 H; galloyl oxygen adjacent methine protons of the gemini chain), 3.66-3.28 (m, 16 H; oxygen adjacent methylene protons of the gemini chain), 1.54-0.80 (m, 46 H; oxygen nonadjacent protons of the gemini chain).

^{13}C NMR of GG (100 MHz, DMSO-d6) δ 165.38, 145.56, 138.54, 119.37, 108.68, 71.41, 70.65, 70.14, 69.33, 68.88, 31.35, 29.09, 29.07, 28.88, 28.77, 25.60, 22.15, 13.98. The ^{13}C chemical shifts of 165.38 ppm corresponded to the signals of the galloyl ester carbons, 145.56 to 108.68 ppm corresponded to the signals of the aromatic carbons of gallic acid, 71.41 to 13.98 ppm corresponded to the signals of the gemini chain carbons.

IR of MG (cm^{-1}) 3450 (s, ν_{O-H}), 3330 (s, ν_{O-H}), 2918 (s, ν_{C-H}), 2849 (s, ν_{C-H}), 1670

(s, $\nu_{C=O}$), 1626 (s, $\nu_{C=C}$), 1608 (s, $\nu_{C=C}$), 1468 (s, δ_{O-H}), 1404 (s, δ_{O-H}), 1300 (s, ν_{C-O}), 1258 (s, ν_{C-O}), 1027 (s, δ_{C-H}), 769 (m, δ_{C-H}). The absorption bands at 3450 cm^{-1} and 3330 cm^{-1} corresponded to the stretching vibrations of the phenolic hydroxyl groups of gallic acid. The absorption band at 1670 cm^{-1} was attributed to C = O stretching vibration, which confirmed esterification of gallic acid and dodecanol.

IR of GG (cm^{-1}) 3420 (m, ν_{O-H}), 2924 (s, ν_{C-H}), 2853 (s, ν_{C-H}), 1632 (w, $\nu_{C=O}$), 1463 (m, δ_{O-H}), 1378 (m, δ_{O-H}), 1083 (w, δ_{C-H}), 721 (w, δ_{C-H}). The absorption band at 3420 cm^{-1} corresponded to the stretching vibration of the phenolic hydroxyl groups of gallic acid. The absorption band at 1632 cm^{-1} was attributed to C = O stretching vibration, which confirmed esterification of gallic acid and the gemini chain.

The identity and purity of GSt and GSi were further investigated by HPLC-MS (**Figure 2**). ESI-MS spectra of MG and GG (negative ion mode) clearly exhibited their corresponding structures: MG, $[M-H]^-$ 337.33 and $[2M-H]^-$ 674.56; GG, $[M-H]^-$ 849.33. Then HPLC-MS analysis revealed that the purities of MG and GG were 99.2% and 93.6%, respectively. The confirmation of structure and purity assured further antioxidant activity studies.

Radical Scavenging Activities of the Mono Gallate and Gemini Gallate Evaluated by DPPH and ORAC Assays. Radical scavenging activities of the mono and gemini gallates (MG, GG) were evaluated by the generally applied DPPH assay, which was developed by Blois who first identified DPPH as a source of stable free radicals.[31] In the study, the unmodified gallic acid and the typical lipophilic

antioxidants including BHA, BHT and TBHQ were also investigated for comparison. As shown in **Figure 3A**, MG and GG exhibited outstanding antioxidant activities compared with other antioxidants, suggesting successful inheritance of the antioxidant activity from GA. Then the EC_{50} value expressed as Trolox equivalent (TE) was employed to evaluate their DPPH radical scavenging activity.[32] According to **Table 1**, the EC_{50} values of the antioxidants were described as follows: BHA (0.794 TE), BHT (0.397 TE), TBHQ (1.059 TE), GA (2.060 TE), MG (1.806 TE) and GG (3.835 TE). The EC_{50} values demonstrated that the antioxidant activities of MG and GG were substantially higher than those of the frequently employed antioxidants, BHA, BHT and even TBHQ. The EC_{50} value of the gemini gallate was around twice of that of the mono gallate, which suggested that the antioxidant behavior of mono and gemini antioxidants was similar in the homogeneous system because the antioxidant activity was just proportional to the molar ratio of the galloyl group (MG, 1 galloyl group; GG, 2 galloyl groups).

