Preparation and Characterization of Astaxanthin-loaded Liposomes Stabilized by Sea Cucumber Sulfated Sterols Instead of Cholesterol

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Abstract: Liposomes are widely used as carrier system for bioactive ingredients and usually need to be stabilized by cholesterol. However, the relationship between cholesterol intake and human health has been controversial. The objective of this study was to develop novel multifunctional nanoliposomes stabilized by sea cucumber sulfated sterols via the thin-film hydration method. The liposomes obtained from this study were obviously stable for more than 27 days at 4°C. Astaxanthin was successfully encapsulated by a novel uniform liposome prepared with a mass ratio of egg yolk lecithin to sea cucumber sulfated sterols at 3:1. The mean particle size was 109.53±0.30 nm with 0.241±0.005 polydispersity index and zeta potential value of -21.13±1.01 mV. Astaxanthin-loaded liposome stabilized by sea cucumber sulfated sterols exhibited significantly higher antioxidant activities in terms of DPPH radical-scavenging activity and reducing power than the mixture of astaxanthin and blank sea cucumber sulfated sterols liposome during storage of 6 and 12 days, respectively. The *in vivo* digestion and absorption results showed that the bioavailability of dietary astaxanthin encapsulated in liposomes could significantly be improved. Being an efficient carrier with multi-functions, the novel liposome stabilized by sea cucumber sulfated sterols had great potential in functional food development and biomedical applications.

Key words: liposome, sea cucumber sulfated sterols, cholesterol, astaxanthin, bioavailability

1 Introduction

In recent decades, liposomes have been commonly used as a carrier system for encapsulation of bioactive ingredients^{1,2}. Phospholipids and cholesterol are generally the main components of traditional liposomes. It is known that cholesterol stabilizes the liposome structure by increasing fluidity and decreasing surface charge in the physiological environment^{1,3-5}. However, the relationship between cholesterol intake and human health has been controversial whether it is associated with an increased risk of cardiovascular disease. Higher consumption of dietary cholesterol resulted in a higher incident risk of cardiovascular disease among US adults has been reported previously⁶. Thus, it is essential to seek cholesterol alternatives to stabilize liposomes.

Sterols, the members of the steroid family, are precursors to some hormones and vitamins in organisms. Phytosterols, zoosterols, and mycosterols are derived from the membranes of plants, animals, and microorganisms, respectively. Phytosterols are commonly used as functional ingredients to reduce cholesterol circulation because of its inhibitory effect on low-density lipoprotein cholesterol absorption^{7,8}. In contrast to phytosterols and cholesterol, the C-3 position of marine-derived sea cucumber sterols is a sulfate group as illustrated in Fig. 1 while the C-3 position of cholesterol and phytosterols is a hydroxy group^{7,9-12}. Our previous works done on chemical structure analysis revealed that sea cucumber sterol has a steroidal ring

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structure with a polar head group (sulfate) at the C-3 site and a branched acyl chain at the C-17 site^{11, 12}. This unique structure may result in unique functional characteristics of sea cucumber sterols. The health benefits of sea cucumber sulfated sterols, such as the alleviation of insulin resistance and inflammation as well as the amelioration of lipid accumulation in high-fat-high-fructose diet-induced obese mice, have been reported related to the sulfate group and the branch chain^{11, 12}. It also has been reported that sterol sulfate could alleviate atherosclerosis by means of mediating hepatic cholesterol metabolism in ApoE^{-/-} mice¹³. These findings have provided insights into the potential use of sterol sulfate as functional ingredients for metabolic syndrome prevention. Despite the great potential of sea cucumber sulfated sterols in the sector of health benefits, there is limited information on the role of a cholesterol substitute to stabilize liposomes since the addition of a very polar group to the structure of steroids may increase the surfactant properties.

