Preparation and Characterization of Vesicles Using Octasubstituted Cyclotetraphosphazene

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A cyclotetraphosphazene derivative with eight chains was synthesized from octachlorocyclotetraphosphazene. The vesicles were prepared using the cyclotetraphosphazene derivative and cholesterol. The resulting vesicles were characterized by TEM and measurements of their encapsulation efficiency. The stability of the vesicles was enhanced with the addition of dihexadecylphosphate. The size and the encapsulation efficiency of the vesicles changed according to the amount of cholesterol added. The size and the encapsulation efficiency of the vesicle were lowest when the mole ratio (cholesterol: the cyclophosphazene derivative) was 0.9.

Key Words : Cyclotetraphosphazene, Vesicle, Cholesterol, Encapsulation efficiency

Introduction

Vesicles are formed with a spherical molecular bilayer membrane containing water. Research on vesicles began with the model system of a biological membrane.[1,2] There are many applications of vesicles because vesicles have a unique structure in which the space is separated by a membrane, and water is contained inside the membrane. These applications include drug carrier system,[1] photochemical solar energy conversion systems,[3,4] reactivity control systems,[5-7] and controlled release systems.[8-12]

Many vesicles have been formed from natural and synthetic surfactants.[13-15] However, most vesicles are formed from an ionic surfactant. A small amount of vesicles have been formed from nonionic surfactants. Nonionic surfactants have lower toxicity than ionic surfactants. Therefore, nonionic surfactants can be used in biological applications. However, the stability of the vesicle from nonionic surfactants is inferior to that from ionic surfactants.

Cyclotriphosphazene has a hexagonal structure and cyclotetraphosphazene has an octagonal structure. Most studies on the cyclotriphosphazene focused on ring opening polymerization of the cyclotriphosphazene ring.[16,17] With the exception of polymerization research, cyclophosphazene derivatives have been examined as potential flame retardant materials,[18-24] lubricants,[25] and star polymers.[26-28]

In this study, a cyclotetraphosphazene derivative was synthesized, and a vesicle was formed using this compound and cholesterol. The formed vesicles were characterized, and the encapsulation efficiency of some compound was measured.

Experimental

Reagents and instruments. Phosphonitrilic chloride oligomer (PCO) was purchased from Shanghai Qichen Chemicals, and octachlorocyclotetraphosphazene (OCCP) was obtained by fractional vacuum sublimation of PCO, followed by recrystallization in hexane. PCO is a mixture containing a trimer, tetramer, and a small amount of oligomer. Cholesterol, 1,6-diphenyl-1,3,5-hexatriene (DPHT), tetrabutylammonium bromide (TBAB), dihexadecyl phosphate (DHPP), Sephadex G-75, Tritron X-100 were purchased from Aldrich Chemical. Tetraethylene glycol monodecyl ether (TGME) was supplied by Fluka Chemical.

Nuclear magnetic resonance spectroscopy (NMR) was performed using a DPX 300 (Bruker), and Infrared spectroscopy (IR) was performed using a FT-IR 680 (Jasco International). Elemental Analysis was performed using EA 1110 (CE Instruments). A ISSK2 (ISS) spectrofluorometer, a Cole-Parmer 4710 250 W sonicator, and a JEOL JEM-2010 transmission electron microscope (TEM) were used for further analysis. The mean size of the vesicles was measured using a Mastersizer 2000 particle size analyzer (Malvern).

Synthesis of the cyclotetraphosphazene derivative containing eight TGMEs (OTCP). NaH 0.784 g (32.8 mmol) was dissolved in 20 mL of anhydrous terahydrofuran (THF) as a suspension state, and a solution of TGME 5.50 g (16.4 mmol) and TBAB 0.0977 g (0.303 mmol) in 20 mL of THF was added slowly to the NaH solution. The mixed solution was heated under reflux for 3 h. A solution of OCCP 0.635 g (1.37 mmol) in 20 mL of anhydrous THF was added slowly to the mixed solution. The resulting solution was heated under reflux for 72 h. The NaCl formed was filtered out and the filtered NaCl was washed several times with CH_2Cl_2. The remaining solution was evaporated in a rotary evaporator. The product was obtained as a viscous liquid state. The product was purified twice by column chromatography (CH_2Cl_2, CH_3OH). The solvent in the first and second column chromatography was CH_2Cl_2:CH_3OH = 80:1 and CH_2Cl_2:CH_3OH = 10:1, respectively.