Furthermore, the antioxidant activities of MG and GG were evaluated by the frequently used ORAC assay. Initially, this method was proposed and named by Cao et al.[33] who took β-phycoerythrin as an indicator protein, AAPH as a peroxyl radical generator and Trolox as a standard. Later fluorescein rather than β-phycoerythrin was applied as the indicator owing to excellent photostability and notable reduction of cost.[34] To study the lipophilic MG and GG antioxidants, a previously developed fat-soluble fluorescent probe, dodecanoyl 5-aminofluorescein (DAF), was employed as the indicator,[27] and Tween-80 was added to promote the aqueous solubility in this

345 investigation. The concentration of the MG was set to twice of that of the GG to
346 facilitate comparison of their antioxidant capacities. As shown in **Figure 3B**, the
347 antioxidant activity (indicated as Net AUC) of GG was basically twice of that of MG
348 during the examined concentration (3.12 to 50 μmol/L). The ORAC values were
349 further expressed as Trolox equivalent (TE) as follows: MG (3.05 TE), GG (6.22 TE).
350 As a result, the antioxidant activity of ORAC assay was proportional to the molar
351 ratio of the galloyl group, demonstrating that there was no antioxidant behavior
352 difference between MG and GG in a sufficiently solubilized system which was
353 consistent with the DPPH assay. Presumably, the difference of self-assembly behavior
354 of MG and GG was masked in a sufficiently solubilized or homogeneous system.
355
356 **Inhibition of β-Carotene Oxidation in O/W Emulsion.** After demonstration of
357 the extraordinary radical scavenging activities of MG and GG, their antioxidant
358 activities in heterogeneous O/W emulsion system were further explored. The
359 β-carotene O/W emulsion was applied as the model to investigate the antioxidant
360 activity in emulsion.[11] Traditionally, linoleic acid was often employed to generate free
361 radicals and bleach the unsaturated β-carotene in emulsion. However, the
362 autooxidation of linoleic acid was not easy to control and so inconvenient for further
363 structural analysis. In previous study, an AAPH-based β-carotene O/W emulsion
364 assay was developed employing AAPH as the aqueous radical initiator.[11] Thermal
365 initiation of AAPH was readily controllable. As a result, AAPH-based β-carotene
366 bleaching assay was applied in this study to facilitate further investigation.

As disclosed in **Figure 4A**, GG exhibited outstanding antioxidant activity in O/W emulsion though slightly lower than TBHQ but higher than other antioxidants. It was not a surprise that the antioxidant activity of more lipophilic MG was lower than those of BHA and BHT at low concentration since the radical was produced in aqueous phase by hydrophilic AAPH. As demonstrated in previous study, the performance of an antioxidant was greatly influenced by either location of the radical source or distribution of the antioxidant.[11] The less lipophilic BHA and BHT were more efficient to scavenge radicals in aqueous phase at low concentration. These results were consistent with the previous study. As expected, MG would be more efficient when the radicals were generated in oil phase as indicated in previous study.[11] Although GG demonstrated eminent antioxidant activity in O/W emulsion, the activity relative to MG was still proportional to the molar ratio of the galloyl group probably owing to that excessive addition of Tween-80 emulsifier diluted the effective interfacial concentration of the antioxidant and also masked the difference of self-assembly behavior.[12-15]

To unmask the difference of MG and GG, their antioxidant activities in β-carotene O/W emulsion were further observed with reduced amount of Tween-80. To our delight, exceptional antioxidant activity of GG (5 to 2 times relative to MG) was unveiled at low concentration (<0.5 mmol/L) in the test (**Figure 4B**). The antioxidant activity ratio (GG to MG: 5, 0.25 mmol/L) was significantly higher than the molar ratio of the galloyl group (GG to MG: 2), demonstrating notable contribution from the difference of self-assembly behavior. The significant promotion of the antioxidant

activity in O/W emulsion under the condition of reduced amount of the emulsifier suggested that the gemini antioxidant more readily assembled at interfacial layers and so increased the interfacial concentration and also helped to maintain the integrity of oil droplets when the emulsifier was limited.[12-15,21] As a result, these results exhibited the superiority of the gemini antioxidant relative to the traditional mono antioxidant especially under the condition of low concentration of emulsifier.