Astaxanthin is a carotenoid pigment derived from many sources, such as plants, microalgae, bacteria, yeasts, protists, and aquatic animals^{14–16}. In this study, astaxanthin was used as a representative of a bioactive compound with remarkable functional characteristics, such as antioxidant activity, oxidative stress and inflammation alleviation, immune response enhancement, cardiovascular disease prevention, as well as immunoreaction promotion^{14, 15, 17, 18}. Astaxanthin is, however strongly sensitive to light, heat, and oxygen and thus susceptible to isomerization, oxidation, and degradation because of its highly unsaturated molecular structure, resulting in bioavailability reduction and application limitation in nutraceutical, agriculture, and medicine^{15, 19}. Therefore, it is needed to develop means to extend the astaxanthin application array. Higher *in vitro* bioavailability of the astaxanthin-loaded liposomes than the free astaxanthin has been previously reported^{2, 14}. The astaxanthin-loaded liposomes stabilized by cholesterol showed advantages in the features of high encapsulation efficiency, small particle size with uniform size distribution¹⁵. The modifications of membrane surface could prevent astaxanthin from oxidation, degradation and enhance the antioxidant capacity of the liposome^{19, 20}.

The aim of this study was to develop a novel multifunctional nanoliposome stabilized by sea cucumber sulfated sterols via the thin-film hydration method. Astaxanthin was chosen as an encapsulated compound, and the antioxidant capacity as well as *in vivo* digestion and absorption characteristics were evaluated. This study will provide a theoretical basis to develop multifunctional liposomes stabilized by sulfated sterols isolated from sea cucumber.

2 Materials and Methods

2.1 Materials

Sea cucumber (*Cucumeria frondosa*) was purchased from Nanshan Aquatic Products Market (Qingdao, Shandong, China). Egg yolk lecithin (L- α -phosphatidylcholine) with the purity of $\geq 85.6\%$ and cholesterol with the purity of $\geq 99.0\%$ were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Phytosterols (purity $\geq 90.0\%$) and astaxanthin (*Haematococcus pluvisialis* extract, purity $\geq 90\%$) were obtained from Shaanxi Kingsci Biotechnology Co., Ltd. (Shaanxi, China). All reagents employed were of analytical grade. Deionized water (Milli-Q Gradient system, Millipore, Bedford, Mass., USA) was utilized for all experiments.

2.2 Preparation of sea cucumber sterol

Sea cucumber sulfated sterols were extracted from sea cucumber (*C. frondosa*) body walls by the modified method previously described¹². Briefly, the freeze-dried sea cucumber body wall powder was constantly stirred with chloroform:methanol (2:1, v/v) at the total material to liquid ratio of 1:15 (w/v) for 24 h. The sea cucumber mixture was filtrated and added with 1/4 volume of reagent solution. The bottom (chloroform) phase was then collected after placing the mixture into a separatory funnel for 24 h. The crude lipid extract was obtained after vacuum evaporation of chloroform solution. The crude lipid extract was later purified via silica-gel column chromatography using chloroform, chloroform:methanol (9:1, v/v), and acetone as eluents. The acetone extract was further suffered by silica-gel column chromatography using chloroform:methanol (5:1, v/v) as eluent. Thin layer chromatography analysis was used to confirm the purified sterol sample. The purity of sea cucumber sterols was determined to be more than 90% by high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD, Waters 93 Corporation, Milford, MA, U.S.A.) according to our previous works done on chemical structure analysis, suggesting that sea cucumber (*C. frondosa*) sterol was a mixture of cholest-5-en-3 β -yl hydrogen sulfate (51.5%) and 24-methylenecholesterol sulfate (40.5%) as shown in Fig. 1^{11, 12}.

2.3 Preparation of liposomes and astaxanthin loaded liposomes

The liposomes and astaxanthin-loaded liposomes were prepared via thin-film hydration method previously described¹⁵. The cholesterol liposome (CL), phytosterol liposome (PL), and sea cucumber sulfated sterol liposome (SL) were prepared by mixing egg yolk lecithin with the weight ratio to sterols (cholesterol, phytosterol, and sea cucumber sulfated sterols, respectively) of 3:1. The astaxanthin-loaded liposomes; that is, astaxanthin-loaded cholesterol liposome (ACL), astaxanthin-loaded phytosterol liposome (APL), and astaxanthin-loaded sea cucumber sul-

