New design of vesicle-forming potential anti-cancer agent†

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A novel amphiphile derivative of the anti-cancer agent PALA (*N*-(phosphonoacetyl)-L-aspartate), has been synthesized: dioctadecyl *N*-(phosphonoacetyl)-L-aspartate (PALAmod). Its aptitude to form bilayers or vesicles has been investigated. The melting temperature T_m of the alkyl chains of PALAmod is comparable with those of the bilayers of distearoyl phosphatidylcholine (DSPC) or distearoyl phosphatidylglycerol (DSPG) which have fatty chains of the same length. Encapsulation of two different hydrophilic probes, carboxyfluorescein (CF) and glucose, was examined, demonstrating the likely existence of vesicular structures either in lecithin–PALAmod mixtures or in pure PALAmod, respectively. The size of the particles has been measured from quasielastic light scattering experiments. The particle diameter at fixed pH (6.9), goes from 74 nm at [PALAmod] 3.6 mM to 52 nm at 14.8 mM.

Nouvelle conception d'un agent anticancéreux potentiel sous forme de vésicule. Le diester carboxylique stéarique de l'acide *N*-(phosphonoacetyl)-L-aspartique (PALAmod) a été synthétisé en vue d'examiner les capacités de ce tensioactif à s'autoorganiser en bicouches ou en vésicules. La température de fusion T_m des chaînes alkyles du PALAmod est comparable à celles des bicouches de la distearoyl phosphatidyl choline (DSPC) ou du distearoyl phosphatidyl glycerol (DSPG), qui possèdent des chaînes grasses de même longueur. La formation de structures vésiculaires constituées soit d'un mélange lécithine–PALAmod, soit de PALAmod pur a été établie à partir de mesures d'encapsulation de sondes hydrophiles, carboxyfluorescéine (CF) ou glucose. La taille des particules a été évaluée par des expériences de diffusion quasiélastique de la lumière. Le diamètre des particules à pH fixé (6.9), varie de 74 nm, à une concentration en PALAmod de 3.6 mM, à 52 nm, à une concentration de 14.8 mM.

Introduction

Research in the field of anti-cancer chemotherapeutic agents includes different conceptual approaches.[1] One of them is based on the inhibition of nucleotide synthesis. Nucleotides are indeed essential metabolites involved in the building of DNA and RNA. Decreasing their production is considered as one of the possible ways to prevent the growth of tumorous cells. The biosynthesis pathway of pyrimidinic nucleotides (uracil, cytosine, thymine) is controlled by an allosteric enzyme, aspartate transcarbamylase (ATCase).[2] Inhibition of this enzyme may be obtained by using substrate analogues showing competitive binding to the catalytic site with respect to the natural substrates (carbamyl phosphate and L-aspartate in the present case).

A bisubstrate analogue, with a chemical structure close to that of the assumed transition-state of the ATCase-catalyzed reaction, has been synthesized by Collins and Stark.[3] This molecule, *N*-(phosphonacetyl)-L-aspartate (PALA), has proved to be a good competitive inhibitor of carbamyl phosphate, as demonstrated by *in vitro* studies[4,5] and then, has influenced numerous other preparations.[6] Unfortunately, the anti-cancer activity of free PALA, which was established from *in vitro* investigations, was not as large as expected when *in vivo* tests were conducted.[5,6e] This was at least partly attributed to the proposed mechanism for cellular uptake and especially to the role of endocytosis and lysosomal pH.[7] A major research objective is aimed at improving the transport of PALA towards its desired target, ATCase.

At the lysosomal pH, which is of the order of 5, the PALA molecule is expected to be essentially in the form of a trianion, when considering the pK_a's of the phosphonic group (2.43 and 7.85 in the case of ethylphosphonic acid) and of the carboxylic functions (2.09 and 3.86 for aspartic acid), respectively.[7] Whether the global electric charge carried by the molecule should be decreased or increased to facilitate its penetration in the cytoplasm is a controversial question.[7,8] Attempts have recently been made to answer this question by modifying the global charge of the molecule. This was achieved, for instance, by substituting the hydrogens in the α-position of the phosphonate group by fluorine atoms, in order to decrease the second pK_a of the phosphonic acid and thus to ensure a full ionization of the molecule at the lysosomal pH. Unfortunately this new molecule has shown, *in vitro*, a decreased affinity for ATCase.[9] So, getting a better insight into the part played by the electric charges in the translocation mechanism (passive diffusion or endocytosis) appears to be a difficult task.

