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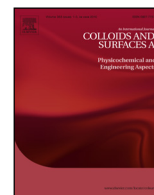

Inter-vesicle polymerization using nonionic oxyethylene-hydrogenated castor oil

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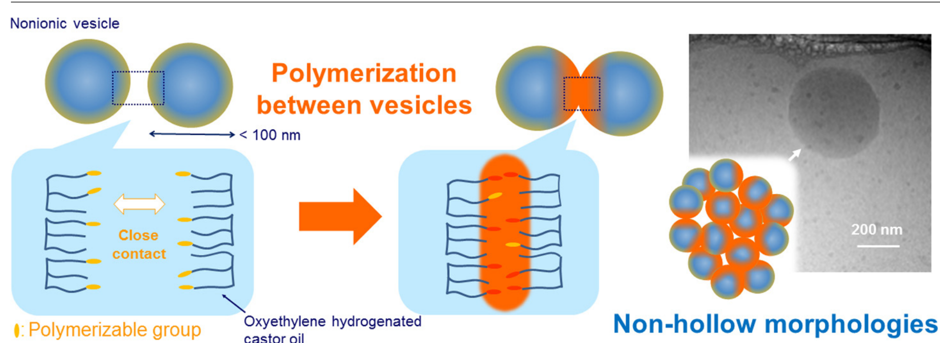
HIGHLIGHTS

- An oxyethylene-hydrogenated castor oil with polymerizable moieties forms vesicles.
- Vesicle polymerization is dominated by temperature and lipid concentration.
- The mechanism of inter-vesicle polymerization is discussed.
- Non-hollow morphologies of the polymerized vesicle were observed by cryo-TEM.

GRAPHICAL ABSTRACT



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ABSTRACT

Oxyethylene-hydrogenated castor oil with acrylate-modified oxyethylene end groups was employed for the preparation of polymerizable vesicles in aqueous solution. ^{1}H NMR spectroscopy was used to investigate the polymerization process using azobis(isobutyronitrile) as an initiator. UV light promoted the vesicle polymerization, and polymerized vesicles were shown to be resistant to disruption by polyoxyethylene(10) octylphenyl ether. The dependence of resistance on vesicle concentration and incubation temperature was suggested to result from the inter-vesicle polymerization. Cryo-transmission electron microscopy provided evidence that unilamellar vesicles with a diameter smaller than 100 nm were distorted by polymerization, thereby exhibiting non-hollow morphologies.

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1. Introduction

Although liposomes and vesicles have displayed useful potential in many applications, including nanoparticle synthesis [1], metal-ion separation [2], and drug-delivery vehicles [3], their inherent lack of storage stability represents a drawback that limits their utilization. To overcome this issue, polymerizable vesicles were introduced and various polymerization methods have been reported [4–9]. To prepare such robust vesicles, lipids with polymerizable moieties need to be synthesized; however, their synthesis often involves complicated and multistep reactions. From the perspective of costs, convenience, and simplicity, nonionic lipids are considered to be promising candidates, because they have been recognized as cost-effective substances with a relatively facile synthesis design of molecular structures. In particular, if nonionic lipids are originated from natural products, they have additional advantages in terms of safety use. Considering these points, we focused our investigation on oxyethylene-hydrogenated castor oils [10,11], which are used in pharmaceuticals, cosmetics,

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toiletries, etc. Owing to their biocompatibility and non-toxicity, oxyethylene-hydrogenated castor oils have attracted much interest and, to some extent, are already commercially available.

In this work, we report the formation of vesicles using lipid **1**, i.e., an oxyethylene-hydrogenated castor oil bearing an oxyethylene end group modified by acrylate (Fig. 1). A large number of lipids carrying polymerizable moieties have been shown to successfully stabilize vesicles through the formation of polymeric networks [12–15]. However, the preparation of robust vesicles is not the only feature of polymerizable vesicles. O'Brien and co-workers [16–18] have reported that a mixture of polymerizable lipids and nonfunctional lipids may cause phase separation in vesicle membranes, which can destabilize the vesicles in some cases. Various types of vesicle polymerization methods are needed for the diverse range of applications of polymerizable vesicle besides membrane stabilization. The copolymerization of monomers inserted in a matrix of polymerizable lipids has been investigated by Jung et al. [19] and they have observed the interesting morphology of thickened and wrinkled vesicle bilayers. Raghavan and co-workers [15] observed "dimpled" of vesicles resulted from the copolymerization of bilayer with a hydrophobic cross-linker. Studies about the morphological changes that result from vesicle polymerization are limited, while several research groups have reported the morphological changes that are induced by the polymerization of monomers in a vesicle matrix such as closed spherical polymer shells [20,21] and the so-called parachute-like morphologies [22,23].

