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ORIGINAL ARTICLE

Investigation of Micellization and Vesiculation of Conjugated Linoleic Acid by Means of Self-Assembling and Self-Crosslinking

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Abstract Bioactive and biocompatible conjugated linoleic acid (CLA) has been only considered as a food or medicine ingredient due to its rare natural occurrence. In this work, the surface activities and pH-induced self-assembling behaviors of the semi-synthetic CLA molecules into micelles or vesicles were systematically investigated. First, the self-assembling of CLA was studied in detail, and it was found that aside from temperature and ionic strength, pH is the prominent factor affecting the self-assembling of CLA. Moreover, stable CLA ufasomes (unsaturated fatty acid liposomes) in uniform size were obtained by self-crosslinking of the CLA ufasomes, and the morphologies of the crosslinked CLA assemblies were recorded by transmission electron microscopy, which made known the pH-induced formation of the CLA ufasomes or the CLA micelles. The crosslinked CLA assemblies presented improved properties such as a higher calcium stability, a lower lime soap dispersing requirement and a better solubilization ability than that of the CLA molecules themselves or the pre-crosslinked linoleic acids. These investigations could be helpful for comprehensively understanding effects of environment factors on self-assembling behaviors of conjugated fatty acids and responsive polymerization of polymerizable surfactants.

Keywords Conjugated linoleic acid · Micelle · Ufasome · Self-assembling · Self-crosslinking · Surface activity · Polymerizable surfactant

Introduction

Over the past decades, polymerizable surfactants have attracted more attention and been used in a variety of application areas [1]. Among these polymerizable surfactants, polymerizable lipids, especially unsaturated fatty acids, are topics of great interest. Conjugated linoleic acid (CLA), a rare natural lipid present in animals and plants, has attracted considerable attention because of its excellent anticarcinogenic [2], antiatherogenic [3] and anti-inflammatory [4] functions. However, the rare natural occurrence of CLA is still the limitation for application. Recently, the chemical synthesis of CLA has been reported, which has now made it possible to use CLA extensively for nutraceuticals. From what has been reported, to date, CLA has mainly been synthesized through dehydration of ricinoleic acid [5], isomerization of linoleic acid (LA) catalyzed by metal [6], enzyme and cultured cells [7], and alkali isomerization of LA [8]. Nevertheless, these chemical synthesis routes have several obvious limitations: (1) the traditional dehydration of ricinoleic acid generally provided low CLA yield and complicates the chemical compositions of products [9]; (2) the metal-catalyzed isomerization may result in some toxic and environmental problems that would undoubtedly impede CLA from being used in food, drugs and cosmetics [10]; (3) though the chemoenzymatic synthesis can obviously enhance the product selectivity, low CLA yield still restricts its availability and further application [11]. Therefore, a semi-synthesis method, the alkali isomerization of LA (Scheme 1), certainly becomes the

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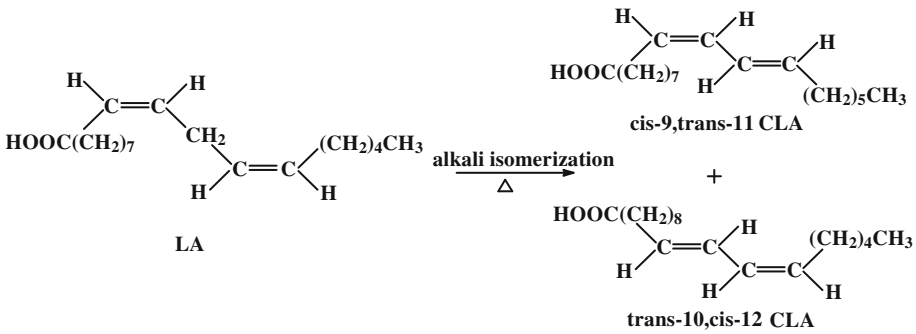
Scheme 1 Semi-synthesis of CLA from LA by alkali isomerization



most promising synthesis strategy, with many advantages such as low-cost, high safety, high product yield and selectivity, which is favorable for eliminating the worry about the availability of CLA [12].

Fatty acid vesicles, especially ufasomes ("unsaturated fatty acid liposomes") assembled from unsaturated fatty acids, have been considered as very attractive carriers for food, drug and cosmetics capsules. Interestingly, many of their assembling processes can be adjusted by pH, temperature, salinity and water hardness [13, 14]; for instance, decanoic acid in water being self-assembled into vesicles at pH 6.8–7.8, and evolved into micelles at pH 11.0 [15–17], as well as oleic acid [18, 19]. Much of the attention regarding CLA has only been focused on its nutrition and bioactivity [20, 21] rather than its surface activities, self-assembling behaviors and further applications as biocompatible and polymerizable surfactants for its rare natural occurrence. However, the colloid properties of CLA and the reactive conjugated double bonds in the CLA molecule could make it a potential capsule wall material through self-assembly followed by self-crosslinking of CLA molecules. Actually, a very interesting phenomenon was previously observed when testing the lime soap dispersing requirement (LSDR) of CLA in our laboratory. The aqueous solution of CLA in hard water gradually turned from cloudy to clear after a long standing time or moderate heating and the aged CLA solution became more resistant to hard water and less sensitive to lowing of the pH than the parent solution. Giving the fact that CLA was easily polymerized by moderate heating [22], we speculated that part of the CLA molecules in the aged solution may self-crosslink to form polycarboxylates that display better calcium stability (CS), and the self-crosslinked CLA, if it exists, could be a new microcapsule material with more stable vesicle structure and a wider environmental tolerance window.

