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The self-crosslinked ufasome of conjugated linoleic acid: Investigation of morphology, bilayer membrane and stability

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ABSTRACT

Unsaturated fatty acid liposomes (Ufasomes) have attracted interests because of the ready availability of unsaturated fatty acids and the simple assembly strategy. However, the colloidal instability of the ufasomes hinders them from applying in the fields of drug delivery and food additives. In the present work, conjugated linoleic acid (CLA) with triple activities of bioactive, assembling and crosslinking was employed as a new molecular building block to construct ufasome and afterwards crosslinked ufasome. First, CLA ufasome was self-assembled from CLA molecules in response to pH variation, and the suitable CLA concentrations and pH ranges were determined by surface tension measurement and acid–base titration. Subsequently, the self-crosslinked CLA ufasome was prepared by intra-ufasomal crosslinking of conjugated double bonds in the CLA molecules. The morphologies of the self-crosslinked CLA ufasomes were imaged using transmission electron microscopy (TEM), from which the size of 20–50 nm and the bilayer thickness of 2.7 ± 0.5 nm were detected. Most importantly, based on the comparison of the bilayer thicknesses of the different fatty acids, the molecular arrangement in the bilayer membrane of the self-crosslinked CLA ufasome is named "side-by-side" model contrary to the ordinary "tail-to-tail" model. The pH stability of the self-crosslinked CLA ufasome was examined in virtue of dynamic light scattering tests. Finally, in vitro release results of 5-fluorouracil from the self-crosslinked CLA ufasome showed that the process was slow and sustainable.

1. Introduction

Colloidal soft matter [1,2], especially molecular assemblies such as liposomes, nanotopes, macromolecular micelles and the relative hollow spheres, have attracted particular attention academically. However, their applications in food additives, drug delivery and sustained release systems are still largely unexplored, which is at least partially due to concerns of the molecular building blocks regarding the special molecular structure, complex constituent, possible toxicity, high cost, etc. Fatty acid vesicles are colloidal suspensions with a closed lipid bilayer membrane that is composed of fatty acids and their ionized species (soap, anionic fatty acids) [3,4]. The formation of unsaturated fatty acid vesicles was first reported by Gebicki and Hicks [5] in 1973 and named as "ufasomes". Recently, various fatty acid vesicles have been preparing from different molecular building blocks [6–12], including saturated fatty acids such as octanoic acid [13] and decanoic acid (DA) [14,15], unsaturated fatty acids such as oleic acid (*cis*-9-octadecenoic acid) and linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid) [5,16], and even highly unsaturated fatty acids as exampled by docosahexaenoic acid (DHA) [8]. It is the excellent biocompatibility, simple constituents and ready availability that become the advantages of fatty acids superior to the aforementioned other molecular building blocks [17]. Nevertheless, since the driving force to form fatty acid vesicles is essentially the co-assembly of fatty acid and their ionized species and dependent on the noncovalent interaction between fatty acid species, the colloidal stability of fatty acid vesicles is inherently weak. Moreover, the applications of fatty acid vesicles in the fields of food additives, drug delivery and sustained release systems are still hindered by the colloidal stability such as pH-, temperature-, divalent cation- and even concentration sensitivity [3,12].

In response to the challenges, various kinds of polymerized assemblies, either surfactant- or lipid-type, have been introduced to overcome the colloidal instability problem [18,19]. One of the

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interesting ways to complete structural stabilization of molecular self-assemblies was chemically tethering the polymerizable molecular building blocks [20]. It has been frequently seen that the similar polymerization manner was widely used in structural fixation of macromolecular micelles [21] and polymerizable surfactant assemblies [22,23], but it has been rarely performed in structural fixation of liposomes and ufasomes. Up to date, an anionic lipid, 11-acrylamidoundecanoic acid (AUA), was demonstrated that it could self-assemble into ufasome in aqueous solution, and then stable vesicle was prepared through intra-ufasomal crosslinking between the terminal double bond in AUA molecules [20,24]. In addition, Lee and coworkers [25] reported that crosslinked 10-undecenoic acid (UDA) vesicle with improved colloidal stability was obtained from UDA ufasome through thermal polymerization in the bilayer membrane of the UDA ufasome. The fact that the isolated double bond in AUA or UDA molecules could be crosslinked demonstrates that the double bond situates at the terminal of hydrophobic chain has small steric hindrance. However, to the best of our knowledge, up to date, there is no report about stable vesicles obtained from natural unsaturated fatty acids. That is because the double bonds in the molecules of oleic acid (OA), linoleic acid (LA) and linolenic acid (LNA) are nonconjugated and located in the middle of the molecules, resulting in large steric hindrance although they are cheap and readily available. Interestingly, it is noteworthy that a novel stable vesicle was reported by introducing 10,12-pentacosadiynoic acid (PCDA) into bilayer membrane of liposomes and chemically tethering the liposome through UV crosslinking of conjugated triple bonds in the middle of PCDA molecule [18]. Recently, liposome of diacetylene lipids were chemically tethered the conjugated triple bonds situated in the middle of diacetylene lipid chain to yield the stable polymerized liposome [19], and similar results have been summarized [26–28]. Nevertheless, all the aforementioned lipids and phospholipids generally used as molecular building blocks are unnatural lipids, and thus possess many disadvantages such as high cost, rare resource, cumbersome synthesis, instability and unsafety.