On the other hand, endocytosis cannot be considered a very efficient process. It has been demonstrated that, even with a large extracellular concentration of PALA, only a very small quantity is transferred across the cell walls.[7] Any improvement of the efficiency of the uptake mechanism should obviously be beneficial to the development of the therapeutic applications. In this perspective, very encouraging results have been obtained by Sharma *et al.*[10] who have demonstrated that

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liposome-encapsulated PALA shows enhanced antitumor activity. They have examined the inhibitory effects on tumor cell growth of different PALA-containing liposome formulations. Depending on the cell line considered, the optimal formulation indicated a 22- to 570-fold greater sensitivity compared to free PALA. Additional work in this direction has been pursued by Kim and Heath[11] who have encapsulated PALA in antibody-directed liposomes, able to recognize specific antigens present at the surface of tumor cells. A spectacular efficiency of PALA was obtained in that case, giving further encouragement for developing the use of that kind of vector. However, a major drawback of the vesicular encapsulation method is that only a very small amount of the initially synthesized PALA (usually less than 5%) is encapsulated, the largest part of the drug being lost.

This has prompted us to imagine a new formulation in which the loss of PALA would be limited and its local concentration would be high. The idea was to confer to the PALA molecule itself an amphiphilic character, so that the modified molecule (PALAmod) can mimic the behaviour of phospholipids in making vesicular structures. These structures could either result from a self-organization of PALAmod or from the association of this molecule with vesicle-forming amphiphiles like phospholipids. In the latter case the local concentration of PALAmod could be tuned by adjusting the composition of the vesicles. Of course the inhibition of ATCase could only be obtained after the release of free PALA, which could be regenerated *in situ* from the esterolysis of PALAmod by lipases present in the cytoplasm.[12] We are not yet at this point, which will have to be examined in a forthcoming step. At the present stage of this work, we had two objectives: (i) to synthesize a modified PALA molecule presenting a strong analogy with natural phospholipids (see Scheme 1), and (ii) to demonstrate that this new molecule has the expected vesicle-forming capacity.

Experimental

Materials

All the chemicals used were commercially available guaranteed reagents unless otherwise stated.

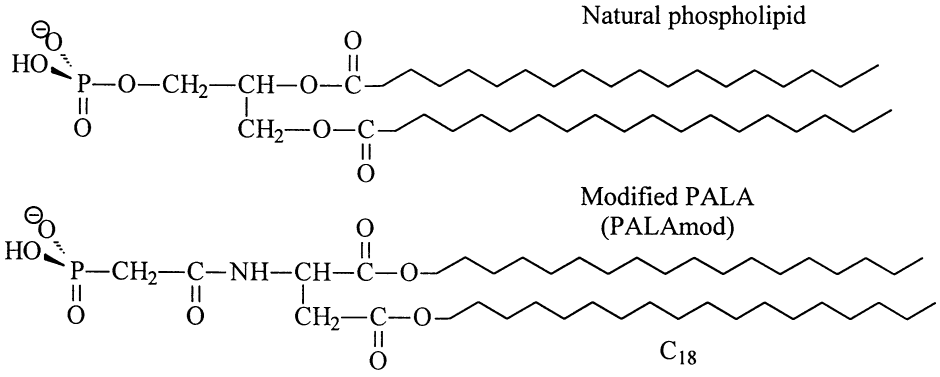
1-Octadecanol, egg yolk lecithins (L-α-phosphatidylcholine type XVI), cholesterol and HEPES buffer were obtained from Sigma. Dibenzyl-L-aspartate was purchased from Bachem, n-BuLi and bromotrimethylsilane were purchased from Aldrich. 5(6)-Carboxyfluorescein (CF) was from Kodak, Sephadex G50 (medium) from Pharmacia Biotech, Triton X-100 from Fluka. The fluorescence polarization probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) and D-glucose were purchased from Fluka and Sigma, respectively.

Diethyl methylphosphonate was prepared from triethylphosphite and iodomethane,[13] and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) was prepared according to the previously reported method.[14] Melting points were determined with an Electrothermal digital apparatus and are uncorrected. Optical rotations were measured with a Bellingam-Stanley Ltd. ADP 220 polarimeter (10 cm, 5 ml, $CHCl_3$ at 25 °C, *c* in g per 100 ml). IR spectra were taken on a Nicolet 210 infrared spectrometer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AC 250 spectrometer. Deuteriochloroform (99.8% atom enriched, Eurisotop) was used as NMR solvent. The NMR chemical shifts are reported in δ (ppm) based on internal $SiMe_4$ ($\delta_H = 0$) or the solvent signal ($CDCl_3$, $\delta_C = 77.0$) or external 1% H_3PO_4 in D_2O ($\delta_P = 0$) as references. FAB mass spectra were measured on a Micromass Manchester Autospec Fitted Cesium gun spectrometer. All reactions, except for the catalytic hydrogenation were carried out in an inert atmosphere. Tetrahydrofuran (THF) was distilled from sodium–benzophenone, diethyl ether and dichloromethane were distilled from P_2O_5. Column chromatography was carried out on Merck Silicagel 60 (particle size 0.040–0.063 mm).