Thus, in this paper, the self-assembling behavior of the CLA molecules induced by changes of pH and ionic strength was firstly investigated by the tensiometry method. Then, thermo-induced self-crosslinking of the CLA assemblies was further studied by UV spectroscopy, FT-IR spectroscopy and transmission electron microscopy (TEM). Subsequently, the CS and the encapsulation of 5-fluorouracil (5-FU) using the self-crosslinked CLA assemblies were tested. These studies could be helpful for comprehensively understanding the effects of environment factors on the self-assembling behaviors of conjugated fatty acids and responsive polymerization of polymerizable surfactants. The investigation of microenvironment driven-self-assembling of the CLA molecules and the resultant self-crosslinked ufasomes would be of benefit to fabricate biocompatible carriers for medicine capsules.

Experimental Section

Materials

Safflower oil was purchased from Cofco Co., Ltd. Urea, ethylene glycol, potassium hydroxide, ammonium persulphate (APS), calcium acetate, calcium chloride, magnesium sulfate, toluene and 5-FU were all analytical reagent grades and purchased from Sinopharm Chemical Reagent Co. Ltd. Sodium dodecyl sulfate (SDS, 99 wt%) was purchased from Acros Organics Co. Ltd. All materials were used without further purification. Ultrapure Millipore water (18.2 MΩ cm) was used throughout.

Methods

Preparation of Linoleic Acid

LA was prepared from saponification of safflower oil and enriched by urea inclusion. Typically, 400 mL sodium hydroxide solution (4 wt% in ethanol) was added dropwise in 4 h into a flask containing 100 g safflower oil. The reaction mixture was stirred at 60 °C for another 2 h, and then cooled to room temperature. The produced sodium soap of fatty acid mixture was obtained by filtration, while the unreacted components were discarded with the filtration solution. The filtrate was acidified with 10 wt% HCl to pH 2, and washed alternatively with distilled water and

saturated saline. The resultant fatty acid mixture was dried over Na_2SO_4. Then, 30 g fatty acid mixture was added dropwise in 2 h into the mixture of 75 g urea in 240 mL ethanol (95 v%) at 80 °C under N_2 atmosphere, and refluxed for another 30 min. The reaction mixture was placed at −18 °C for 12 h; the produced crystal was obtained after filtration and acidified again with 10 wt% HCl to pH 2. The acidified mixture was extracted with anhydrous diethyl ether, and the ether phase was then washed with distilled water and saturated salt water alternatively and repeatedly, and finally dried over Na_2SO_4 to obtain purified LA (97.6 wt%) after remove the ether.

Semi-Synthesis of Conjugated Linoleic Acid

CLA was semi-synthesized by alkali isomerization of LA with KOH and ethylene glycol. 10 g LA was dropped in 30 min into a flask containing 5 g KOH and 15 mL ethylene glycol at 170 °C under N_2 atmosphere, and reacted for another 4 h. The reaction mixture was acidified with 10 wt% HCl to pH 3, and extracted with anhydrous diethyl ether. The ether phase was then washed with distilled water and saturated salt water alternatively, and finally dried over Na_2SO_4. A light yellow liquid of CLA was obtained after removal of the ether. The obtained CLA was characterized with a FTLA2000-104 infrared spectrometer (ABB Bomem Co. Ltd., Canada), a Persee T6 UV–Vis spectroscopy (Beijing Pgeneral Co. Ltd.), a 400 MHz Avance III NMR spectrometer (Bruker, Germany) and a FULI 9790 gas chromatograph (Fuli Analytical Instrument Co. Ltd.) equipped with PEG 20000 capillary column and FID detector, respectively. IR: 3,015, 1,709, and 1,654 cm^{-1}, =CH, C=O and C=C, respectively; 946 and 982 cm^{-1}, conjugated double bond of *trans*-, *cis*-CLA and *cis*-, *trans*-CLA, respectively [23]. The characteristic absorption peak of conjugated double bond at 234 nm [24] was observed in UV spectrum. ^1H NMR ($CDCl_3$, ppm) δ: 5.68 (m, 4H), 2.34 (t, 2H), 2.12 (q, 4H), 1.63 (m, 2H), 1.32 (m, 16H), 0.89 (t, 3H).

Self-Assembling of CLA

One hundred milliliters of the aqueous CLA solution (3 mmol/L) was prepared in a 250-mL flask by dissolving CLA in a NaOH–borate buffer (20 mmol/L) and adjusting the solution to pH 10.5 with a little NaOH solution (0.1 mol/L). The flask then stood at room temperature overnight to let CLA molecules self-assemble into CLA micelle.

The procedure of preparation of CLA ufasome is similar to the above preparation process of CLA micelle. 100 mL of the aqueous CLA solution (3 mmol/L) was prepared in a 250-mL flask by dissolving CLA in a boric–borate buffer (20 mmol/L) and adjusting the solution to pH 8.6 with a little borate solution (0.2 mol/L). Then the flask stood at room temperature overnight to let CLA molecules self-assemble into CLA ufasomes.

Self-Crosslinking of CLA Assemblies

After adding initiator APS (5 wt% of CLA) into the above CLA micelle solution and bubbling N_2 for 30 min, the mixture was heated to 70 °C for 24 h under N_2 atmosphere, initiating the self-crosslinking of the CLA micelles.

Similarly, APS (5 wt% of CLA) was added into the above CLA ufasomes solution, and N_2 was bubbled through the mixture for 30 min; it was heated to 70 °C for 24 h under N_2 atmosphere, initiating the self-crosslinking of the CLA ufasomes.

