Asymmetric lamellar phospholipid aggregates bearing fluorocarbons

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(Received June 11, 1993; accepted December 30, 1993)

Abstract

Phospholipid-encapsulated fluorocarbons form vesicle-like aggregates. Because of the lipophilic nature of fluorocarbons in the core, the number of surrounding lipid layers is consequently uneven. The lamellar structures of droplet coating have been confirmed by physical methods such as NMR spectroscopy, optical and cryo-electron microscopy, zeta potential, etc. The observed line broadening of signals in the ^1H NMR spectra has been shown to be a result of hindered segmental motions in PFC-saturated lamellar structures. The existence of a negative surface charge as supported by the zeta potential contributes to an increase in the dispersion stability. Whereas light microscopy confirmed the isotropic nature of the dispersion, cryo-electron micrographs allowed the film thickness of the lamellae to be measured. The conditions under which such particles are prepared, especially the nature of the phospholipids used, are described. In addition, some new applications based on these aggregates are discussed.

1. Introduction

The term vesicle (liposome) is commonly used to describe spherical aggregates which are formed from phospholipid (PL) bi- or multi-layers in aqueous solution. The nature of such vesicles is determined by the polarity of the PL bilayer, where the hydrophilic head groups are directed towards the aqueous bulk phase as well as towards the water-containing vesicle core.

This description of vesicles (or liposomes) is widely used in membrane science, biochemistry, pharmacology and medicine. It is also used in cases where lipophilic compounds, e.g. drugs, are taken up by the PL carrier. These substrates are thought to be dissolved exclusively in the hydrophobic part of the lipid. However, because of phospholipid saturation, the capacity is small. Larger quantities of hydrophobes (lipophiles) are dispersed by other mechanisms. One is oil stabilization, in which a conventional oil/water emulsion is formed with a monolayer of single phospholipid molecules. In such circumstances, under controlled conditions, lamellar structures are formed leading to multilamellar, but asymmetric, structures.

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2. Experimental details

Fluorocarbon-filled PL vesicles were obtained from aqueous PL vesicles by the application of external energy (high-speed mixing, ultrasound, high-pressure homogenization). Dispersion volumes of between 10–100 ml were prepared by agitation of the liquid mixture with an ultrasonic disintegrator applying an energy output of up to 300 W cm^{-2}. Sonication was conducted over a period of 2–12 min. All samples were ice-cooled.

Commercial grade soy bean lecithins purchased from Fluka (Asolectin) and Sigma und Nattermann (Phospholipon) were used. The fluorocarbons covered a broad range of perfluorinated molecules including perfluorodecalin (PFD), perfluorodibutylmethylamine (F-DBMA), perfluorocyclohexylmethylmorpholine (F-CMM), perfluoro-octyl bromide (PFOB), perfluoro-n-hexane and others. With the exception of F-DBMA (99.1%) and F-CMM (99%), which were prepared in the authors' laboratory, the fluorocarbons were purchased from Fluorochem Ltd., Old Glossop, UK. All liquids were of high purity.

Electron micrographs were obtained by the standard method of negative staining with uranyl acetate. Optical investigations were undertaken using a Zeiss polarization microscope with a magnification of 1000–1200. Specialized cryo-electron micrographs were recorded by a transmission technique from thin frozen aqueous films.

Elsevier Science S.A.
SSDI 0022-1139(94)03065-8

NMR spectra were recorded using a Varian XL-300 spectrometer at 121 MHz (^{31}P), 300 MHz (^{1}H) and 282 MHz (^{19}F), respectively. Chemical shifts are relative to phosphoric acid, TMS and Freon-11 as standards, negative values to high field. All data were reproducible.

Zeta potentials and electrophoretic mobilities were measured using a Malvern system 4700c instrument combined with a Zeta Sizer 3 unit. Particle diameters and size distributions were obtained by photon correlation spectroscopy (PCS) using a Coulter N4 Nanosizer. The data were processed by unimodal analyses (Gaussian distribution) for which a deviation of c. ± 10% was found independently.