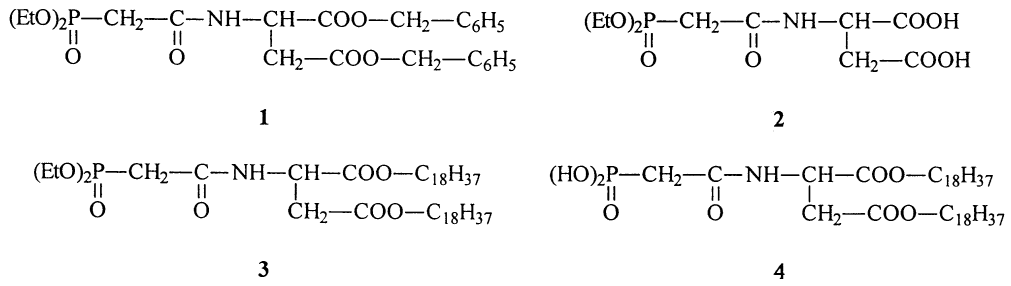
Syntheses

Dibenzyl *N*-(diethylphosphonoacetyl)-L-aspartate 1.[15] To a stirred solution of the *p*-tosylate salt of H-Asp(OBn)-OBn (10 g, 20 mmol) in dichloromethane (100 ml) was added triethylamine (2 g, 20 mmol), followed by the successive addition of diethyl phosphonoacetic acid[16] (3.9 g, 20 mmol) in dichloromethane (100 ml), BOP (8.83 g, 20 mmol) in dichloromethane (50 ml) and triethylamine (2 g, 20 mmol). After stirring for 1.5 h at room temperature the reaction mixture was diluted with dichloromethane (100 ml). The organic layer was washed sequentially with 2 N H_2SO_4 (3 × 100 ml), 5% $NaHCO_3$ (3 × 100 ml), and saturated NaCl (100 ml) solutions. The dichloromethane layer was dried ($MgSO_4$), filtered and concentrated. The crude oil was purified by rapid filtration on silica gel with pure ethyl acetate as eluent to give the product **1** (8.55 g, 82%) as a yellow oil. ^1H NMR (250 MHz, $CDCl_3$): δ 1.30 (t, $J = 7.0$ Hz, 3H), 1.31 (t, $J = 7.0$ Hz, 3H), 2.90 (dd, $J = 4.70$ Hz, $J = 17.1$ Hz, 1H), 2.87 (d, $J = 20.7$ Hz, 2H), 3.08 (dd, $J = 4.67$ Hz, $J = 17.1$ Hz, 1H), 4.08–4.18 (m, 4H), 4.85–5.00 (m, 1H), 5.08 (s, 2H), 5.13 (s, 2H), 7.29–7.35 (m, 10H), 7.43 (d, $J = 8.0$ Hz, 1H). ^{13}C NMR (62.53 MHz, $CDCl_3$): δ 16.28 (d, $J = 7.1$ Hz), 35.31 (d, $J = 142.0$ Hz), 36.27, 49.00, 62.71 (d, $J = 5.6$ Hz), 62.80 (d, $J = 5.6$ Hz), 66.82, 67.53, 128.27, 128.40, 128.56, 164.07, 170.03, 170.36. ^{31}P NMR (101.25 MHz, $CDCl_3$): δ 19.23. IR (neat): 1025, 1250, 1675, 1740 cm^{-1}.

***N*-(Diethylphosphonoacetyl)-L-aspartic acid 2.** To a solution of **1** (2.5 g, 5 mmol) in AcOEt (20 ml) was added 10% Pd–C (0.5 g). The solution was stirred for 20 h under 30 bars of hydrogen at room temperature. The mixture was filtered through Celite and the Celite was washed with AcOEt. The filtrate was concentrated and the resulting *N*-(diethylphosphonoacetyl)-L-aspartic acid **2** was used directly in the next step. The product **2** (1.52 g) was obtained as a



Scheme 1



Scheme 2

brown oil in 98% yield. ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.32 (t, J = 7.0 Hz, 6H), 2.80–3.20 (m, 4H), 4.09–4.22 (m, 4H), 4.80–5.00 (m, 1H), 7.88 (d, J = 8.0 Hz, 1H). ^{13}C NMR (62.53 MHz, $CDCl_3$): δ 16.13 (d, J = 6.0 Hz), 34.99 (d, J = 131.4 Hz), 35.83, 48.82, 63.49 (d, J = 5.5 Hz), 63.57 (d, J = 5.5 Hz), 164.66, 173.38, 174.17. ^{31}P NMR (101.25 MHz, $CDCl_3$): δ 20.48. IR (neat): 1025, 1200, 1660, 1730, 3100–3600 cm^{-1}.