Microscopic Investigation of Antioxidant Activity in O/W Emulsion. To further understand details of the antioxidant behavior in O/W emulsion, critical micelle concentrations (CMC) of the antioxidants were determined by a steady-state fluorescence method employing pyrene as the fluorescent probe. The ratio of fluorescence intensity (I_1/I_3) reflected the hydrophobicity of the microenvironment of pyrene. A surface-active compound would cause an abrupt change of the curve of I_1/I_3 at the point of micelle formation. As shown in **Figure 5**, there were no inflection points observed for lipophilic BHA, BHT, TBHQ and hydrophilic GA, which confirmed that they were not surface-active and so had no micellization activity. Notable surface activity was observed for MG, and the CMC of MG was determined as 0.13 mmol/L through calculation of the intersection of the trend lines. The CMC of GG determined as 0.04 mmol/L was significantly smaller than that of MG, which confirmed superior micellization activity of the gemini antioxidant. The superior micellization capacity presumably increased effective interfacial concentration of GG and promoted antioxidant activity accordingly.[12-15]

411 Then the microstructure of β-carotene emulsions of MG and GG with normal and
412 reduced amount of Tween-80 was explored by optical microscopy and transmission
413 electron microscopy (TEM) to further understand the antioxidant behavior. As
414 revealed in microscopic bright field images, both MG emulsion (**Figure 6a1, 6b1**)
415 and GG emulsion (**Figure 6a2, 6b2**) had a consistent dispersion of the oil droplets in
416 aqueous phase. The oil droplets in GG emulsion were generally larger than those in
417 MG emulsion. Moreover, the oil droplets in MG or GG emulsion with normal amount
418 of Tween-80 (**Figure 6a1 or 6a2**) were larger than those in the corresponding
419 emulsion with reduced amount (**Figure 6b1 or 6b2**). The details of the microstructure
420 of MG and GG emulsions were further scrutinized by TEM. The darker inner area of
421 oil droplets in GG emulsion (**Figure 6a4**) clearly demonstrated better encapsulation
422 of β-carotene relative to MG emulsion (**Figure 6a3**). Under the condition of reduced
423 amount of Tween-80, the oil droplets in GG emulsion (**Figure 6b4**) exhibited
424 significantly better encapsulation of β-carotene compared with MG emulsion (**Figure**
425 **6b3**). Hence, the microscopic images of the O/W emulsions confirmed great
426 micellization ability of GG which resulted in the outstanding antioxidant
427 performance.

428 Therefore, these results proved the concept that gemini architecture would improve
429 antioxidant performance in O/W emulsion presumably through increase of interfacial
430 concentration, thus referred to as gemini interfacial antioxidant. The great
431 micellization capacity of the gemini interfacial antioxidant could be attributed to the
432 tighter connection enforced by the ethylene glycol chain through elimination of the

433 steric interaction between bulky galloyl moieties.[21] The outstanding micellization
434 capacity of the gemini interfacial antioxidant eventually brought about preferable
435 location at interfacial layer and enhancement of antioxidant performance in
436 heterogeneous system. Reduction application of food additives in a food system was
437 generally welcome because food additives often produced undesired off-flavor. The
438 gemini interfacial antioxidant was of the activity of emulsifier and so highly desirable.
439 Hence, the gemini interfacial antioxidant was very useful to lower the amount of
440 emulsifier and particularly as the low concentration of emulsifier was required. The
441 developed concept of gemini interfacial antioxidant would be useful for design of
442 antioxidants in complicated settings.
443
444 In conclusion, the gemini gallate interfacial antioxidant was designed and
445 successfully prepared by Steglich esterification with an improved purification
446 protocol through a prepacked column and peristaltic pump based purification system.
447 The gemini gallate had excellent radical scavenging activity and superior antioxidant
448 activity in O/W emulsion relative to the mono gallate especially under the condition
449 of reduced amount of emulsifier. Further studies revealed that the extraordinary
450 antioxidant performance of the gemini interfacial antioxidant in O/W emulsion was
451 presumably attributed to increased interfacial concentration of the antioxidant
452 enforced by the gemini molecular architecture. The superior antioxidant activity of the
453 gemini antioxidant hold a great promise for antioxidation in O/W emulsion.
454

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Notes

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Figure 1. (A) Synthetic route of the gemini gallate and (B) the prepacked column and peristaltic pump based purification system.

Figure 2. HPLC-MS of (A) the mono gallate (MG) and (B) the gemini gallate (GG).

Figure 3. (A) DPPH radical scavenging activities and (B) Net AUC of ORAC assay.

Table 1. EC_{50} Values of BHA, BHT, TBHQ, GA, MG, and GG in DPPH Assay.