Fig. 1 Chemical structures of sulfated sterols isolated from the sea cucumber. A: Cholest-5-en-3 β -yl hydrogen sulfate, B: 24-methylene-cholesterol sulfate.

fated sterol liposome (ASL) were prepared by mixing egg yolk lecithin with the weight ratio to sterols and astaxanthin of 3:1:1. The mixtures were solubilized in chloroform/methanol (3:1, v/v), and evaporated in a vacuum rotary evaporator (SHB-III, Zhengzhou great wall scientific industry, and trade Co., Ltd, Henan, China) at 45°C to form a thin and dry lipid layer in a pear-shaped spinner flask. The liposome suspension was obtained by hydrating the lipid layer with phosphate-buffered saline (PBS, pH 7.2). The liposome suspension was then sonicated for 10 min (3 s on and 3 s off) at 25°C using an ultrasonic cell crusher noise isolating chamber (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd, Zhejiang, China). All nanosuspensions were flushed with nitrogen gas and then stored at 4°C in darkness.

2.4 Characterization of liposomes

2.4.1 Particle size, poly dispersity index (PDI), and zeta potential

The samples pretreated with a ten-fold dilution were analyzed for particle size, PDI, and zeta potential by dynamic light scattering (DLS) technique using a Zetasizer (Nano ZS90, Malvern Instruments, Worcester, UK) at 25°C, where the detector angle was at 90°.

2.4.2 Encapsulation efficiency

Encapsulation efficiency of astaxanthin-loaded liposomes (ACL, ASL, and ASL) was determined by the modified method¹⁵. Briefly, 400 μ L of astaxanthin-loaded liposome was mixed with 5 mL petroleum ether and stirred at 30°C for 5 min. The supernatant was collected, evaporated, and solubilized in 5 mL chloroform. The percentage of encapsulation efficiency (%EE) was calculated by the following Equation (1).

Equation (1):

$$\%EE = \frac{[\text{Total Astaxanthin content (mg)} - \text{Free Astaxanthin (mg)}]}{\text{Total Astaxanthin content (mg)}} \times 100 \quad (1)$$

2.5 Morphology of astaxanthin-loaded sea cucumber sulfated sterol liposome

The morphology of astaxanthin-loaded sea cucumber sulfated sterol liposome was done by negative staining with phosphotungstic acid solution on copper grids for 2 min. The excess amount of phosphotungstic acid solution was removed with filter paper. The air-dried stained samples were imaged by a transmission electron microscope (TEM, JEM-1200EX, JEOL, Japan) at 100 kV.

2.6 Antioxidant activity

The antioxidant activities of free astaxanthin mixed with sea cucumber sulfated sterol liposome (A + SL) and astaxanthin-loaded sea cucumber sulfated sterol liposome (ASL) were comparatively determined as follows.

2.6.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH radical scavenging activity of A + SL and ASL samples were assessed by the method described previously with some modifications²¹. Briefly, 1 mL of liposome sample was mixed with 2 mL of 0.1 mM DPPH in ethanol, incubated at ambient temperature in darkness for 30 min. The mixture was then centrifuged at 1,000 g for 10 min. The absorbance of the supernatant was measured at 517 nm using UV-visible spectrophotometer (Evolution 300 LC, Thermo fisher scientific, USA). Deionized water was used instead of the sample to prepare blank samples. Ethanol was used to prepare the control instead of the DPPH solution. The DPPH radical scavenging activity was calculated according to Equation (2).

$$\% \text{ Scavenging activity} = \left[1 - \frac{(\text{A517 sample} - \text{A517 control})}{\text{A517 blank}} \right] \times 100 \quad (2)$$

2.6.2 Reducing power

The reducing power of A + SL and ASL samples was determined by the previously reported method with some modifications¹⁵. In short, 1 mL of liposome sample was mixed with 1 mL of 1% potassium ferricyanide and incubated for 20 min at 50°C. The mixture was cooled down to ambient temperature and added with 1 mL of 10% trichloroacetic acid. The mixture was further centrifuged at 20,000 g for 10 min at 25°C and 2 mL of supernatant was

mixed with 2 mL deionized water and 400 μL of 0.1 % ferric chloride. The absorbance was measured at 700 nm against a blank, deionized water, by UV-visible spectrophotometer.