Dioctadecyl *N*-(diethylphosphonoacetyl)-L-aspartate 3. 1-Octadecanol (stearic alcohol) (3.5 g, 13 mmol), BOP (5.7 g, 13 mmol), and triethylamine (658 mg, 6.5 mmol) in dichloromethane were stirred for 1 h at room temperature followed by the slow addition (4 h) of *N*-(diethylphosphonoacetyl)-L-aspartic acid **2** (1.0 g, 3.2 mmol) diluted in dichloromethane (75 ml). After stirring for 15 h the reaction mixture was diluted with dichloromethane (75 ml). The organic layer was washed sequentially with 2 N H_2SO_4 (3 × 50 ml), 5% $NaHCO_3$ (3 × 50 ml), and saturated NaCl (100 ml) solutions. The dichloromethane layer was dried ($MgSO_4$), filtered and concentrated. The crude oil was purified by column chromatography eluting with hexane–ethyl acetate (100 : 0–10 : 90). Dioctadecyl *N*-(diethylphosphonoacetyl)-L-aspartate **3** (1.93 g, 74%) was obtained as a white powder (R_f 0.75, hexane–ethyl acetate 10 : 90). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (t, J = 6.5 Hz, 6H), 1.20–1.80 (m, 64H), 1.32 (t, J = 7.3 Hz, 3H), 1.33 (t, J = 7.3 Hz, 3H), 2.83 (dd, J = 4.6 Hz, J = 17.1 Hz, 1H), 2.90 (d, J = 20.5 Hz, 2H), 2.99 (dd, J = 5.2 Hz, J = 17.1 Hz, 1H), 4.07 (t, J = 7.0 Hz, 4H), 4.08–4.23 (m, 4H), 4.89–5.16 (m, 1H), 7.38 (d, J = 8.0 Hz, 1H). ^{13}C NMR (62.53 MHz, $CDCl_3$): δ 14.07, 16.25 (d, J = 6.0 Hz), 22.65, 25.74, 25.82, 28.41, 28.46, 29.24, 29.32, 29.51, 29.66, 31.89, 35.18 (d, J = 131.0 Hz), 36.08, 48.95, 62.77 (d, J = 5.5 Hz), 62.85 (d, J = 5.5 Hz), 65.29, 66.04, 164.14, 170.38, 170.77. ^{31}P NMR (101.25 MHz, $CDCl_3$): δ 19.32. IR (neat): 1025, 1240, 1660, 1735, 2850, 2920, 2955 cm^{-1}.

Dioctadecyl *N*-(phosphonoacetyl)-L-aspartate 4 (PALAmod). Dioctadecyl *N*-(diethylphosphonoacetyl)-L-aspartate **3** (2.0 g, 2.5 mmol) and bromotrimethylsilane (5 ml, 38.6 mmol) in 1,2-dichloroethane (10 ml) were stirred for 3 h at room temperature, then the solvent was evaporated under vacuum. The resulting brown powder was washed with wet acetone, then dried *in vacuo*. Compound **4** (1.85 g, 97%) was obtained as a white solid. ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (t, J = 6.0 Hz, 6H), 1.10–1.80 (m, 64H), 2.90–3.30 (m, 4H), 4.00–4.30 (m, 4H), 4.80–5.00 (m, 1H). ^{13}C NMR (62.53 MHz, $CDCl_3$): δ 14.08, 22.67, 25.82, 25.89, 28.41, 28.49, 29.36, 29.74, 31.91, 35.99, 36.12 (d, J = 140.0 Hz), 49.32, 65.49, 66.23, 167.07, 170.49, 171.00. ^{31}P NMR (101.25 MHz, $CDCl_3$): δ 18.44. MS (negative FAB): m/z 758.5 ($M^+ - 1$). IR (neat): 1200, 1655, 1735, 2850, 2920, 2950 cm^{-1}. Mp = 74–76 °C. $[\alpha]_D$ = + 7.5° (c = 1). Calc. for $C_{42}H_{82}NO_8P$: C, 66.37; H, 10.87; N, 1.84; P, 4.08. Found: C, 65.7; H, 10.96; N, 1.86; P, 3.88%.

