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Vesicle formation from hexasubstituted cyclophosphazenic derivatives☆

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Abstract

Hexakis[butoxytris(ethoxy)]cyclophosphazene (**3a**), hexakis[dodecyloxytetrakis (ethoxy)]cyclophosphazene (**3b**) and hexakis[hexadecyloxyeicosanekis(ethoxy)]cyclophosphazene (**3c**) were synthesised and their ability to form niosomes was studied. All synthesised compounds in the presence of cholesterol were shown to form vesicles, which aggregated strongly. To prevent aggregation, dicetylphosphate was used. The capacity of the sonicated and unsonicated niosomes to encapsulate hydrophile and lipophile molecules was also studied using carboxyfluorescein and diphenylhexatriene.

Keywords: Hexasubstituted cyclophosphazenes; Vesicles; Niosomes; 1,6-Diphenyl-1,3,5-hexatriene; 5(6)-Carboxyfluorescein

1. Introduction

Vesicular systems are of considerable interest because they can be used as membrane models, in chemical reactivity studies (Fendler, 1982) or in drug delivery and targeting (Lasic, 1993). The most used vesicular carrier systems are liposomes prepared from a wide variety of natural and synthetic phospholipids, but also numerous ionic and non-ionic amphiphiles have been used to form multilamellar and/or unilamellar vesicles (Vanlerberghe et al., 1972; Fendler, 1982). In particular, non-ionic surfactant vesicles (NSVs) or niosomes are being extensively studied (Florence, 1993). These aggregates are widely studied as an alternative to liposomes because they have similar physical properties and can be prepared in the same way as phospholipid vesicles, but they generally

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show higher chemical stability. Vesicle forming properties of an ever increasing number of amphiphilic lipids, which have different chemical structures and composition of the hydrophilic and hydrophobic moieties, are being studied (Vanlerberghe et al., 1972; Vanlerberghe and Handjani-Vila, 1975; Vanlerberghe et al., 1978; Okahata et al., 1981; Echegoyen et al., 1988; Munoz et al., 1993; Schenning et al., 1993, 1994; Ghosh et al., 1996).

During the last 30 years, the polyorganophosphazenes and their low molecular weight cyclic homologues, cyclophosphazene derivatives, have been extensively studied and comprehensively reviewed (Allcock, 1990). In fact, polyphosphazene-based ligand systems and cyclophosphazenes have been shown to possess interesting biomedical properties and diverse applications such as biomedical materials, membrane hydrogels, biologically active agents and phase transfer catalysts (Allcock, 1994). Recently, some of us reported that cyclophosphazenic polypodands, a new class of acyclic many-armed polyethers are particularly efficient phase-transfer catalysts, which combine higher complexing capability in low polarity solvents with excellent chemical stability and ready availability (Landini et al., 1995).

Within our studies on cyclic phosphazenes, we focused our attention on the capacity shown by some of them to form vesicles. Here the aggregation properties of some cyclophosphazenic molecules (hexakis[butoxytris(ethoxy)] cyclophosphazene (**3a**), hexakis[dodecyloxytetrakis (ethoxy)] cyclophosphazene (**3b**) and hexakis[hexadecyloxyeicosanekis(ethoxy)]cyclophosphazene (**3c**)) containing six hydrophilic and lipophilic chains are reported.

2. Materials and methods

2.1. Materials

Cholesterol, dicetylphosphate, 5(6)-carboxyfluorescein (CF) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemicals (Milan, Italy); hexachlorocyclotriphosphazene, triethylene glycol monobutyl ether, tetraethylene glycol monododecyl ether and polyethylene glycol monohexadecyl ether were purchased from Fluka (Milan) and used as received. All solvents were high performance liquid chromatography grade.

2.2. Synthesis

Compounds **3a–c** were synthesised according to a literature procedure (Podda, 1988) by reacting the hexachlorocyclotriphosphazene (**1**) with a molar excess (10 moles per mole) of the appropriate polyethylene glycol monoalkyl ether (**2a–c**) in THF in the presence of sodium hydride as a base and of catalytic amounts of tetrabutylammonium bromide under solid liquid phase-transfer catalysis conditions.