Yield 48.2% (1.88 g, 0.660 mmol), ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 24H, 8 CH_3), 1.06-1.51 (m, 128H, CH_2), 3.27-4.15

(m, 144H, O-CH_2), IR (cm^{-1}) 2930, 2870 (stretching C-H), 1150 (stretching C-O), 1120 (stretching -P=N-), elemental analysis $C_{144}H_{296}N_4O_{40}P_4$, calculated: C 60.73 H 10.48 N 1.97, observed: C 60.51 H 10.58 N 1.92.

Formation of vesicles. 0.150 g (0.0502 mmol) of OTCP, 0.0194 g (0.0502 mmol) of cholesterol, and 2.7 mg (4.94 μmol) of DHPP were dissolved in 10 mL of methanol in a 50 mL round-bottomed flask. The methanol was evaporated from this solution, and a very thin membrane layer remained at the bottom of the flask. 3.5 mL of distilled water was added to the flask, and vortex mixed for 10 min. The formed suspension contained multilamellar vesicles (MLV). This suspended solution was sonicated for 20 min, resulting in the formation of unilamellar vesicles (ULV). A Cole-Parmer 4710 250 W sonicator was used, and the power was set to 50% amplitude.

Measurement of encapsulation efficiency. The encapsulation efficiency was measured using DPHT. DPHT was added during the course of forming the vesicle solution, so that the final DPHT concentration was 20 mM. The vesicle part was separated from the free DPHT in the solution using gel filtration with Sephadex G-75. The separated vesicle was destroyed using Triton X-100, and then the fluorescence was measured at an excitation and emission wavelength of 350 nm and 425 nm, respectively.

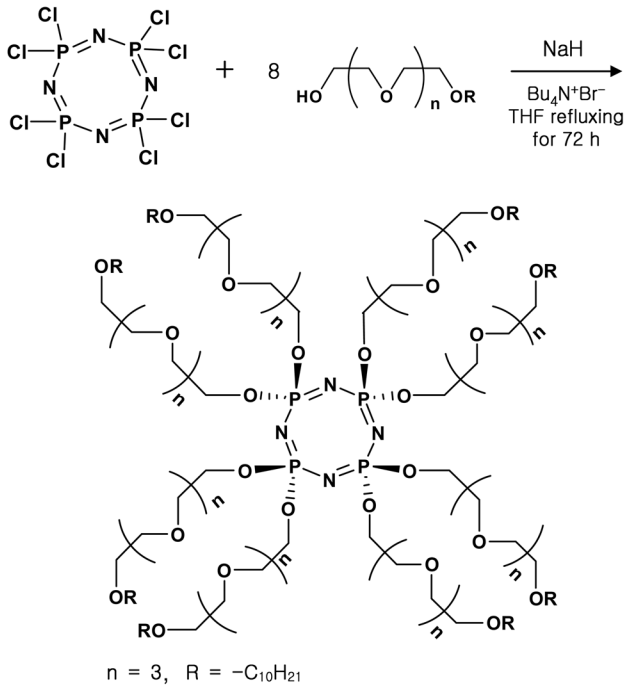
Results and Discussion

Synthesis of cyclotetraphosphazene derivative. The chlorocyclophosphazene used in this study was a mixture containing the trimer and tetramer. The pure tetramer was obtained from fractional vacuum sublimation, followed by recrystallization in hexane. The IR spectrum of hexachlorocyclotriphosphazene (trimer) showed an absorption of -P=N- at 1218 cm^{-1}. However, the absorption of -P=N- in OCCP (tetramer) was observed at 1310 cm^{-1}. The IR spectrum of the trimer showed almost no absorption at 1310 cm^{-1} and there was almost no absorption at 1218 cm^{-1} by the tetramer. Therefore, it is possible to estimate the relative ratio of the trimer and tetramer in a mixture using the IR spectrum.

In the reaction of OCCP with TGME, the TGME: OCCP mole ratio used was 12 in order to improve the yield. The yield of the reaction was greatly increased by adding TBAB as a phase-transfer catalyst. Scheme 1 summarizes the reaction process.