Techniques

The vesicle dispersions were prepared following a procedure derived from that described by Cleij *et al.*[17] The surfactants (either pure PALAmod, or a lecithin–PALAmod mixture, eventually including cholesterol, depending on the case) were dissolved in methylene chloride or in a solution of DPH in methylene chloride for the fluorescence polarization experiments. The solvent was evaporated and the resulting solid was dried for 4 h under vacuum. HEPES buffer (10 mM, pH 7.4 unless otherwise indicated) and NaOH (usually 1 equivalent per PALAmod molecule) were then added to the film which was left over night under agitation to allow its hydration. The obtained dispersion was sonicated for 5 min at 40 °C (Branson sonifier 200 W, tip 13 mm, 40–45% power output). The vesicle dispersion was filtered at room temperature through 0.45 μm Millipore membranes (HATF type, *i.e.* surfactant-free) in order to remove the titanium particles coming from the immersion probe. For the encapsulation experiments either CF (50 mM) or glucose (0.5 M) were introduced into the dispersion before sonication.

The size of the particles was measured at 25 °C using a home-assembled quasielastic light scattering (QELS) apparatus coupled with a Malvern autocorrelator. When necessary the dispersions were diluted with the HEPES buffer, so as to adjust the scattering intensity. Filtration through 0.45 μm Millipore membranes was systematically performed just before the scattering experiments. We checked that dilution, after sonication, does not affect significantly the value or the hydrodynamic radius of the particles.

For the encapsulation experiments, steric exclusion chromatography (SEC) on Sephadex G50 was used to separate the vesicle-encapsulated probes from those which remained free. Some of the preliminary experiments involving the CF probe were carried out with visual examination of the eluting solution, which was strongly colored, followed by fluorescence analysis of the collected fractions. The majority of the experiments (especially those involving glucose) were performed with a Biologic LP chromatography system (Bio-Rad) equipped with a model 2128 fraction collector and a double trace recorder (Tracelab BD 41). Both the absorbance (or turbidity) measured at 254 nm and the conductivity of the eluted solution were recorded. The void volume of the Sephadex column was calibrated using Blue Dextran 2000 kDa and found to be of the order of 13 ml for 4 g of Sephadex. During the preequilibration of the column, the absence of change of conductivity of the eluent was used as a test to ensure a good conditioning of the column. The eluent used for the chromatography of the dispersions of vesicle-encapsulated CF was a solution containing 10 mM HEPES buffer (pH 7.0) and 0.1 M NaCl. This was assumed to limit osmotic pressure variations between the inner and outer vesicle compartments and thus to avoid osmotic swelling. In the case of vesicle-encapsulated glucose, HEPES buffer was used to maintain the pH, without any other added solute. Under these conditions a partial breaking of the vesicles, due to osmotic stress, cannot be completely ruled out.

The fluorescence measurements were carried out on a Shimadzu FR 540 spectrofluorimeter. For carboxyfluorescein the excitation and emission wavelengths were 490 and 520 nm, respectively. To measure the concentration of encapsulated

CF, 0.5 ml of Triton X-100 at concentration 0.16 M were added to 0.5 ml of the vesicular dispersion and diluted with the buffer (HEPES 0.01 M, NaCl 0.1 M, pH 7.4) to a final volume of 3 ml. The CF concentration was determined from a standard curve, with all the caution necessary to be sure that the dilution is large enough so that there is no fluorescence quenching.

For polarization measurements with DPH (3×10^{-5} M), polarizers were introduced in the light beam before and after the fluorescence cell. The excitation and emission wavelengths were 360 and 430 nm, respectively. The chain melting temperature was taken to be equal to the temperature at mid-transition.

For glucose trapping experiments the analysis of the encapsulated and free glucose respectively was based on the usual enzymatic reaction.[18] The glucose was oxidized by glucose oxidase leading to a production of hydrogen peroxide. The latter reacts with *o*-dianisidine (Sigma) to form a coloured product absorbing at 435 nm. A Varian Cary 3E spectrophotometer was used for the measurements. The glucose content was determined from a calibration curve obtained with known concentrations of glucose. Triton X-100 was added to the dispersions, in conditions similar to those reported above in the case of CF, to ascertain the breaking of the vesicles.

Results and discussion

Synthesis of PALAmod 4

The synthesis of PALAmod **4** was based on the preparation of the precursor **1** from a classical coupling between diethylphosphonoacetic acid[16] and H-Asp(OBn)-OBn using BOP as the coupling reagent (82%).[15] Benzyl esters of **1** could be chemoselectively hydrogenated with a Pd–C catalyst to give **2** keeping the diethylphosphonic ester intact (98%). Reaction of **2** with an excess of stearic alcohol (4 equiv.) and BOP as coupling reagent led to dioctadecyl *N*-(diethylphosphonoacetyl)-L-aspartate **3** (74%). Chemoselective deprotection of the diethylphosphonic ester was then achieved with a large excess of bromotrimethylsilane (15 equiv.) followed by washing of the resulting brown solid with wet acetone and afforded the target compound **4** (97%).