Figure 4. (A) Inhibition of β-carotene oxidation in O/W emulsion and (B) ratio of the antioxidant activity of GG to MG with reduced amount of Tween-80.

Figure 5. Critical micelle concentration (CMC) of the antioxidants determined by pyrene fluorescent probe.

Figure 6. Images of O/W emulsions in β-carotene bleaching assay: (a1-a4) the emulsions with normal amount of Tween-80; (b1-b4) the emulsions with reduced amount of Tween-80; (a1, b1) micrographs of MG emulsions under bright field; (a2,

587 b2) micrographs of GG emulsions under bright field; (a3, b3) TEM images of MG
588 emulsions; (a4, b4) TEM images of GG emulsions.
589

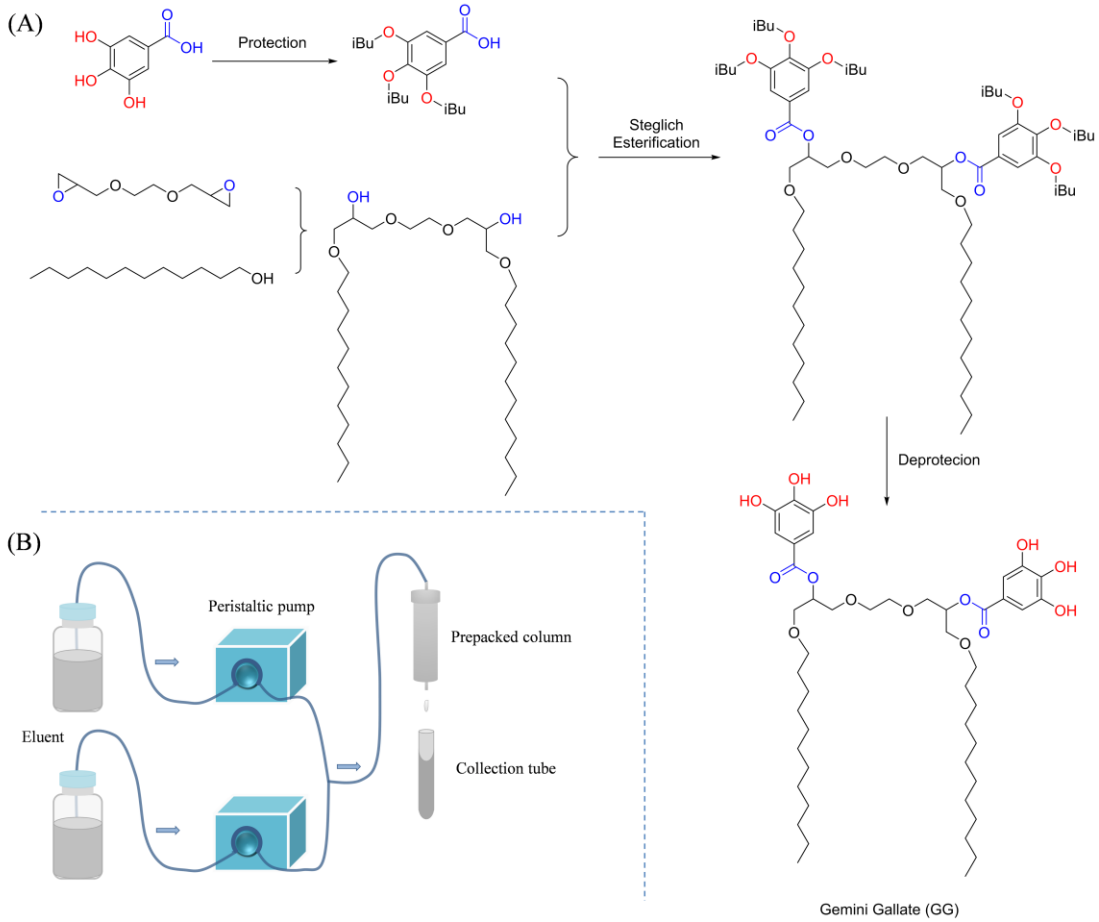
(A)
Protection
Steglich
Esterification
Deprotecion
Gemini Gallate (GG)
(B)
Peristaltic pump
Prepacked column
Eluent
Collection tube