Formation of vesicles. It was difficult to form vesicles using only OTCP but vesicles could be formed when the same mole ratio of cholesterol was added. The same mole ratio of OTCP and cholesterol were dissolved in methanol. The methanol was then evaporated, and a very thin membrane layer was formed at the bottom of the flask. Distilled water was added to the flask, and the multilamella vesicles (MLV), which had a several layered structure, were formed after the vortex mixing was conducted.[1,2] Figure 1 shows TEM images of the MLV formed.

Figure 1 showed sphere shaped vesicles with a diameter of



Scheme 1. Synthetic route to octasubstituted cyclotetraphosphazene.

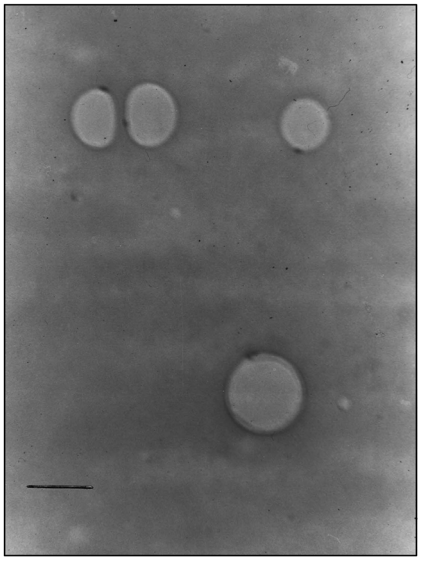


Figure 1. TEM images of the MLVs obtained from octasubstituted cyclophosphazene (bar represents 500 nm).

400-500 nm. The MLV solution was sonicated to form unilamella vesicles (ULV) solution. Figure 2 shows TEM images of the formed ULV, which have a diameter of 150-180 nm with an almost uniform size.

The stability of the formed vesicle was not so good. When the formed vesicle solution remained at room temperature, small amount of precipitation was observed in the solution after 2-3 days. In order to improve the stability of the vesicle, a small amount of DHPP was added during the course of vesicle formation. In this experiment, using only DHPP, 2.7 mg per OTCP 0.15 g improved the stability of the

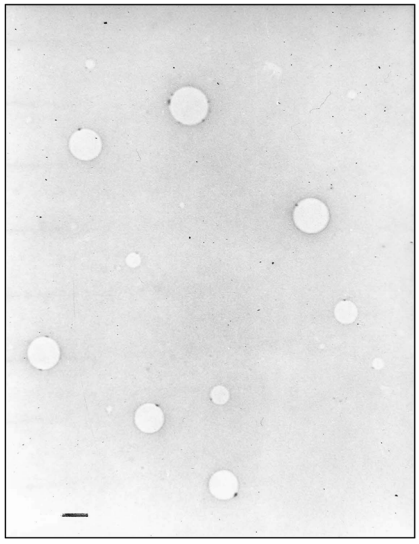


Figure 2. TEM images of ULVs obtained from octasubstituted cyclophosphazene (bar represents 200 nm).

vesicle greatly. The precipitation was not formed even after 20-30 days. In case of the vesicles where DHPP had been added, there was almost no change in the TEM images after 15 days had passed. However, in case of the vesicle where DHPP had not been added, TEM showed an increase in the vesicle size to 300-400 nm after 15 days as well as some agglomeration. This phenomenon is observed frequently in the process of vesicle precipitation. A comparison of the TEM photographs of the vesicle with or without DHPP showed no difference in the shape and size of the vesicle, which means that DHPP had no effect on the size of the vesicle, but only affected the stability. The reason for the increasing stability is that DHPP can form an anionic outer surface and increase the hydrophilic character in the vesicle, that can prevent agglomeration.

Measurement of encapsulation efficiency. DPHT was used to measure the encapsulation efficiency. A vesicle containing DPHT inside the vesicle was formed by adding DPHT during the process to form the vesicle. The concentration of DPHT in the overall solution was 20 mM. The free DPHT that remained outside of the vesicle in solution was separated from the vesicle part using the gel filtration with Sephadex G-75. Tritron X-100 was used to destroy the membrane in the vesicle. The amount of DPHT encapsulated in the vesicle was determined by measuring the fluorescence.

Table 1 shows that 39% of DPHT was encapsulated in the vesicle in case of the MLV. However, 30% of DPHT was encapsulated in the vesicle in case of the ULV.