Preparation and characterization of vesicles

Stability. Different kinds of dispersions including PALAmod or a mixture of PALAmod and lecithin have been prepared by sonication as indicated in the Experimental section. The composition of these dispersions are indicated in the different Tables. We have first checked that, with PALAmod alone, stable dispersions presenting a slight opalescence, are obtained in a pH range of 2.4 to 11.8. The following experiments were performed at neutral pH, so as to meet physiological conditions. The influence of the salinity was also investigated. With monovalent salts (0.1 M of NaCl or KBr), added before the sonication in a 3 mM dispersion of PALAmod, a spectroscopic observation of the turbidity indicates that the dispersions are stable for more than a week. However, a salt addition after the sonication induces a destabilization of the dispersion. This can be assumed to be due to the osmotic stress resulting from the difference between the intra- and extra-vesicular electrolyte concentrations, respectively.

Transition temperature. A well-known property of vesicular systems is the melting temperature T_m of the alkyl chains of the bilayer, which characterizes the transition between the gel (rigid) phase β and the fluid (liquid crystalline) phase α. The value of T_m was measured from the fluorescence polarization of DPH,[19] a lipophilic fluorescent probe whose motion is very sensitive to the fluidity of the amphiphilic membrane. The variation of fluorescence polarization as a function of temperature is represented in Fig. 1. The results indicate a transition temperature $T_m = 62\,°C$. Taking into account the fact that the polar heads are not identical, this value is roughly comparable to the values measured[20] for distearoyl phosphatidylcholine (DSPC) or distearoyl phosphatidylglycerol (DSPG), 55 °C, which have fatty chains of the same length. This result is in line with the idea that vesicles are formed. However we cannot completely rule out at this point the possibility of having open fragments of bilayers, as reported in other circumstances.[21] For this reason, in order to demonstrate the existence of closed structures, we have undertaken encapsulation experiments.

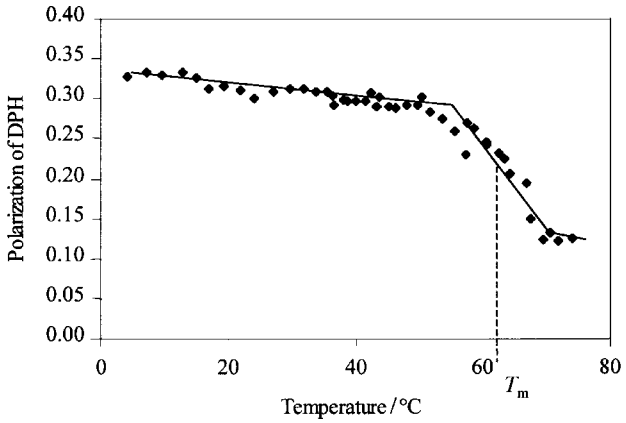


Fig. 1 DPH fluorescence polarization *vs.* temperature in vesicles of PALAmod. [DPH] = 3×10^{-5} M; [PALAmod] = 1×10^{-3} M.

Encapsulation. We have considered two different hydrophilic probes which have been extensively used in the literature: carboxyfluorescein[22] (CF) and glucose.[23,24] The former is very convenient because its fluorescence is quenched when its concentration is above 1.5×10^{-5} M. So if we are able to encapsulate a 50 mM concentration of CF, purify the obtained vesicles and provoke their rupture in one way or another, we should see a fluorescent enhancement due to the dilution. With the second probe the determination of the amount of encapsulated glucose is more tedious and an enzymatic reaction associated with a colorimetric method must be used. However, the fact that the latter probe is neutral constitutes a significant advantage compared to CF (the double negative charge may cause problems when electrostatic interactions with the vesicular particle can occur).

Whatever the type of molecule used to probe the encapsulation, steric exclusion chromatography (SEC) has been used to separate the encapsulated product from the free one. Once the vesicles have been separated, the amount of encapsulated product was determined after breaking of the vesicles by addition of Triton X-100.[22] An illustration of the results obtained is given in Fig. 2, which is relative to glucose.

We have collected in Table 1 some of the information gained from CF encapsulation. Different compositions have been tested. When PALAmod was used alone (C dispersion) or in a mixture containing 25% lecithin (D dispersion), no encapsulation could be measured because the systems were unstable. Increasing the proportion of lecithin to 50% (E dispersion) or 75% (F dispersion) gives stable dispersions with an unchanged percentage of encapsulated CF of 0.21. The addition in the D dispersion of cholesterol, which is expected to rigidify the membrane[25] (G dispersion) improves the stability and leads to a quite similar encapsulation: 0.19%. These