Figure 1

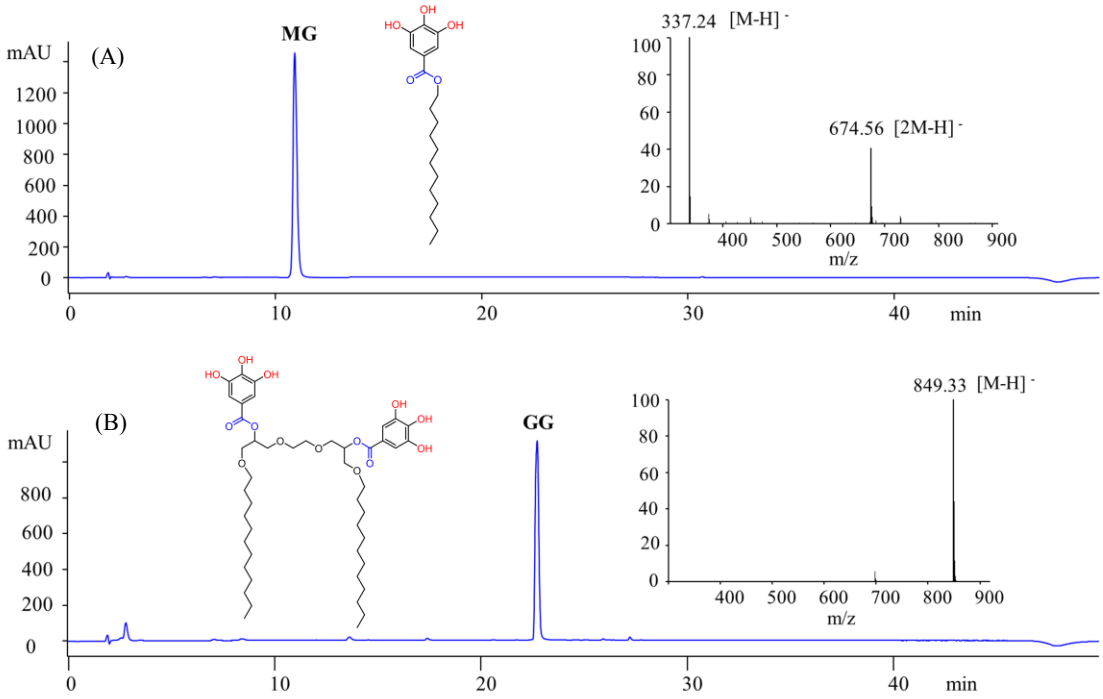
(A)
MG
mAU
1200
1000
800
600
400
200
0
0
10
20
30
40
min
337.24 [M-H]⁻
674.56 [2M-H]⁻
100
80
60
40
20
0
400
500
600
700
800
900
m/z
(B)
GG
mAU
800
600
400
200
0
0
10
20
30
40
min
849.33 [M-H]⁻
100
80
60
40
20
0
400
500
600
700
800
900
m/z

Figure 2

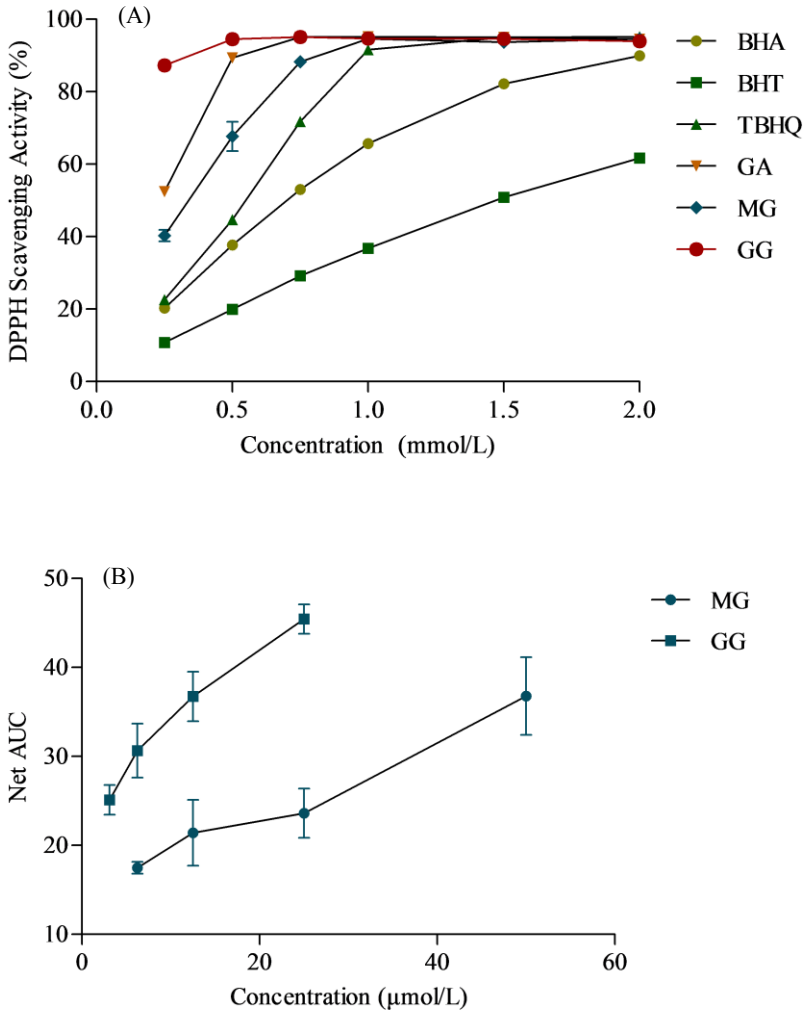
(A)
DPPH Scavenging Activity (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Concentration (mmol/L)
BHA
BHT
TBHQ
GA
MG
GG
(B)
Net AUC
50
40
30
20
10
0
20
40
60
Concentration (μmol/L)
MG
GG