The absorbance of the reaction solution at 234 nm was used to monitor the reaction progress. The morphologies of self-crosslinked CLA assemblies were imaged with TEM (JEM-2100, 200 kV, JEOL Ltd., Japan).

Measurement of Surface Tension

The surface tensions (γ) of the aqueous CLA solution at different pHs and temperatures were measured using the pendant drop method with an optical contact angle tensiometer (OCA 40 micro, DataPhysics Instruments GmbH, Germany). The pH values of all the tested solutions were adjusted with NaOH–borate or boric acid–borate buffer, and ionic strength was adjusted with NaCl. The critical micelle concentration (CMC) and the critical vesicular concentration (CVC) were determined according to the turning points of γ–log c curves.

Calcium Stability

Calcium stability is defined as the maximum concentration of calcium ion with which the sample solution could remain clear. CS was determined using a modified Hart's method [25]. Concretely, 40 mL of the tested sample solution (3 mmol/L) was titrated with a 1 wt% calcium acetate solution in a 100-mL beaker at pH 10.5. The degree of developed turbidity, which just obscured the typewriter print on paper fastened to the other side of the breaker as viewed horizontally through the solution, was taken as the end point of the titrations. CS was expressed as ppm of $CaCO_3$ and calculated as Eq. (1):

$$\text{Calcium tolerance (ppm)} = \frac{0.01V \times \frac{100}{158}}{V + 40} \times 10^6 \quad (1)$$

where V is the consumed calcium acetate solution (mL); 158 and 100 the molecular weight of calcium acetate and calcium carbonate, respectively; 0.01 the concentration of

calcium acetate solution (1 wt%); and 40 is the volume of tested sample solution (mL).

Lime Soap Dispersing Requirement

Lime soap dispersing requirement is a numerical measure of the effectiveness of lime soap dispersants. It is the minimum amount of dispersant causing dispersion of the lime soap deposit. In this paper, LSDR was tested with a modified LSDP assay [26] in which SDS was used as the given dispersant, and the tested soap samples were used instead of sodium oleate. LSDR was similarly defined as the minimum amount of SDS required to completely disperse the resultant lime soap deposit formed by 100 g of the tested soap samples in hard water (1,000 ppm) at pH 10.5 and 30 °C, which was calculated as Eq. (2). Thus, a soap sample with a low LSDR is better than one with a high LSDR.

$$\text{LSDR}\ (\%) = \frac{V_1 \times 2.5}{V_2 \times 5.0} \times 100 \tag{2}$$

where V_1 and V_2 are the volume (mL) of SDS solution and sample solution, respectively; and 2.5 and 5.0 are the concentration (g/L) of SDS and the tested sample, respectively.

Solubilization Capacity

Briefly, 5 mL of the tested soap samples (3 mmol/L) was mixed with 0–0.12 mL toluene in nine volumetric flasks (10 mL), respectively. After shaking (80 r/min) in a thermostat oscillator at 30 °C for 1 h, the absorbance of the sample solution was recorded at 560 nm with a Persee T6 UV–Vis spectroscopy. According to the absorbance–toluene volume curve, the maximum amount of toluene solubilized in the CLA assemblies was determined based on the fact that the absorbance increases suddenly upon reaching the solubilization limitation. The solubilization capacity (A_M) was calculated as the following Eq. (3) [27].

$$A_M = \frac{A}{V \times C} \tag{3}$$

where A_M is solubilization capacity (L/mol); A the maximum amount of solubilized toluene corresponding to turning point (mL) in the curve of absorbance versus toluene volume; V the volume of the tested soap sample solution (mL), and C is the concentration of the testing soap sample solution (mol/L).

Microencapsulation and Sustained Release of 5-FU Using Crosslinked CLA Ufasome

0.0153 g 5-FU was mixed with 10 mL of crosslinked CLA assemblies solution (3 mmol/L, pH 8.6) at 37 ± 0.5 °C, shaken in a thermostat oscillator at 80 rpm for 24 h, followed by dialysis using a membrane with 3,500 molecular weight cut-off against PBS buffer (0.1 mol/L, pH 7.4) for 1 h. The dialysis tube was then relocated in 200 mL fresh PBS buffer, and incubated at 37 ± 0.5 °C and 80 rpm in a thermostat oscillator for the following sustained releasing test. The UV absorbance (266 nm) of the solution was recorded every 15 min. Fresh PBS was recruited to make up the solution volume. The cumulative release was calculated based on the calibration of standard 5-FU solution. The entrapment efficiency (EE) of 5-FU from the crosslinked CLA assemblies was also investigated according to the above dialysis method and calculated as the following Eq. (4):

$$\text{EE}\ \% = \frac{m_1 - m_2}{m_1} \times 100 \tag{4}$$

where m_1 is the total amount of 5-FU in the crosslinked CLA assemblies solution (g); and m_2 is the amount of free 5-FU unencapsulated in the crosslinked CLA assemblies (g). m_2 was calculated based on the calibration of standard 5-FU solution.