2.7 Digestion and absorption *in vivo*

Digestion and absorption study of free astaxanthin and encapsulated astaxanthin were performed by animal experimental procedures previously described¹⁾ according to the guidelines of the Ethical Committee of Experimental Animal Care at the Ocean University of China. Balb/c male mice were offered with commercial feed for mice chow diet and tap water and were maintained at $65 \pm 15\%$ relative humidity at 25°C with a 12 h light-dark cycle facility. The animals were acclimated for 1 week before the experiment. The mice had a commercial diet and free access to tap water throughout the acclimatization period. For experiment set up, they have been fasted overnight with free water access. They were randomly allocated into 3 groups (with 8 mice each) administered with astaxanthin (A), astaxanthin-loaded sea cucumber sulfated sterol liposome (ASL), and astaxanthin-loaded cholesterol liposomes (ACL) accordingly. The samples were administered by oral

gavage at 100 mg/kg body weight of astaxanthin equivalent in a single dose. After gavage, the mice were sacrificed by cervical dislocation at a time interval of 2, 4, 6, 8, 12 and 14 h. The whole blood was collected into EDTA-containing tubes by removal of the eyeball, and plasma was obtained by centrifugation at 5,600 g for 15 min at 4°C . The serum samples were aliquoted and stored at -80°C prior to analysis by liquid chromatography-mass spectrometry (Exion UPLC-QTRAP 6500 PLUS, SCIEX, USA).

2.8 Statistical analysis

The experiment was done in triplicate. All data are presented as means $\pm$ standard deviation. One-way analysis of variance (ANOVA) was performed. Duncan's New Multiple Range Test was used for mean comparison at 95% confidence level.

3 Results and Discussion

3.1 Mean particle size, PDI, and zeta potential

In the beginning, regarding empty liposomes (CL, PL, and SL), the mean particle size varied from 95.50 ± 1.38

Fig. 2 The particle size distribution and polydispersity index (PDI) of empty liposomes stabilized by cholesterol (A), phytosterols (B), sea cucumber sulfated sterols (C), and astaxanthin-loaded liposomes stabilized by cholesterol (D), phytosterols (E), and sea cucumber sulfated sterols (F).

Fig. 3 The zeta potential values of empty liposomes stabilized by cholesterol (CL), phytosterols (PL), sea cucumber sulfated sterols (SL), and astaxanthin-loaded liposomes stabilized by cholesterol (ACL), phytosterols (APL), and sea cucumber sulfated sterols (ASL). Different letters indicated significant difference at $p < 0.05$.

nm to 156.33 ± 2.51 nm, while for the corresponding astaxanthin loaded liposomes (ACL, APL, and ASL), sizes ranged from 95.54 ± 1.08 nm to 125.23 ± 1.37 nm (Fig. 2). Moreover, the PDI varied from 0.133 ± 0.038 to 0.241 ± 0.005 for all empty liposomes and astaxanthin-loaded liposomes. The results from this study showed that the sterol type significantly affected the mean particle size and PDI of the liposomal formulations. Notably, the mean particle size and PDI of all liposome formulations made by the thin-film hydration method were below 200 nm and less than 0.3, respectively, which was in good agreement with the previous findings^{1, 22}. It is known that the PDI value of 0.3 or below is considered to be acceptable for a monodisperse and homogenous population of phospholipid particles, such as nanoliposomes in drug delivery applications^{1, 23}.