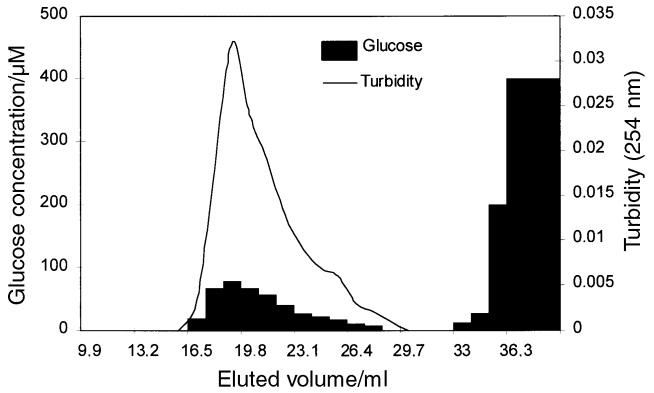


Fig. 2 Gel permeation chromatographic separation of glucose-containing vesicles of PALAmod from free glucose.

results can be compared with those obtained with pure lecithin (B dispersion) or with a mixture of lecithin and cholesterol (A dispersion): 0.25 and 0.44% respectively.

The negative result obtained with PALAmod alone is obviously related to the problems of electrostatic interactions between the negatively charged surfactant and CF. A dilution of PALAmod in lecithin (zwitterionic surfactant) favors the formation of stable vesicles, provided that the mixture contains a sufficient amount of lecithin. This amount can be reduced in the presence of cholesterol. We also notice that the encapsulated amount is independent of the molar ratio between PALAmod and lecithin when stable dispersions are obtained (see E and F dispersions). This can be taken as proof that the encapsulation cannot be due to vesicles of pure lecithin should they coexist with particles of PALAmod.

We have tested the stability of the G dispersion over a period of two weeks. The measured variations of the amount of encapsulated CF are almost within the accuracy of the experiments: starting from a value of 0.19% we found 0.16 after 24 hours, 0.17 after 48 hours and 0.16 after 2 weeks. These small variations indicate that leakage of the encapsulated probe is almost negligible.

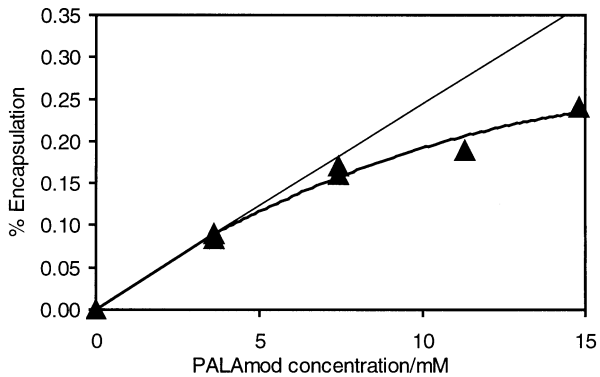


Fig. 3 Variation of the amount of encapsulated glucose (%) *vs.* PALAmod concentration.

The results obtained with the second probe (glucose) demonstrate that vesicles of pure PALAmod are truly formed in the absence of CF. As shown in Table 2 and in Fig. 3, the percentage of encapsulated glucose varies from 0.09 to 0.24% when the PALAmod concentration goes from 3.7 to 14.8 mM. We have also indicated in Table 2 the size of the particles measured from quasielastic light scattering experiments. The decrease of the size is perfectly consistent with the non-linear behaviour observed in Fig. 3. Indeed a rough estimation of the encapsulated volume E_V can be obtained as follows:

$$E_V = \frac{4}{3}\pi\frac{{D_w}^3}{8}N \quad (1)$$

where D_w is the diameter of the water core of the particles and N their number.

$$N = \frac{c \cdot N_A \cdot a_o}{2\pi {D_w}^2} \quad (2)$$

where c is the amphiphile concentration, N_A the Avogadro number, a_o the surface per polar head and the factor $\frac{1}{2}$ takes into account the distribution of the amphiphile between the outside and inside layers, respectively (this is only a crude approximation as indicated above, since for geometrical reasons the partitioning of the amphiphiles between the two layers is in the order of 60 : 40 rather than 50 : 50).[26,27] Introducing eqn. (2) into eqn. (1) and multiplying by 100 to express the value in percent of the total volume leads to

$$E_V\,(\%) = \frac{25}{3}c \cdot N_A \cdot a_o \cdot D_w \quad (3)$$

The values experimentally measured are approximately six times smaller than those calculated from this eqn. (see below), but a more interesting thing is the variation of E_V experimentally observed when the PALAmod concentration is varied. From eqn. (3) we expect a linear dependence of E_V with c only if D_w remains unchanged. This is not the case according to the particle size measurements reported in Table 2. At fixed pH (6.9 ± 0.2), the particle diameter D goes from 74 nm at [PALAmod] 3.6 mM to 52 nm at 14.8 mM. For a fixed concentration the ratio of the E_V values obtained for two different sizes of particles should be such that