Results and Discussion

Microenvironment Manipulated Self-Assembly at Surface Layer and in Aqueous Solution of CLA

Although CLA is insoluble in water; sodium conjugated linoleate (SCL) is an amphiphilic surfactant that can solubilize and self-assemble in water, and thus the mixture of CLA and SCL is expected to assemble together and to cause the variation of surface activity parameters of CLA solution. As reported [19, 28, 29], most assemblies of unsaturated fatty acid can undergo a transformation from micelles to ufasomes responsive to pH variation, and the transformation point mostly relies on pKa of the relevant fatty acids. Since the disassociation constant (pKa) of CLA was 8.5 obtained by acid-base titration, at pH 8.6 where the amount of $C_{17}H_{31}COO^-$ in solution is comparable to that of $C_{17}H_{31}COOH$, it is possible to induce a unique self-assembling of an equal amount of $C_{17}H_{31}COO^-$ and $C_{17}H_{31}COOH$. When the pH is increased to 10.5, a popular pH value usual for soaps in applications, the amount of $C_{17}H_{31}COO^-$ in the solution would be 100 times greater than that of $C_{17}H_{31}COOH$. Upon further increasing the pH to 13, the amount of $C_{17}H_{31}COO^-$ in the solution would be 10,000 times greater than that of $C_{17}H_{31}COOH$, which means $C_{17}H_{31}COO^-$ is almost the exclusive CLA species in the solution. Thereby, an investigation of the pH-induced self-assembling of CLA was carried out at these aforementioned three typical pH values along with various

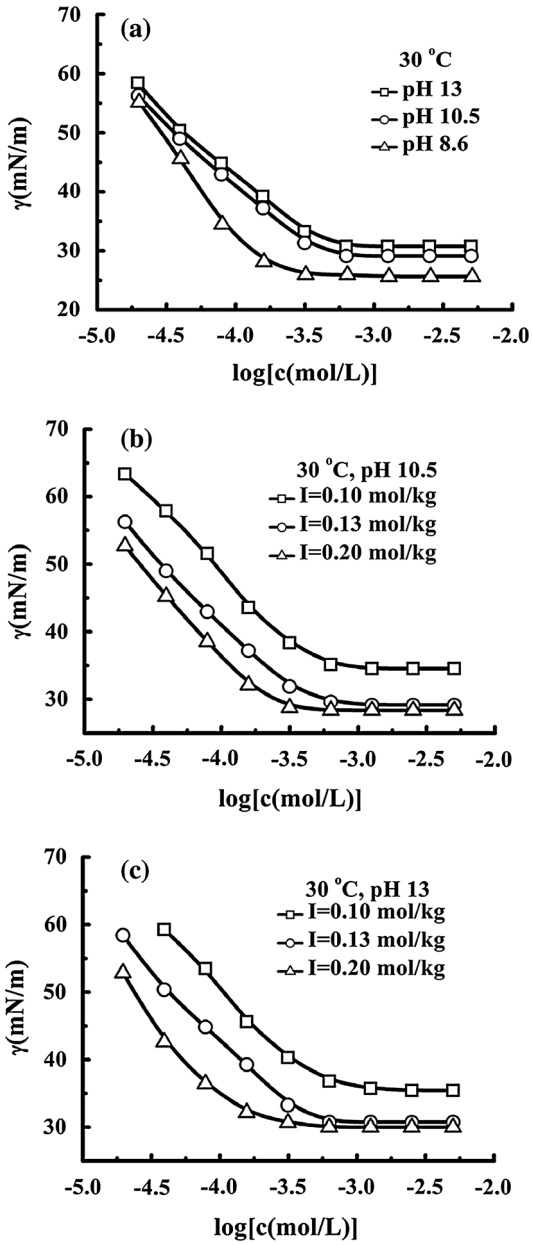


Fig. 1 Microenvironmental influence on the self-assembly of CLA

ionic strengths and temperatures, and the γ–log c curves of CLA solutions are indicated in Figs. 1 and 2.

As SCL is a typical anionic surfactant composed of an active anion (B^-) and a counter cation (A^+), the Gibbs equation is:

$$\begin{aligned} d\gamma = -RT\Big[\Gamma^{(1)}_{A^+} d(\ln a_{A^+}) + \left(\Gamma^{(1)}_{B^-} + \Gamma^{(1)}_{HB}\right) d(\ln a_{B^-}) \\ + \left(\Gamma^{(1)}_{HB} + \Gamma^{(1)}_{B^-} - \Gamma^{(1)}_{A^+}\right) d(\ln a_{H^+})\Big] \end{aligned} \quad (5)$$

In the presence of excess electrolyte that provides same counterion and buffering effect, a_{A^+} and pH will be constants, and $\Gamma^{(1)}_{A^+} = \Gamma^{(1)}_{B^-} = \Gamma^{(1)}$, $a_{B^-} = a$, therefore,

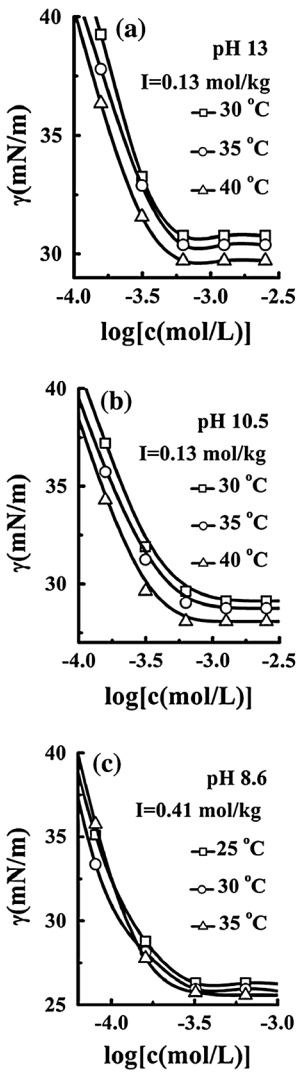


Fig. 2 γ–log c curves of CLA at pH 13 (**a**), pH 10.5 (**b**) and pH 8.6 (**c**) at different temperatures

$$d\gamma = -RT\left[\left(\Gamma^{(1)}_{B^-} + \Gamma^{(1)}_{HB}\right) d(\ln a)\right] \quad (6)$$

In a dilute solution, $d(\ln a) \approx d(\ln x) \approx d(\ln c)$, c is concentration of the surfactant in mol/L. Assuming $\Gamma_m = \Gamma^{(1)}_{B^-} + \Gamma^{(1)}_{HB}$, then, the maximum adsorbed quantity (Γ_m, mol/cm^2), and minimum area per molecule (a_{min}, Å^2), can be calculated as following:

$$\Gamma_m = -\frac{1}{2.303RT}\frac{d\gamma}{d(\log c)} \quad (7)$$

$$a_{min} = \frac{10^{20}}{N_A \Gamma_m} \quad (8)$$

where R is the gas constant, 8.314 J/(mol K); T the absolute temperature in K, and N_A is the Avogadro constant.