The negative zeta potential values of empty liposomes and astaxanthin-loaded liposomes stabilized by different kinds of sterols after fresh preparation are illustrated in Fig. 3. No significant difference was found in the zeta potential values of SL (-22.33 ± 1.35 mV) and ASL (-21.13 ± 1.01 mV) ($p > 0.05$), which were significantly higher than those stabilized by cholesterol as of CL (-6.30 ± 0.78 mV) and ACL (-5.61 ± 0.38 mV), as well as plant sterol as of PL (-4.72 ± 0.68 mV) and APL (-5.80 ± 0.21 mV). The higher the absolute value of zeta potential indicated higher stability of the particle system in general^{1, 24}. According to the previous finding, the zeta potential of liposomes was mainly attributed to the lecithin compositions due to the lack of charge for sterols²⁵. While, in the present study, the higher magnitude of zeta potential of sea cucumber sulfated sterol stabilized liposomes might be resulted from the sulfate group at C-3 position of sea cucumbers derived sterols as illustrated in Fig. 1, which is different from cholesterol and phytosterols where the C-3 position is a hydroxyl group^{7, 9–12}.

Table 1 Mean particle size and polydispersity index (PDI) of empty liposomes stabilized by cholesterol (CL), phytosterol (PL) and sea cucumber sterol (SL) during storage at 4°C.

Samples	Droplet diameter (nm)									
	Polydispersity index									
	0d	3d	6d	9d	12d	15d	18d	21d	24d	27d
CL	95.50 ± 1.38^{df}	134.00 ± 0.60^{db}	149.70 ± 2.74^{db}	230.30 ± 2.15^{aA}	—	—	—	—	—	—
	0.133 ± 0.038^{ab}	0.145 ± 0.011^{bc}	0.181 ± 0.017^{abb}	0.223 ± 0.028^{aAB}	—	—	—	—	—	—
PL	156.33 ± 2.51^{ba}	153.07 ± 0.21^{ba}	153.37 ± 1.85^{bb}	220.83 ± 2.64^{ab}	—	—	—	—	—	—
	0.163 ± 0.025^{aAB}	0.149 ± 0.014^{bc}	0.172 ± 0.006^{abb}	0.195 ± 0.002^{abc}	—	—	—	—	—	—
SL	118.10 ± 0.98^{bc}	119.30 ± 0.70^{ba}	120.00 ± 0.20^{bc}	175.40 ± 1.16^{cd}	209.03 ± 2.02^{cA}	12.17 ± 0.93^{bc}	216.93 ± 0.47^b	214.73 ± 2.93^c	290.43 ± 6.45^{aB}	201.63 ± 3.84^d
	0.240 ± 0.015^{bA}	0.238 ± 0.015^{bA}	0.236 ± 0.020^{bA}	0.166 ± 0.010^{bc}	0.173 ± 0.026^{dB}	0.154 ± 0.022^f	0.216 ± 0.018^{cd}	0.201 ± 0.003^{de}	0.254 ± 0.004^b	0.295 ± 0.005^a

Note: Different small letters indicate significant differences within groups ($p < 0.05$). Capital letters indicate significant differences between groups ($p < 0.05$). '—' indicated that the liposomes exhibited non-uni form particle size.

Fig. 4 The stable days of empty liposomes stabilized by cholesterol (CL), phytosterols (PL), sea cucumber sulfated sterols (SL), and astaxanthin-loaded liposomes stabilized by cholesterol (ACL), phytosterols (APL), and sea cucumber sulfated sterols (ASL). Different letters indicated significant difference at $p < 0.05$.

3.2 Physical stability of liposome formulations

The main objective of the present study was to obtain a physically stable liposome system to deliver functional food ingredients. The stability of liposome formulations stabilized by a different type of sterols was analyzed by determining the droplet size and polydispersity index with the time of storage at 4°C (Table 1). There were significant differences in the stable days between liposomes stabilized by different sterol types ($p < 0.05$). The empty liposome formulations stabilized by sea cucumber sulfated sterols maintained physically stable during storage at 4°C, with little change in mean particle diameter and polydispersity index for more than 27 days, compared with ones stabilized by traditional cholesterol and plant sterol as of 9 days ($p < 0.05$) (Table 1, Fig. 4). Liposome formulations stabilized by sea cucumber sulfated sterols demonstrated more stable systems than the other liposome formulations stabilized by traditional cholesterol and plant sterol ($p < 0.05$), which was consistent with the results of PDI as shown in Fig. 3. Notably, the stable days of astaxanthin-loaded liposomes were significantly shorter than those of empty liposomes ($p < 0.05$), which might result from interference of astaxanthin insertion in the arrangement of phospholipids in bilayers leading to the negative effect on the stability of liposomes according to the previous finding for curcumin-loaded liposomes^{19, 26}.