The size and the encapsulation efficiency of the vesicles

Table 1. Encapsulation Efficiency of Vesicles Obtained from Octasubstituted Cyclotetraphosphazene

Vesicles	Encapsulation efficiency
MLV	39 ± 2%
ULV	30 ± 2%

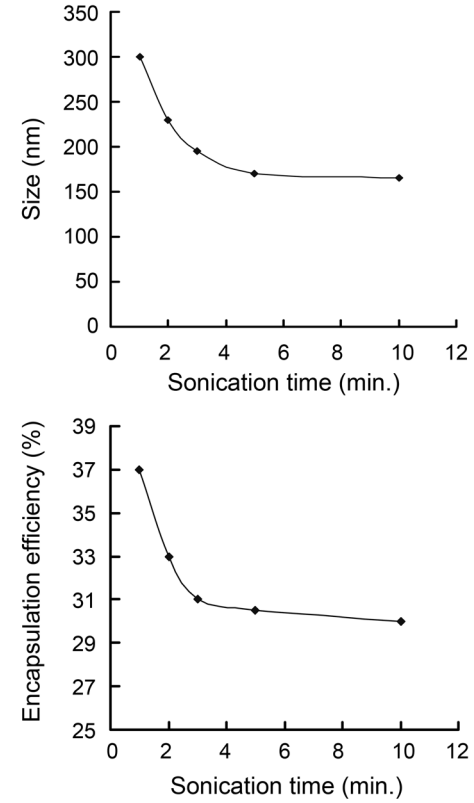


Figure 3. The size and the encapsulation efficiency of the vesicles according to sonication time.

according to the sonication time were investigated. The results are shown in Figure 3.

According to the sonication time, the size of the vesicles

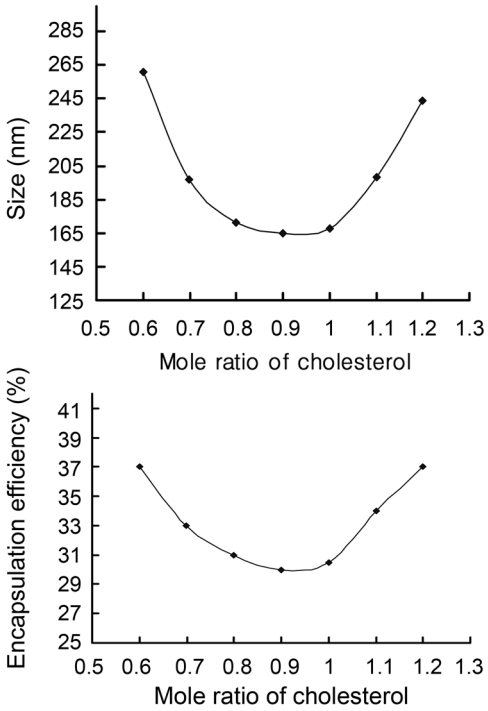


Figure 4. The size and the encapsulation efficiency of the vesicles according to the mole ratio of cholesterol to OCTP.

decreased to 165 nm with a concomitant decrease in the encapsulation efficiency of the vesicles.

The size and encapsulation efficiency of the vesicles (ULV) according to the mole ratio of cholesterol to OCTP were investigated. The results are shown in Figure 4.

The vesicles could be formed in a limited range of cholesterol concentrations. The results showed that when the cholesterol to OCTP mole ratio was 0.9, the vesicle (ULV) had the smallest size. In addition, the encapsulation efficiency of the vesicles (ULV) was also lowest. This means that when the cholesterol to OCTP mole ratio was 0.9, the vesicle showed the most well packed structure.

Conclusions

In this research octasubstituted cyclotetraphosphazene derivative was synthesized using OCCP. The vesicles were formed from this compound with cholesterol at a mole ratio of almost 1:1. TEM showed that the vesicles had a round shape. The stability of the vesicle was increased by adding DHPP. The encapsulation efficiency of the vesicle was examined with DPHT. The encapsulation efficiency of the ULV and MLV was 30% and 39%, respectively. The size of the vesicles and the encapsulation efficiency decreased with increasing sonocation time. The vesicles were smallest and had the lowest encapsulation efficiency at a cholesterol: OCTP mole ratio of 0.9.

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