A decreased tendency of both CMC (or CVC) and γ_{CMC} of CLA with decreasing pH is observed in Fig. 1a. As

Table 1 Parameters of the surface self-assembly/the micellization (or the vesiculation) of the aqueous CLA solution at different pH

Parameter	pH 13			pH 10.5			pH 8.6		
T (K)	303.15	308.15	313.15	303.15	308.15	313.15	298.15	303.15	308.15
I (mol/kg)	0.13	0.13	0.13	0.13	0.13	0.13	0.41	0.41	0.41
γ_{CMC} (mN/m)	30.77	30.39	29.72	29.15	28.75	28.02	26.30	25.85	25.52
Γ_m (10^{-6} mol/m^2)	3.42	3.51	3.58	3.62	3.74	3.80	6.83	6.90	6.98
a_{min} (Å^2)	48.6	47.3	46.4	45.9	44.4	43.7	24.3	24.1	23.8
CMC (mmol/L)	0.42	0.38	0.34	0.41	0.37	0.35	0.20[a]	0.18[a]	0.17[a]
d(log CMC)/dT	0.0091			0.0066			0.0061		
ΔG_m^Θ (kJ/mol)	−59.43	−60.95	−62.46	−59.53	−60.97	−62.25	−62.14[b]	−63.70[b]	−65.04[b]
ΔH_m^Θ (kJ/mol)	32.02	33.09	34.17	23.23	24.00	24.78	20.76[b]	21.47[b]	22.18[b]
ΔS_m^Θ [kJ/(mol K)]	0.3017	0.3052	0.3086	0.2730	0.2757	0.2779	0.2781[b]	0.2810[b]	0.2830[b]

[a] CVC

[b] Thermodynamic parameters at CVC

shown in Fig. 1b and c, both higher surface activities [lower CMC (or CVC) and γ_{CMC}] and more compacter aggregation (higher Γ_m and lower a_{min}) result from increasing temperature and increasing ionic strength. All the surface activity parameters of the aqueous CLA solution at different combinations of pH, temperature and ionic strength are listed in Table 1. Nevertheless, pH is the prominent factor affecting the self-assembling of CLA, and is superior to temperature and ionic strength. At pH 13, the CLA molecules mainly present in $C_{17}H_{31}COO^-$ species (SCL), and the strong electrostatic repulsion among the SCL ions favors forming loose molecular assembly structures, thus leading to a large a_{min}. This signifies small curvature radius of the self-assembly structures and benefits to form spherical micelle. At pH 10.5, the values of Γ_m and a_{min} are similar with those at pH 13, which indicates a similar surface molecular arrangement and a similar self-assembly, and may be reasonably explained based on the occurrence of the pH-dependent species. Whereas, at pH 8.6, the values of a_{min} corresponding to the CLA species is much closer to those of fatty acids at the insoluble monolayer film (20.5 Å^2) [30]. This means that the co-assembly of an equal proportion of $C_{17}H_{31}COOH$ molecules and $C_{17}H_{31}COO^-$ ions may cause a sharp decrease of electrostatic repulsion between the ions and molecules, and then the space between the $C_{17}H_{31}COOH$ molecules and the $C_{17}H_{31}COO^-$ ions drops down dramatically, which is favorable for the formation of a large assembly, such as of rod-like micelles, lamellar micelles or vesicles. As shown in Fig. 2 and Table 1, the values of Γ_m increase and the values of both CMC (or CVC) and γ_{CMC} decrease slightly with increasing temperature. This is because the molecular motion of CLA is enhanced and electrostatic repulsion between hydrophilic head groups of the CLA molecules is screened with increasing temperature, resulting in a tight arrangement of hydrophobic chains.

In addition to the surface activity parameters, the thermodynamic parameters of micellization or vesiculation of CLA at different pHs are calculated according to log CMC–T curves of CLA (Fig. 3) based on the pseudo-phase separation model [31]. Under constant temperature and pressure t, the standard free energy change of micellization ΔG_m^Θ, standard enthalpy change of micellization ΔH_m^Θ, and standard entropy change of micellization ΔS_m^Θ, are calculated, respectively, as following:

$$\Delta G_m^\Theta = 4.606RT \log \frac{\mathrm{CMC}}{\omega} \tag{9}$$

$$\Delta H_m^\Theta = -4.606RT^2 \frac{\mathrm{d}(\log\ \mathrm{CMC})}{\mathrm{d}T} \tag{10}$$

$$-T\Delta S_m^\Theta = \Delta G_m^\Theta - \Delta H_m^\Theta \tag{11}$$

where ω is the concentration of H_2O in mol/L. The thermodynamic parameters of micellization or vesiculation of CLA at different pHs are also listed in Table 1.

As one can see, ΔG_m^Θ is negative and decreases with increasing temperature in all the experimental temperature ranges, which is beneficial to spontaneous micellization and vesiculation. The corresponding ΔS_m^Θ and ΔH_m^Θ are all positive and $T\Delta S_m^\Theta$ is obviously greater than ΔH_m^Θ, indicating that the common driving forces of the micellization and vesiculation of the CLA solution are entropy increases.