3. Results and discussion

In practice, fluorocarbon droplets stabilized by phospholipids are much more stable than comparable systems using conventional emulsifiers [1]. This is certainly not a result of emulsifier surface activity. As we have reported earlier, asymmetric lamellar phospholipid aggregates (ALPHA particles) were formed when fluorocarbons were encapsulated in lipids [2]. Maximum stabilization was achieved only under well-defined preconditions: (i) the lipid used was a highly enriched phosphatidylcholine of soy lecithin (> 90%) with minor parts consisting of negatively charged phosphatides; (ii) PL concentrations were in the range 5%–10%; (iii) fluorocarbons with concentrations ranging from 10%–100% w/v were employed; and (iv) addition of polyols (glycerol, propylene glycol) was necessary to enhance the formation of lamellae. Such polyols also prevented the growth of micro-organisms in the vesicle solution.

It was found that the energy input for producing fresh fluorocarbon droplet surfaces may be best controlled by the use of high-pressure homogenization facilities. Thus, optimizing the PL composition to include a high degree of unsaturation in the acyl groups of phosphatidylcholine, as occurs in natural lecithins, is advantageous because such acyl groups have the ability to reduce the viscosity of aqueous vesicle solutions and because of a remarkable increase in droplet stability [1].

The formation of fluorocarbon (FC)-filled lamellar aggregates occurs via a stepwise process (Fig. 1). Starting from aqueous PL vesicles, an intermediate oil/water emulsion state is formed via molecular phospholipids. The application of external energy generates fresh FC surfaces, which are then covered by single PL molecules. The two fatty acid tails of the lipid 'interact' with the surface of the FC droplet depending on the lipid solubility of the FCs [3]. Excess phospholipid favours multilayer film formation because of the inherent water

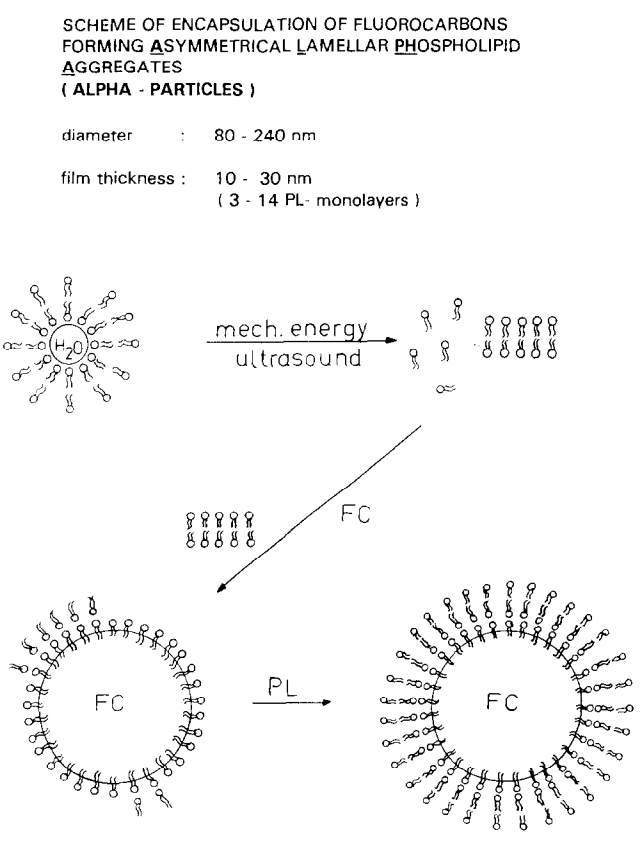

Fig. 1. Process of fluorocarbon encapsulation by phospholipids.

insolubility generated and the thermodynamic gain in entropic energy. Although the interaction forces between lamellae are weak, the overall particle stability in solution is high because of the multilayer structure and the negative surface charge. Friberg and co-workers [4] have shown convincingly that association structures at the oil/water interface reduce the van der Waals energy of coalescence to a remarkable degree.