The reaction mixture, easily purified by column chromatography (silica gel, eluent CH_2Cl_2/MeOH) afforded **3a–c** as viscous liquids. Mass spectra were obtained on a VG ZAB 2F instrument operating in electron ionisation (EI)conditions (70 eV, 200 μA) and with an ion source of 200°C. The samples were introduced directly in the source and heated to 180°C. The ^{1}H-NMR and ^{31}P-NMR spectra were measured on a Varian Unit 300 spectrometer using deuteriochloroform as solvent, tetramethylsilane as the internal standard and 85% phosphoric acid as the external standard. The IR spectra were recorded on a Perkin Elmer 1310 spectrophotometer.

2.3. Vesicle preparation

Multilamellar vesicles (MLV) were prepared according to the thin film hydration method. Compounds **3a–c** (47.5 μmoles), cholesterol (47.5 μmoles) and dicetylphosphate (5 μmoles) were completely dissolved in about 10 ml of methanol. The solvent was vacuum evaporated to form a thin film inside the vessel. Distilled water (2.5 ml) was added and the mixture was shaken with a mechanical stirrer in a water bath at 20°C for about 1 h. Small unilamellar vesicles (SUV) were prepared starting from MLV dispersions which were sonicated in a Soniprep 150 apparatus

(MSE, Crowley) equipped with an exponential microprobe, operating at 14 KHz and an amplitude of 6 μm. Sonication was carried out under a nitrogen stream, for 30 min (six times 5 min).

The CF-entrapped vesicles were prepared according to the procedure described but hydrating the thin lipidic film with 2.5 ml of 25 mM CF solution in sodium hydroxide (pH 7.5 with HCl). The DPH-entrapped vesicles were prepared by dissolving the lipophilic dye in methanol together with the lipidic phase and hydrating the obtained thin film with distilled water to obtain a final 2×10^{-4} M DPH concentration.

2.4. Transmission electron microscopy

Negative stain micrographs were prepared on copper grids covered with a carbon film. The vesicle dispersion pipetted onto the grids and stained with 1% phosphotungstic acid, were viewed and photographed with a Jeol 100 S or a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV.

2.5. Photon correlation spectroscopy

Photon correlation spectroscopy measurements of the hydrodynamic size of the vesicles were performed using a Nicomp 370 autocorrelator (Argon laser, $\lambda = 488$ nm)

2.6. Encapsulation efficiencies

Encapsulation efficiencies of CF and DPH were determined by gel filtration chromatography. An aliquot of probe-entrapped MLV and SUV dispersions was gel chromatographed on a Sephadex G-75 column (10 ml) to remove the extravesicular dye, while another was left intact. After disruption of vesicles with methanol or 1% Triton X-100 or lyophilization, both solutions were analysed for their fluorescence by means of a Perkin Elmer LS-3 fluorimeter at an excitation wavelength of 490 (CF) or 350 nm (DPH) and an emission wavelength of 514 (CF) or 425 nm (DPH).

3. Results and discussion

3.1. Synthesis

Compounds **3a–c** (Scheme 1) were synthesised according to the literature procedure (Podda, 1988). The products, viscous liquids, were dispersible in water at room temperature. The structure of compounds **3a–c** was assigned on the basis of analytical and spectroscopic data (IR, NMR and MS) and the characteristics of the synthesised hexasubstituted cyclophosphazenes are summarised in Table 1. As can be seen, all the ^{31}P-NMR spectra of compounds **3a–c** showed the typical singlet peak at $\delta = 19$–19.4 ppm (Gobbi et al., 1994). Furthermore, MS measurements confirmed the assigned structures of compounds **3a–c**: all spectra showed well detectable molecular ions $[M]^{+\cdot}$.

3.2. Vesicle formation

All synthesised compounds in the presence of an equimolar amount of cholesterol were shown to form vesicles on hydration followed by mechanical stirring and/or sonication. In the absence of a charge inducer, vesicles aggregated strongly. To prevent aggregation, dicetylphosphate (DCP) was added to the lipidic phase. Best results were obtained when the lipidic phase was formed by equimolar amounts of compounds **3a–c** and cholesterol and 5% DCP: the resulting dispersions showed high stability for weeks at room temperature. On the contrary, in the absence of cholesterol, none of these compounds were able to form vesicles.

3.3. Vesicle characterisation

The MLV and SUV dispersions were characterised using transmission electron microscopy and light scattering. In the first analysis, the transmission electron micrographs provided confirmation of the formation of vesicular structures (Fig. 1).