Thermo-Induced Self-Crosslinking of CLA Assemblies

After confirmation of the proposed microenvironment manipulated self-assembling of CLA, the self-crosslinking of the CLA assemblies is then reasonably expected. The CLA assemblies were heated in aqueous phase separately at 50 and 70 °C, and were referred to as CLA-50 and CLA-70, respectively. The thermo-induced self-crosslinking of the CLA assemblies was verified by UV and IR spectra

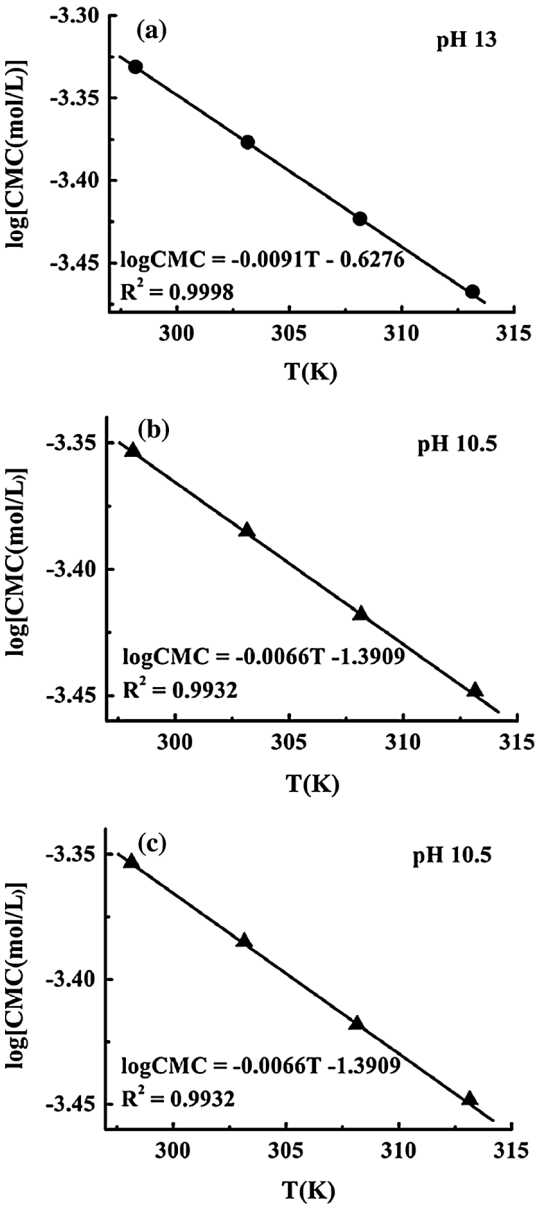


Fig. 3 log CMC–*T* curves of CLA at pH 13 (**a**), pH 10.5 (**b**) and pH 8.6 (**c**)

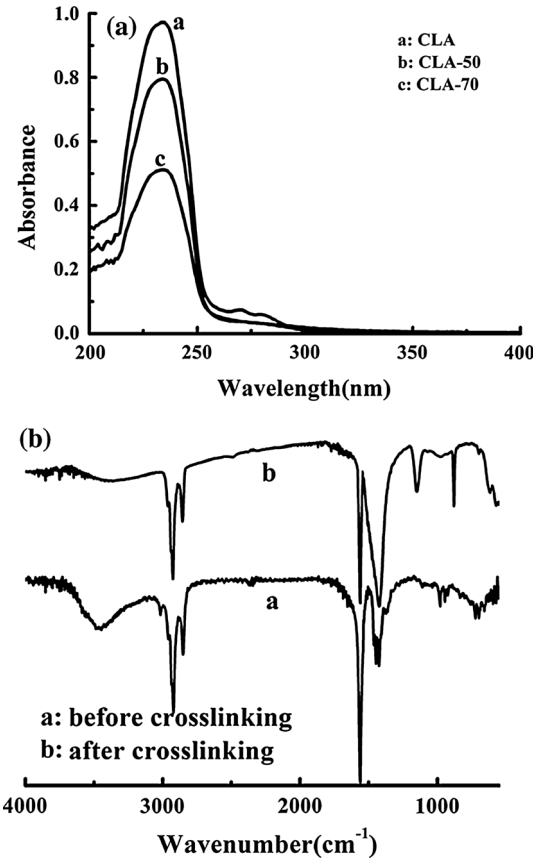


Fig. 4 UV (**a**) and IR spectra (**b**) of CLA before and after crosslinking at pH 10.5

(Fig. 4). With increasing temperature, the characteristic absorption peak of the conjugated double bond at 234 nm is shrunk, which is usually interpreted as the disappearance of the conjugated double bond in the CLA molecules. Additionally, when the crosslinking degree of double bonds is approximately completed, the peaks corresponding to the conjugated double bonds, i.e., 3,015, 1,654, 982 and 946 cm^{-1}, are no longer presented in the FT-IR spectrum.