In order to detect the above-mentioned lamellar structures, we have carried out electrical measurements, ^{31}P and ^{1}H NMR investigations, PCS as well as optical and cryo-electron microscopy. To obtain information on the surface charge, zeta potentials and specific charge densities of aqueous ALPHA particle solutions have been measured. Charge densities were obtained by titration using a cationic polyelectrolyte (1×10^{-3} N poly-Dadmac). The results are listed in Table 1. Both solutions studied contained polyols. Obviously, decreasing the FC/PL ratio increases the surface charge.

It is known that saturation of lipid lamellae with some lipophilic PFCs leads to a broadening of the NMR lines of the lipid [1]. When PFD is dissolved in the lipid bilayer, it could perturb the ordering of the hydrocarbon chains of the lipid. Under these circumstances, both the ^{19}F NMR fluorocarbon shift and its signal shape would not be affected. However, ^{1}H NMR

TABLE 1. Surface charge of ALPHA particles

Fluorocarbon (% w/v)	Phospholipid (%)	Diameter, d (nm)	Zeta potential (mV)	Charge density (μequiv. g^{-1})
56% F-TPA	5	286	−56.07	5.35
62% PFD/DBMA	7.7	244	−61.07	7.36

TABLE 2. ^{1}H NMR data for ALPHA particles in D_2O

Site	Proton chemical shift (ppm)	Relative intensity	Line width (lamellae without PFD) (Hz)
CH_3–N	3.24	12	17.7 (12.7)
CH_2–N	2.03	5	31.8 (18.4)
CH_3 chain	0.88	8	30.9 (23.8)
CH_2 chain	1.29	36	48.6 (41.2)

spectroscopy should give more interesting results. Accordingly, we have studied the ALPHA-particle system (30% w/v PFD, 6.5% Phospholipon 80) prepared in deuterated water by sonication using ^{1}H NMR resonance methods (Table 2).

For all proton signals, the peak areas correspond to the appropriate number of protons when account is taken of multiple unsaturation in the CH_2 chains. To effect a comparison, aqueous lamellar PL vesicles without PFD were prepared under conditions identical to those described above. The ^{1}H NMR line width of such solutions differed from those of solutions containing ALPHA particles (Table 2) by 28%, 42%, 23% and 14%, respectively. The corresponding ^{31}P NMR spectra consisted of one line only, with a chemical shift of +0.1 ppm. In this case, the line width in the spectrum of the solution containing ALPHA particles (same system as listed in Table 2) was 93.7 Hz (without PFD, the line width was 57.7 Hz). It should be noted that according to Barenholz et al. [5], the line width of methylene protons in lipid chains of aqueous unilamellar vesicles obtained by sonication is 20 Hz.

The largest difference in line width was observed for $-CH_2-$ units linked to nitrogen, i.e. the hydrophilic head group of the lipid. Obviously, restrictions of segmental motions due to conformational changes are greatest at this molecular site. Accordingly, changes in the NMR parameters are also the greatest.

In order to decide between isotropic lamellar aggregates and anisotropic liquid crystalline phases, we have carried out investigations using a light microscope (magnification ×1000) with crossed polarizers. For such studies, we specially prepared particles of c. 10 μm diameter in the system F-hexane/PL/water by high-speed mixing. All the vesicle-like solutions obtained were isotropic, indicating that the PFC core of the particles had been surrounded by a spherical unresolved multilayer of c. 1 μm thickness.

Electron microscopy with negative staining gave only limited information concerning the nature of particles. However, the existence of homogeneity and the particle diameter can be derived from the micrograph depicted in Fig. 2. More structural information was obtained from the cryo-transmission electron micrographs of frozen amorphous films (Fig. 3) [6], which illustrate the lamellar coating of the fluorocarbon core. The number of monolayers involved in the coating could be calculated from the thickness of the spherical multilayer. Riess and co-workers [7] have recently confirmed the existence of such a lamellar arrangement by freeze fracture microscopy.