Here, we report the polymerization of nonionic vesicles that contain lipid **1** and show a new type of vesicle polymerization, which result in morphological changes. The acrylate moieties were polymerized in a vesicular suspension, using UV light in the presence of a suitable initiator that can generate free radicals. The stabilization of the polymerized vesicle was investigated by the addition of a bilayer-disrupting agent. The polymerization mechanism is discussed in terms of its dependence on the concentration of lipid **1** and incubation temperature. Cryo-transmission electron microscopy (cryo-TEM) revealed remarkable changes in the morphology of the polymerized vesicles.

2. Experimental

2.1. Materials

Lipid **1** was kindly provided by Daiichi Kogyo Seiyaku Co. (Kyoto, Japan) and used as-received. The numbers of the oxyethylene groups and the acryl groups of lipid **1** were determined by ^{1}H NMR by comparing the peak area of the methyl group (0.88 ppm) with those of oxyethylene (3.63–3.66 ppm) and acryl (5.83–6.46 ppm). It was found that the number of the oxyethylene groups and the acryl groups was 7 and 1.5, respectively. Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol. A bilayer-disrupting detergent, e.g., polyoxyethylene(10) octylphenyl ether (Triton X-100) and D_2O

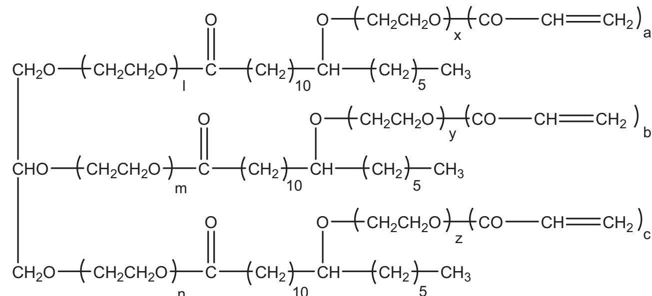

Fig. 1. Chemical structure of polymerizable oxyethylene-hydrogenated castor oil (lipid **1**).

(99.9–%) for the ^{1}H NMR measurements were obtained from Wako Chemical Co. (Kyoto, Japan) and used as-received. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Invitrogen and used as-received. Other materials were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Vesicle preparation and polymerization

Lipid **1** and AIBN were dissolved in benzene, and the solvent was removed with a rotary evaporator at 35 °C to leave a thin film on the wall of a flask. Further traces of the solvent were removed by drying the film under vacuum for at least 2 h. Millipore water was added to this and the dispersion was vortexed. D_2O was used instead of Millipore water in the ^{1}H NMR experiments. Unilamellar vesicles were obtained after 10 freeze (liquid nitrogen)–thaw (25 °C water bath) cycles. The concentration of lipid **1** in the obtained dispersion was 43 mM. The AIBN concentration was 7.2 mM, which was optimized via a separate experiment (for further details, see dependence of polymerization on AIBN concentration in the Supplementary data). To prepare the samples of various vesicle concentrations, Millipore water was added to the original vesicle dispersion (43 and 7.2 mM of lipid **1** and AIBN, respectively). Exposure to light was avoided during all vesicle-preparation procedures. Prior to polymerization, oxygen was removed by bubbling argon through the sample dispersion. The vesicle samples were then sealed and incubated for 45 min. UV irradiation (<330 nm) was carried out during the incubation with gently stirring by using a xenon lamp (500 W) equipped with a photoguide tube and a Toshiba UV-D33S filter.

2.2.2. Lysis of vesicles

Triton X-100 was injected into the incubated vesicle sample to reach a 20-fold molar excess Triton X-100 to lipid **1**. The sample was stirred and kept at 25 °C for 24 h. The optical density at 800 nm (OD_{800}) of each sample was then used to describe the resistance to the bilayer disruption. The experiments were carried out at 25 °C with a Shimadzu UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan), using a cuvette with an optical-length path of 1 mm for the measurements of the wide range of the optical density. More than four experiments were conducted for each sample.

2.2.3. Vesicle aggregation assay

Vesicle aggregation was followed by optical-density (turbidity) measurements at 800 nm with a Shimadzu UV-3600 spectrophotometer. The temperature of the turbidity measurements was controlled by an external thermostat. The turbidity was recorded 10 min after the temperature change, while the sample was gently stirred in a cuvette with an optical-length path of 10 mm. The baseline turbidity was provided by monitoring OD_{800} of the vesicle sample containing 0.83 mM of lipid **1** at 1 °C ($OD_{800} = 1.3 \pm 0.1$). The vesicles that underwent neither irradiation nor incubation were used for the aggregation assay; this experiment was repeated eight times.

2.2.4. Cryo-TEM

A copper grid (200-mesh) was dipped into the sample dispersion, and the excess sample was blotted with filter paper to leave a thin film on the grid. This film was vitrified in liquid ethane and observed at approximately −176 °C using a JEM-3100FEF (JOEL, Tokyo, Japan) operating at 300 kV.

2.2.5. ^{1}H NMR

The experiments were conducted at 25 °C. ^{1}H NMR spectra were acquired using a JNM-270 instrument (JOEL, Tokyo, Japan)

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