Preliminary light scattering experiments showed that the size of vortexed vesicles were 400–460 nm in diameter, while SUV were 120–150 nm. Samples always showed an irregular size distribution:

Table 1
Structural and spectral data for compounds **3a–c**

Compound	Molecular formula	Molecular weight	Yield (%)	IR neat (cm^{-1})	^{1}H-NMR $CDCl_3$ δ	^{31}P-NMR $CDCl_3$ δ
3a	$C_{60}H_{126}N_3O_{24}P_3$	1366.60	29	2940, 2860 (stretching C–H), 1120 1110 (stretching C–O) 1060 (stretching –P=N)	4.04–3.25 (m, 84 H, 42 O–CH_2) 1.68–1.09 (m, 24 H, 12 CH_2) 0.86 ppm (t, 18 H, 6 CH_3)	19 (s, OR_2)
3b	$C_{120}H_{246}N_3O_{30}P_3$	2304.23	61	2930, 2860 (stretching C–H) 1150, 1120 (stretching C–O) 1050 (stretching –P=N)	4.16–3.28 (m, 108 H, 54 O–CH_2) 1.50–1.06 (m, 120 H, 60 CH_2) 0.83 ppm (t, 18 H, 6CH_3)	19.44 (s, (OR_2)
3c	$C_{336}H_{678}N_3O_{126}P_3$	6870.01	70	2930, 2880 (stretching C–H) 1160, 1100 (stretching C–O) 1050 (stretching –P=N)	4.16–3.25 (m, 480 H, 240 O–CH_2) 1.60–1.05 (m, 180 H, 90 O–CH_2) 0.80 ppm (t, 18 H, 6 CH_3)	19.20 (s, OR_2)

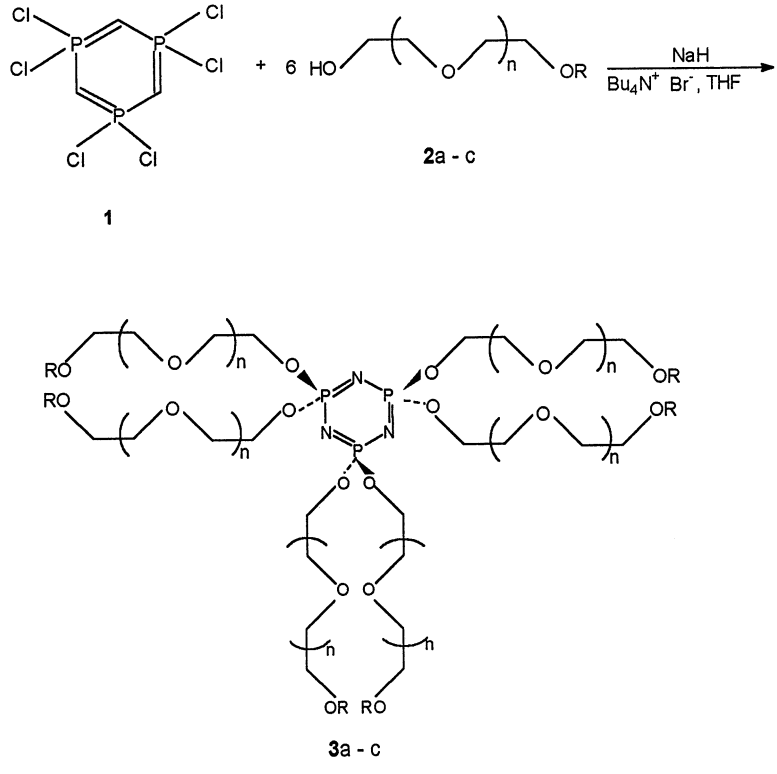
all vesicle preparations gave a polydispersity index that was greater than 0.2. Samples prepared using compound **1c** also showed a small amount (6–10%) of particles, size 9–10 nm, which could be micelles.

Finally, the encapsulation properties of vesicles in a molar ratio of compounds **1a–c**/CHOL/DCP = 1/1/0.1 were studied using a hydrophilic and a hydrophobic fluorescent probe: 5(6)-CF and DPH, respectively. The entrapment efficiencies, calculated as a percentage of the dye entrapped, are shown in Table 2.

As can be seen, the amount of CF that could be encapsulated into cyclophospazenic vesicles is low, but the low encapsulation ability of NSVs for CF has already been reported (Baillie et al., 1984; Schenk et al., 1989).