Asymmetric lamellar PL aggregates are excellent gas-transporters in the blood stream because of the ex-

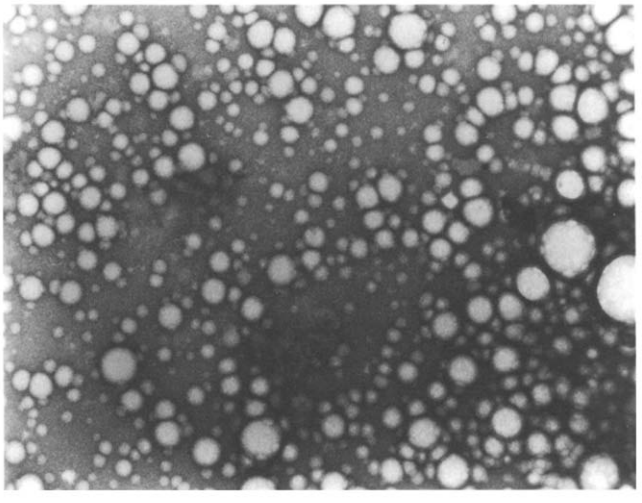

Fig. 2. Electron micrograph of ALPHA particles at ×47 000 original magnification.

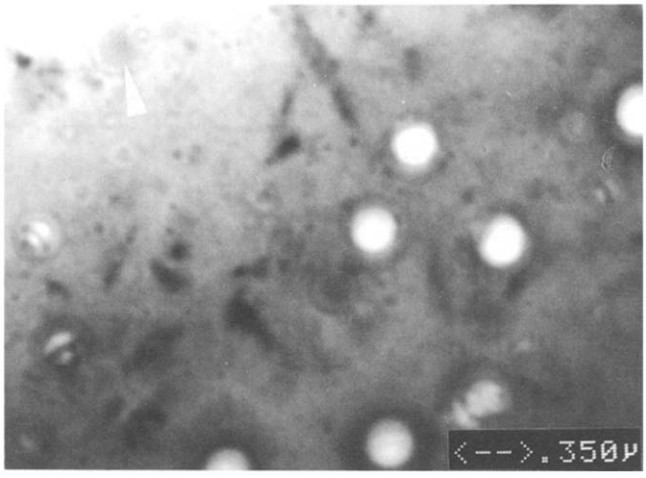

Fig. 3. Cryo-transmission electron micrograph of frozen films of ALPHA particles (arrows).

tremely high encapsulated PFC content. In addition, the lamellar particle structure allows penetration into skin tissue, an important property principle in cosmetics and dermatics where the oxygen supply is influenced by controlled penetration into different skin planes.

The particle size and lipophilicity of PFCs determine the rate of penetration and the half-life time in tissue [8]. Since ALPHA particles are capable of carrying lipophilic drugs, vitamins, diagnostic agents, etc. after topical administration, the use of spin-labelled PL particles with fluorocarbon radicals possessing a long life-time could enable the skin penetration process to be demonstrated by EPR tomography [2].

Acknowledgement

The authors wish to thank Dr J. Röding for helping with the cryo-electron microscopy.

References

1 U. Groß, L. Kolditz, G. Papke and S. Rüdiger, *J. Fluorine Chem., 53* (1991) 163.
2 U. Groß, S. Rüdiger and W. Radeck, DE Patent 41 27 442, 1991.
3 U. Groß, G. Papke and S. Rüdiger, *J. Fluorine Chem., 61* (1993) 11.
4 S. Friberg, P. Jansson and E. Cederberg, *J. Colloid Interface Sci., 55* (1976) 614.
5 Y. Barenholz, S. Amselem and B. Lichtenberg, *FEBS Lett., 99* (1979) 210.
6 J. Röding, *Proc. 47th Annu. Meet. Electron Microsc. Soc. Am.*, San Francisco, 1989, p. 744.
7 M. Postel, P. Chang, J.-P. Rolland, M.-P. Krafft and J.G. Ricss, *Biochim. Biophys. Acta 1086* (1991) 95.
8 U. Groß, H. Röding, K. Stanzl and L. Zastrow, DE Patent Appl. 42 21 255, 1992; 42 21 256, 1992; 42 21 268, 1992; 42 21 269, 1992.
9 U. Groß, N. Groth and T. Herrling, *Xth Eur. Symp. Fluorine Chem.*, Padua, 1992; Abstract of paper B18, *J. Fluorine Chem., 58* (1992) 196.