3.3 Encapsulation efficiency of liposome formulations

The percentage of encapsulation efficiency (% EE) of astaxanthin-loaded liposomes stabilized by cholesterol (ACL), phytosterols (APL), and sea cucumber sulfated sterols (ASL) were not significantly different ($p > 0.05$) as shown in Fig. 5. The % EE of astaxanthin-loaded liposomes in this study were found to be in a range of $94.55 \pm 5.36\%$ to $97.89 \pm 5.34\%$, which were comparable to the

Fig. 5 The percentage of encapsulation efficiency (% EE) of astaxanthin-loaded liposomes stabilized by cholesterol (ACL), phytosterols (APL), and sea cucumber sulfated sterols (ASL).

previous report on optimization study of astaxanthin-loaded liposome preparation as of $97.68 \pm 0.34\%$ ¹⁵, and a range of $91.42 \pm 0.78\%$ to $97.13 \pm 0.14\%$ ²⁷. The high % EE of liposomes obtained from this study suggested a potential use of them as drug delivery systems.

3.4 Liposome morphology

Transmission electron microscope images of astaxanthin-loaded sea cucumber sulfated sterol liposomes (ASL) with a scale bar of 20 nm and a scale bar of 200 nm are presented in Fig. 6. Liposomes were observed to be spherical and oval with a smooth surface similar to images previously reported for astaxanthin-loaded liposomes^{15, 27}, cisplatin-loaded liposomes²⁸, sea cucumber-derived saponin liposomes¹, and breviscapine-loaded liposomes²⁹. The particle size of liposomes was approximately 100-150 nm in size, which was in good agreement with the mean size particle measured by Zetasizer (Table 1).

3.5 Antioxidant activity

Figure 7A illustrates DPPH scavenging activity profiles of free astaxanthin mixed with sea cucumber sulfated sterol (A + SL) and astaxanthin-loaded sea cucumber sulfated sterol (ASL) liposomes during storage of 6 days at 4°C. The % DPPH scavenging activity of the A + SL sample significantly decreased to a higher extent than the ASL sample over storage time. DPPH scavenging ability of astaxanthin is resulted from two β -ionone rings and eleven conjugated double bonds in the structure of astaxanthin¹⁵. Figure 7B presents reducing power profiles of the A + SL and the ASL samples during storage of 12 days at 4°C. In the same manner, as % DPPH scavenging activity, the reducing power of the A + SL sample significantly decreased at an expansively higher degree than the ASL sample over storage time. The reducing power of astaxanthin was affected by the conjugated double bond that provides electrons to reduce Fe^{3+} to Fe^{2+} , and by the molecular steric

Fig. 6 Transmission electron micrographs of astaxanthin-loaded liposomes stabilized by sea cucumber sulfated sterol (ASL) with scale bar of 20 nm (A) and 200 nm (B).

Fig. 7 DPPH radical scavenging activity (A) and reducing power (B) of a mixture of astaxanthin and empty liposomes stabilized by sea cucumber sulfated sterols (A + SL), and astaxanthin-loaded liposomes stabilized by sea cucumber sulfated sterols (ASL) during storage.

hindrance effect. The higher magnitude of the reducing power of the ASL sample expressed over the A + SL sample might be attributed to the reduction in molecular steric hindrance effect when astaxanthin was incorporated into the liposome membrane, resulting in an increase in reducing power¹⁵⁾. In general, liposomes, stored as aqueous dispersions, are unstable since the encapsulated material tends to leak out of the bilayer structure³⁰⁾, affecting astaxanthin stability during storage. The astaxanthin insertion into the lipid bilayer not only decreased membrane fluidity but also increased micropolarity in the membrane, leading to an increase in antioxidant activity³¹⁾. The result of this study showed that the antioxidant activity of astaxanthin-loaded liposome stabilized by sea cucumber sulfated sterols was remarkably enhanced as compared to free astaxanthin mixed with sea cucumber sulfated sterol liposome in terms of % DPPH scavenging activity and reducing power profiles, respectively.