In summary, the desired essential features of the molecular building blocks used to assemble stable ufasomes are strong activity of assembling and crosslinking, safety, low cost and ready availability. In the present work, conjugated linoleic acid (CLA) is chosen as an excellent candidate of the molecular building blocks for constructing the stable ufasomes, because CLA possesses many advantages: (1) it has similar self-assembling activity to the ordinary fatty acids due to the carboxyl group; (2) it has strong crosslinking activity due to the conjugated double bonds, which can eliminate the ill-effect caused by the dissatisfactory location of double bonds; (3) it is bioactive and biocompatible since it is naturally present in plants and animals and used for nutraceuticals; and (4) it is readily available by semisynthesis from natural LA with high yield and low cost. Therefore, in this paper, CLA was firstly prepared by semisynthesis method through alkali isomerization reaction of LA to provide a novel molecular building block combining with triple activities of bioactive, assembling and crosslinking in one molecule. And then, the CLA ufasome was gained from self-assembly process of CLA molecules induced by pH variation. Subsequently, the self-crosslinked CLA ufasome was obtained by structural fixation of the CLA ufasome through intra-ufasomal thermal-crosslinking of the conjugated double bonds in CLA molecules, and the pH stability of the self-crosslinked CLA ufasome was examined in virtue of dynamic light scattering tests. Finally, in vitro release of 5-fluorouracil from the self-crosslinked CLA ufasome was also preliminarily investigated. These above investigations are significant for fabricating and stabilizing the ufasome constructed by CLA as molecular building blocks and using the stable ufasome as molecularly ordered microcapsules.

2. Materials and methods

2.1. Materials

Safflower oil was purchased from Cofco Co. Ltd. Boric acid, sodium borate, dibasic sodium phosphate (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and 5-fluorouracil (5-FU) were all analytical reagent grades and purchased from Sinopharm Chemical Reagent Co. Ltd. Ammonium persulfate (APS) was purchased from Sinopharm Chemical Reagent Co. Ltd. and further purified by recrystallization before use. Ultrapure Millipore water (18.2 MΩ cm) was used throughout.

2.2. Preparation and characterization of CLA

LA was prepared from saponification of safflower oil and enriched by urea inclusion. Typically, 400 mL of sodium hydroxide solution (4 wt% in ethanol) was added dropwise for 4 h into a flask containing 100 g of 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, washed with distilled water and saturated saline alternatively. The resultant fatty acid mixture was dried over Na_2SO_4. Then, 30 g of fatty acid mixture was added dropwise in 2 h into the mixture of 75 g urea in 240 mL of ethanol (95 vol%) 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. 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 after removal of the ether.

CLA was semi-synthesized by alkali isomerization of LA with KOH and ethylene glycol. Ten grams of LA was dropped in 30 min into a flask containing 5 g of KOH and 15 mL of 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, 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., China) using ethanol as solvent and a 400 MHz Bruker Avance III NMR spectrometer (Bruker, Germany), respectively. IR: the peaks of 3015, 1709 and 1654 cm^{-1} were attributed to stretching vibration of =CH, C=O and C=C, respectively. Compared to LA, the out-of-plane bending vibration bands at 946 and 982 cm^{-1} were corresponded to *trans*-, *cis*-CLA and *cis*-, *trans*-CLA, indicating the presence of conjugated double bond [29]. The characteristic absorption peak of conjugated double bond at 234 nm [30] 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). In comparison with LA, the peak at 2.05 ppm assigned to hydrogen of C11 in LA molecule disappeared. The percentages of the enriched LA and the obtained CLA were analyzed using a FULI 9790 gas chromatograph (Fuli Analytical Instrument Co. Ltd., China) equipped with PEG 20000 capillary column and FID detector, and the temperatures of the column and the detector were 220 and 270 °C, respectively. The percentages of the enriched LA and the obtained CLA were up to 97.6 and 96.0%, respectively.