Figure 3

Table 1

Antioxidant	EC_{50} (TE)*
BHA	0.794 ± 0.002 e
BHT	0.397 ± 0.001 f
TBHQ	1.059 ± 0.012 d
GA	2.060 ± 0.005 b
MG	1.806 ± 0.019 c
GG	3.835 ± 0.022 a

* The values are given as mean ± SD (n=3). The lowercase letters (a-f) indicate the significant difference ($p<0.05$).

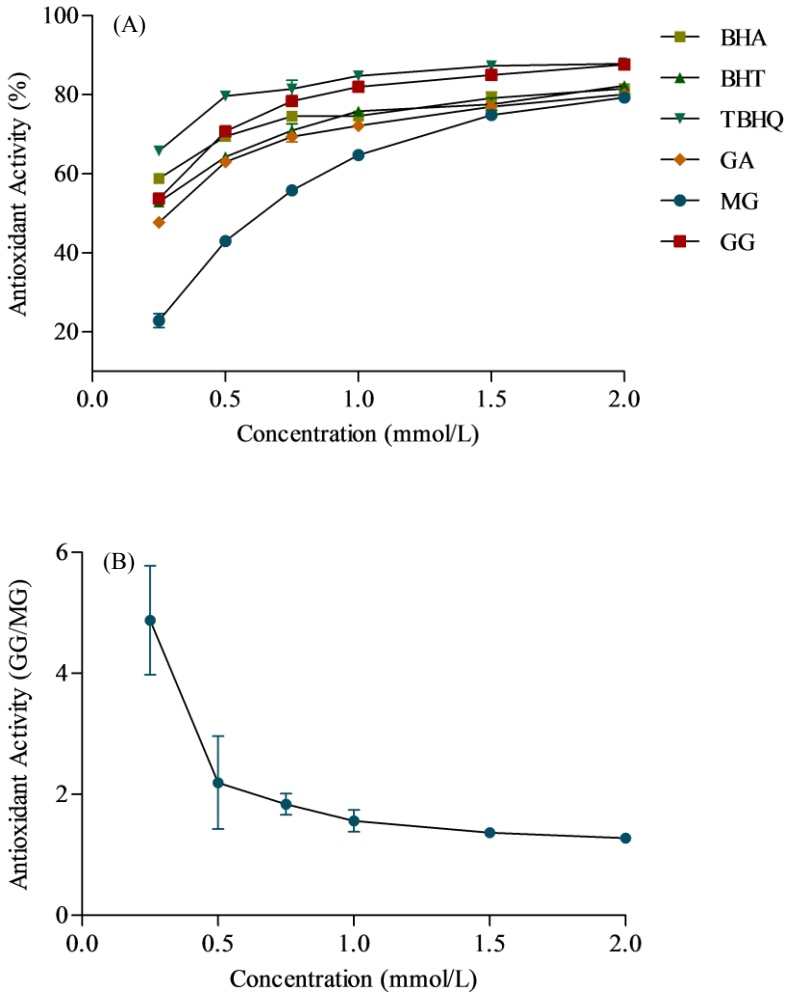
(A)
Antioxidant Activity (%)
100
80
60
40
20
0.0
0.5
1.0
1.5
2.0
Concentration (mmol/L)
BHA
BHT
TBHQ
GA
MG
GG
(B)
Antioxidant Activity (GG/MG)
6
4
2
0
0.0
0.5
1.0
1.5
2.0
Concentration (mmol/L)