Good entrapment efficiencies were observed for DPH, especially for vesicles prepared with compound **3b**, which shows the lowest hydrophilic–lipophilic balance. Sonicated vesicles always showed the lowest efficiencies.

In a previous paper (Varnek et al., 1993), some of us carried out a molecular mechanics study of cyclophospazenic polypodands to investigate the catalytic activity of these ligands in solid–liquid phase transfer reactions. The number of possible conformers of these 'octopus-like' molecules is very large, but the most stable conformer that can explain their catalytic ability is shown in Fig. 2, where each polyethereal chain forms a chelating bonding site. This conformer, with hydrophilic chains around the planar centre and assembled lipophilic moieties, could help in understanding how these unusual shaped molecules can organise themselves in a vesicular bilayer that is formed only in the presence of cholesterol.



Scheme 1. The synthesis of hexasubstituted cyclophosphazenes **3a–c**; (a) $n = 2$, $R = C_4H_9$; (b) $n = 3$, $R = C_{12}H_{25}$; (c) $n = 19$, $R = C_{16}H_{33}$.

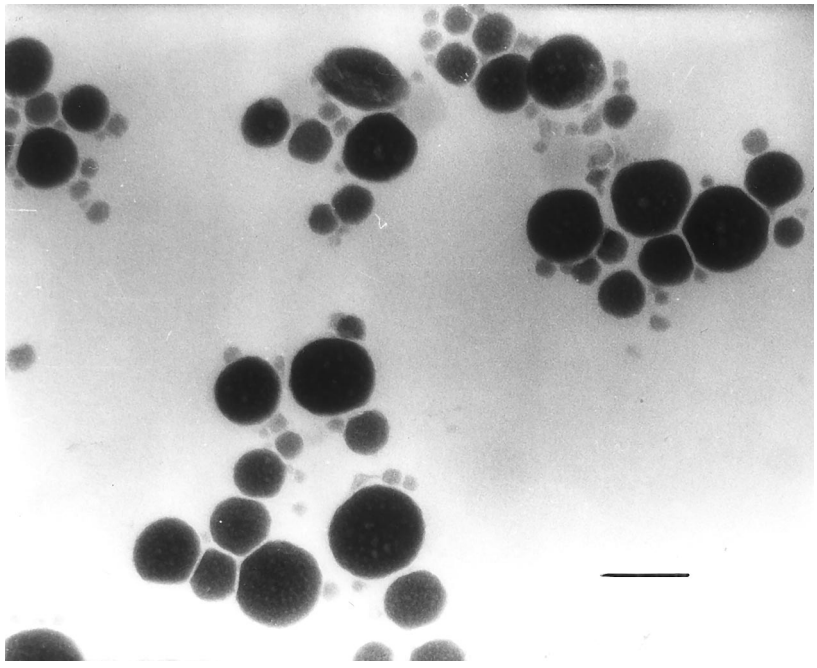


Fig. 1. Negative-stain transmission electron micrographs of sonicated vesicles of **3a** (× 90 000 magnification); bar, 100 nm.

Table 2
HLB values ($20 \times MW_{Hydrophilic}/MW_{Total}$) and entrapment efficiency of hexasubstituted cyclophosphazenic vesicles

Compound	HLB	Vesicles	Entrapment efficiency (%CF ± SD)	Entrapment efficiency (%DPH ± SD)
3a	13	SUV	0.89 ± 0.20	17.72 ± 0.35
		MLV	1.20 ± 0.30	31.30 ± 0.53
3b	10	SUV	1.03 ± 0.25	36.00 ± 0.45
		MLV	2.22 ± 0.30	41.88 ± 0.44
3c	15.5	SUV	0.90 ± 0.26	22.76 ± 0.30
		MLV	1.47 ± 0.31	29.05 ± 0.65

4. Conclusions

The results obtained show that the studied compounds **3a–c** are able to form vesicles in which both hydrophile and lipofile compounds can be entrapped even if the encapsulation efficiency in CF is very low. However, this fact is quite common for NSVs, which enclose a smaller volume of water, having smaller sizes than phospholipidic vesicles.

The DPH entrapment capability of these polypodands shows good agreement with their catalytic efficiency reported in the literature (Landini et al., 1995).

This new type of vesicle appears to be suitable for use as a carrier for bioactive material.

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Fig. 2. Plot of the most stable conformer of cyclophosphazenic polypodands with long polyethereal chains according to Varnek et al. (1993).

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