Furthermore, based on the aforementioned self-assembling behavior of CLA, the CLA assemblies were further attempted to be chemically tethered through self-crosslinking at pH 10.5 and pH 8.6, respectively. From TEM images of the thermo-induced self-crosslinked CLA assemblies, one can clearly find the stable spherical micelles at pH 10.5 (Fig. 5a), and the stable ufasomes with diameters of ~48 nm at pH 8.6 (Fig. 5b). The fact shows that the CLA species self-assemble into micelles and ufasomes corresponding to different pH values, and then structural fixation of the CLA assemblies is completed by the self-crosslinking among the conjugated double bond in the CLA molecules. Interestingly, the micelles obtained at pH 10.5 are quite larger than classical micelles. It may be that the thermo-inducement procedure disturbs the normal micelle formation and results in formation of the large particles, and thus a less disturbing method is being studied in our laboratory to obtain the fixed normal micelle. Moreover, the bilayer thickness of the crosslinked CLA ufasome is greater than the length of an extended CLA molecule but much shorter than twice the length of CLA chain, suggesting that the molecular arrangement within the bilayer membrane of the CLA vesicle is not the normal "tail-to-tail" model. This is possibly because there is intercalation of fatty acid chains from the outer and the inner bilayer leaflets, or the fatty acid chains in the bilayer membrane are considerably distorted due to the conjugated

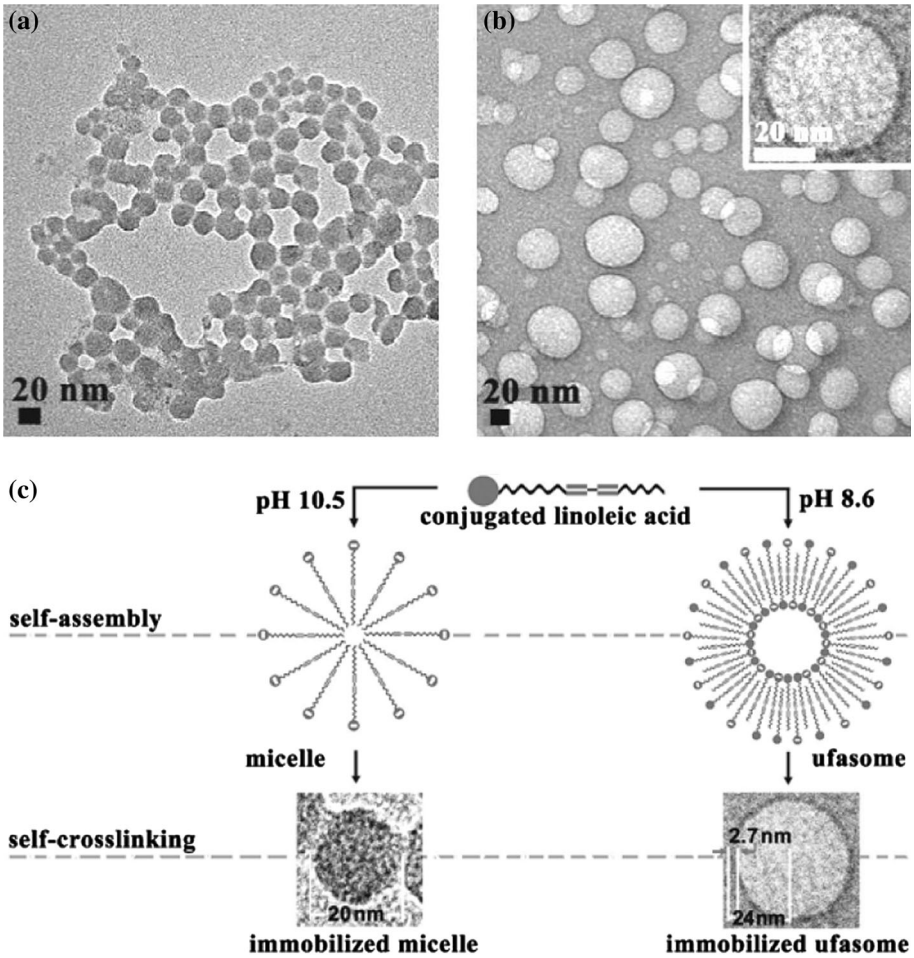


Fig. 5 TEM images of the self-crosslinked spherical micelles at pH 10.5 (**a**) and the ufasomes at pH 8.6 (**b**) prepared by self-crosslinking at 70 °C, and the illustration of the pH-induced formation of the micelle or the ufasome of CLA (**c**)

double bonds situated in the middle of the hydrophobic chain. The size distribution and the molecular arrangement of CLA molecule within the bilayer membrane of the crosslinked CLA ufasome are similar to those of the docosahexaenoic acid (DHA) vesicle [14]. Thus, a tentative mechanism is proposed to account for the formation of the spherical micelles and the ufasomes induced by pH changes as shown in Fig. 5c. At high pH, the $C_{17}H_{31}COO^-$ ions are the main species and result in the formation of spherical micelle in solution; with decreasing pH, $C_{17}H_{31}COO^-$ ions are protonated to form $C_{17}H_{31}COOH$ molecules that are expected to weaken the interaction among $C_{17}H_{31}COO^-$ ions and cause the formation of the ufasomes of CLA. Moreover, it should be emphasized that the transition between micelles and ufasomes occurs only before self-crosslinking.

Other Miscellaneous Properties

The other miscellaneous properties, such as CS, dispersing ability, and solubilization ability of CLA-50 and CLA-70, were examined and the results are exhibited in Fig. 6. At the same time, CLA, LA and pre-crosslinked LA (named as LA-50 and LA-70, obtained by heating LA solution at 50 and 70 °C with the same reaction conditions as in "Methods", respectively) were taken as the controls. Their dispersing abilities and solubilization abilities were investigated over lime soap and toluene, respectively. From the results shown in Fig. 6, it can be found that CS (Fig. 6a), LSDR (Fig. 6b) and solubilization ability (Fig. 6c) of the crosslinked CLA assemblies are superior to those of all LA species, and the higher crosslinking degree of CLA assemblies is, the better the above properties are. More importantly, the above three properties of CLA-50 and CLA-70 are better than that of CLA itself, which is consistent with the aforementioned phenomenon that the aged CLA solution became less sensitive to pH and was accompanied by an increase of resistance to hard water.