3.6 *In vivo* digestion and absorption

The absorption process, *in vivo* stability and release profile, are all very important factors to the oral bioavailability of astaxanthin. The result of this study showed that the concentrations of astaxanthin in mice serum after oral administration of astaxanthin-loaded liposomes either stabilized by cholesterol (ACL) or sea cucumber sulfated sterols (ASL) were remarkably enhanced as compared to free astaxanthin (A) as shown in Fig. 8. The concentrations of astaxanthin in mice serum slightly increased and peaked at 0.130 ± 0.010 $\mu\text{g/mL}$ at 6 h after oral administration of free astaxanthin (A) and decreased to be 0.031 ± 0.009 $\mu\text{g/mL}$ at 14 h. The bioavailability in a range of 10–50% of the given dose when astaxanthin was administered orally according to the previous report with the amount of astaxanthin recovered of 0.140 ± 0.0102 $\mu\text{g/mL}$ and 0.0575 ± 0.0106 $\mu\text{g/mL}$ in plasma at 8 h and 24 h, respectively, after oral administration at a dose of 100 mg/kg in rats³²⁾. On the other hand, the concentrations of astaxanthin in mice serum rapidly increased during the first 4 h after oral administra-

Fig. 8 The concentration-time curves of astaxanthin in mice serum after oral administration of free astaxanthin (A), astaxanthin-loaded liposomes stabilized by cholesterol (ACL) and astaxanthin-loaded liposomes stabilized by sea cucumber sulfated sterols (ASL).

tion of astaxanthin-loaded liposomes either stabilized by cholesterol (ACL) or sea cucumber sulfated sterols (ASL) and gradually decreased after 4 h to be 0.352 ± 0.011 µg/mL and 0.298 ± 0.010 µg/mL for the ACL and the ASL samples, respectively, at 14 h. The concentrations of astaxanthin in mice serum after oral administration of astaxanthin-loaded liposomes either stabilized by cholesterol (ACL) or sea cucumber sulfated sterols (ASL) were not significantly different ($p > 0.05$). The release of astaxanthin in mice serum of astaxanthin-loaded liposomes was prolonged because of slow disintegration and solid structure of lipid carriers according to previous reports^{33, 34}. Moreover, only liposomes that exist in the gastrointestinal tract and be able to enter into the mucus layers can reach the intestinal epithelia and can be absorbed together with the loaded materials^{29, 35}, suggesting that dietary astaxanthin in liposome form could significantly improve the bioavailability.

4 Conclusion

In the present study, a novel multifunctional nanoliposomes stabilized by sea cucumber-derived sulfated sterols rather than cholesterol was successfully developed using the classical thin-film dispersion method. The result showed that the sterol type used to stabilize liposomes significantly affected the mean particle size, PDI, and zeta potential values of the liposomal formulations. Liposome formulations stabilized by sea cucumber sulfated sterols either empty or astaxanthin-loaded ones demonstrated a higher magnitude of zeta potential values than the other liposome formulations stabilized by traditional cholesterol and plant sterols, resulting in longer stable days. The anti-

oxidant activity of astaxanthin-loaded liposome stabilized by sea cucumber sulfated sterols was remarkably enhanced compared to the free astaxanthin mixed with sea cucumber sulfated sterol liposome. In addition, the digestion and absorption results *in vivo* showed that dietary astaxanthin in liposome form could significantly improve the bioavailability. The result from this present study suggested that the use of nanoliposomes as an astaxanthin delivery system could be an effective method to improve the level of astaxanthin supplied in the body. As an efficient carrier with multi-functions, the novel liposome stabilized by sea cucumber-derived sulfated sterols had great potential in functional food developments and biomedical applications.

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Conflicts of Interest

The authors declare no conflicts of interest.

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