Figure 4

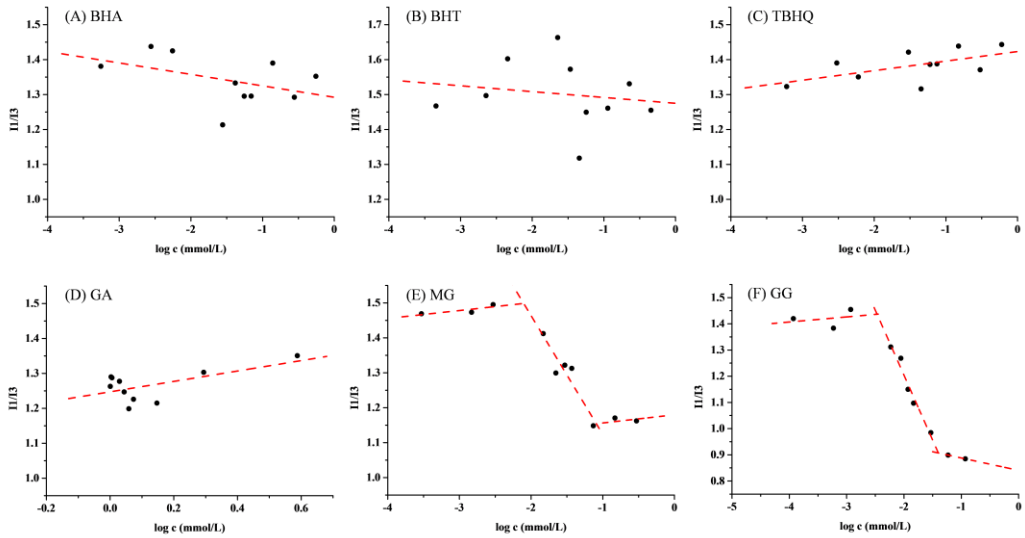

Figure 5

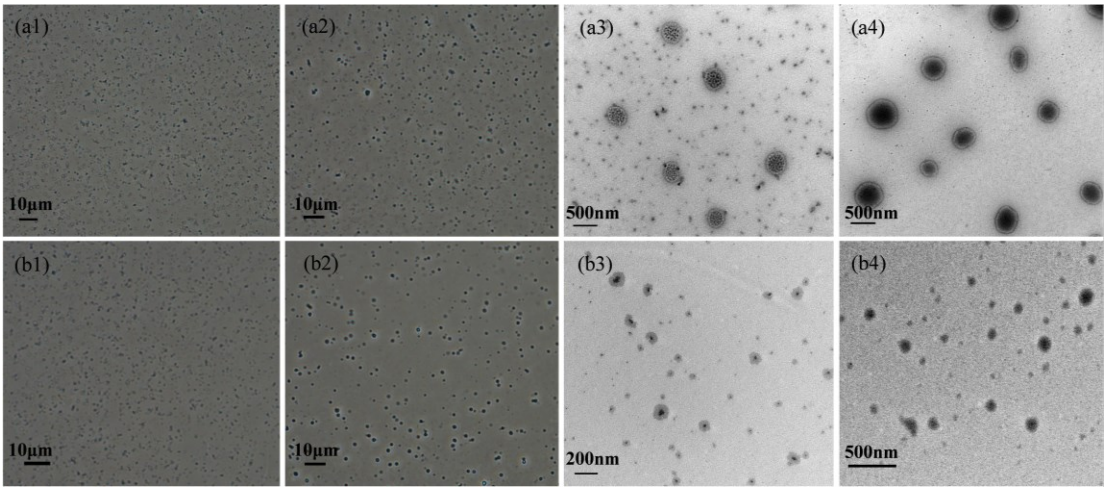
(a1)
10μm
(a2)
10μm
(a3)
500nm
(a4)
500nm
(b1)
10μm
(b2)
10μm
(b3)
200nm
(b4)
500nm

Figure 6

Graphic Abstract