Additionally, the microencapsulation and the sustained release of 5-FU were assayed using the crosslinked CLA ufasome fabricated at pH 8.6 and 70 °C. The cumulative release of 5-FU from the crosslinked CLA ufasome is 51.4 % in 1 h, compared to the 95.7 % of cumulative release of 5-FU in the unencapsulated control. Nevertheless,

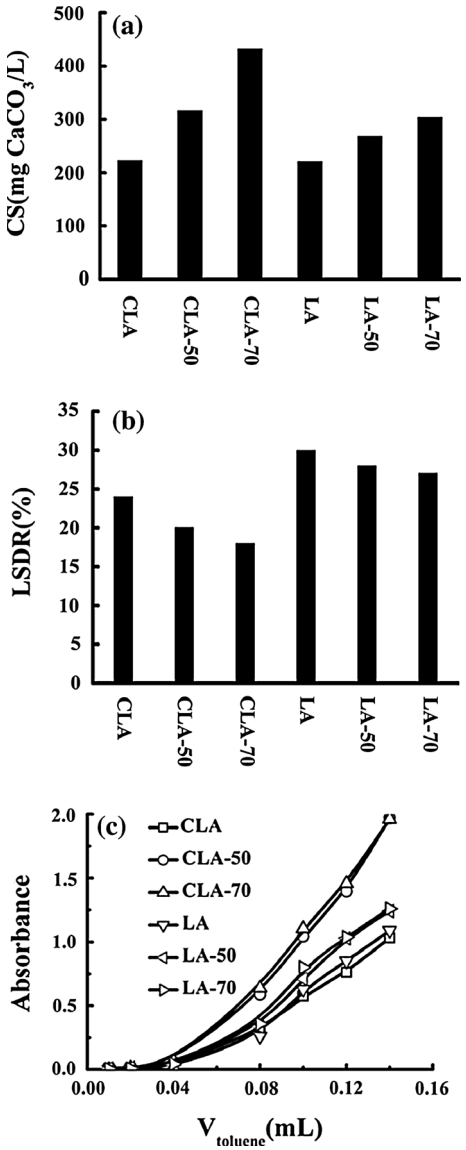


Fig. 6 Calcium stability (**a**), lime soap dispersing requirement (**b**) and solubilization ability (**c**) of various CLA species

the non-crosslinked CLA ufasome collapsed in PBS buffer at pH 7.4, and the cumulative release of 5-FU was unmeasured. Moreover, the ratio of entrapped volume is one of the most significant properties of vesicles. In this paper, the ratio of entrapped volume of the crosslinked CLA ufasome can be abstractly calculated according to the size and the bilayer thickness of the crosslinked CLA ufasome obtained from TEM results and expressed as $V_2/(V_1 - V_2)$. The volumes of the crosslinked CLA ufasome and the internal aqueous compartment are named as V_1 and V_2, respectively. Then the ratio of entrapped volume was calculated as 2.3, indicating that the volume of the internal aqueous compartment is greater than that of the external compartment. So large a ratio indicates that the crosslinked CLA ufasome looks like a vesicle rather than a micelle. In addition, the entrapment capacity (EC, mol/mol) of vesicles is usually represented as a mass ratio or a mole ratio of solute and lipid, and it was expressed as the mole ratio of solute and lipid in this paper. The EC was calculated based on the encapsulation results and using the following equation:

$$\begin{aligned} \mathrm{EC} &= \frac{\text{moles of entrapped } 5-\mathrm{FU}}{\text{moles of CLA}} \\ &= \frac{\text{gross weight of } 5-\mathrm{FU} \times \mathrm{EE} \ / M_{5-\mathrm{FU}}}{\text{moles of CLA}} \end{aligned} \tag{12}$$

where $M_{5\text{-FU}}$ is the molar mass of 5-FU (g/mol). From the microencapsulation and the sustained release results of 5-FU, the EE was determined and calculated according to Eq. (4) as 39.7 %. Then the EC was calculated as 1.6, suggesting that the mole of solute is higher than that of lipid, which supports the above deduction that the crosslinked CLA ufasome is vesicle-like.

Conclusions

In present experiments, a semi-synthetic lipid CLA was obtained by alkali isomerization of LA, and then its self-assembling behaviors at surface layer and in solution were investigated by surface tension strategy. It was found that pH, temperature and ionic strength obviously influenced the surface self-assembling behaviors and micellization (or vesiculation) of CLA, but pH mainly manipulated the self-assembling of the CLA molecules. Furthermore, the TEM images revealed that the stable, homogeneous and cross-linked CLA assemblies were obtained from pH dependent ufasomes by self-crosslinking of the CLA molecules. The crosslinked CLA assemblies are micelles at pH 10.5 and ufasomes at pH 8.6, respectively, because at pH 10.5 the $C_{17}H_{31}COO^-$ ions are the main species of CLA that trend to assemble into micelles, whereas pH 8.6 is slightly higher than the p*K*a of CLA and one-half of CLA species in the solution are the CLA molecules that are prone to form ufasomes. The experimental results showed that the crosslinked CLA assemblies possessed superior CS, LSDR and solubilization ability to CLA itself and all the LA species, and the higher crosslinking degree induced better miscellaneous properties. These findings will help to enrich colloid chemistry properties of unsaturated lipids and reveal the interesting potential to generate new stable ordered microcapsules from ufasomes through combining pH-induced self-assembling and self-crosslinking.

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