$$\frac{E_{V1}}{E_{V2}} = \frac{D_{w1}}{D_{w2}} \quad (4)$$

This is almost exactly verified with the results reported in Fig. 3, if we compare the E_V value obtained from a linear extrapolation of the results at low concentration we obtain at [PALAmod] 14.8 mM, $E_{V1} = 0.36\%$, whereas the experimental value E_{V2} is 0.24%. The ratio between the two values is of the order of 1.5 : 1. If now we estimate the value of D_w from

$$D_w = D - 2l \quad (5)$$

where l is the thickness of the bilayer (about 50 Å), we obtain $D_{w1}/D_{w2} = 64/42 = 1.52$. So the drop of E_V compared to the linear behaviour is almost exactly what can be expected from the variation of the size of the particles. This can be taken as additional proof that PALAmod vesicles are really formed.

Table 1 Percentages of encapsulated carboxyfluorescein

	Composition of dispersions						
	A	B	C	D	E	F	G
[PALAmod]/mM	0	0	5	3.9	2.6	1.3	3.9
[α-Lecithin]/mM	5.5	5	0	1.3	2.6	3.9	1.3
[Cholesterol]/mM	3.5	0	0	0	0	0	1.2
Stability	Stable	Stable	Unstable	Unstable	Stable	Stable	Stable
(%) Encapsulated	0.44	0.25	0	0	0.21	0.21	0.19

Table 2 Percentage of glucose encapsulation and particle diameters

Experiment number	Amphiphile nature and concentration/ mM	Sonicated solution	Final pH (after sonication)	Eluent composition	Glucose encapsulation (%)	Particle size/ nm
B2	PALAmod 3.7 mM	HEPES 10 mM Glucose 0.5 M	4.80	HEPES 10 mM (pH = 7)	0.09	95
B3	PALAmod 3.6 mM	HEPES 10 mM Glucose 0.5 M NaOH 2.7 mM	6.70	HEPES 10 mM (pH = 7)	0.09	74
B4	PALAmod 3.7 mM	HEPES 10 mM Glucose 0.5 M NaOH 3.7 mM	7.00	HEPES 10 mM (pH = 7)	—	72
B7	PALAmod 7.4 mM	HEPES 10 mM Glucose 0.5 M NaOH 7.4 mM	6.80	HEPES 10 mM (pH = 7)	0.17	65.5[a]
B8a	PALAmod 7 mM	HEPES 10 mM Glucose 0.5 M	6.90	HEPES 10 mM (pH = 7)	0.17	58
B8b		NaOH 7.4 mM	6.90	HEPES 10 mM (pH = 7) + 0.13 mM Lecithin	0.22	58
B8c			6.90	HEPES 10 mM (pH = 7) + 0.13 mM PALAmod	0.17	58
B9a	PALAmod 11.3 mM	HEPES 10 mM Glucose 0.5 M	7.09	HEPES 10 mM (pH = 7)	0.19	62
B9b		NaOH 11.3 mM	7.09	HEPES 10 mM (pH = 7) + 0.13 mM Lecithin	0.32	62
B10	PALAmod 14.8 mM	HEPES 10 mM Glucose 0.5 M NaOH 14.8 mM	7.00	HEPES 10 mM (pH = 7)	0.24	52
B12[b]	PALAmod 7.2 mM	HEPES 10 mM Glucose 0.5 M NaOH 7.2 mM	7.00	HEPES 10 mM (pH = 7)	0.07	65
U	Phosphatidylcholine 3.6 mM	HEPES 10 mM Glucose 0.5 M	7.00	HEPES 10 mM (pH = 7) + 0.13 mM Lecithin	0.25	—
A3	Phosphatidylcholine 7.2 mM	HEPES 10 mM Glucose 0.5 M	7.00	HEPES 10 mM (pH = 7) + 0.13 mM Lecithin	0.31	100

[a] Average over two different experiments. [b] Sonication time was 15 minutes instead of 5 as for the other experiments.

However the low absolute value of E_V compared to the theoretical prediction remains a puzzling problem. Indeed, assuming $a_o = 61$ Å^2, as previously reported for phosphatidylcholine at the inner surface of vesicles,[26] one obtains 0.58% at PALAmod concentration 3 mM ($D_w = 64$ nm) and 1.9% at 14.8 mM ($D_w = 42$ nm), when the experimental values are 0.09 and 0.24% respectively. Taking into account the dissymmetry between the two amphiphile layers would reduce the theoretical values by less than 20%. For the sake of comparison we have performed glucose encapsulation experi-

Table 3 Analysis of a series of collected fractions: glucose concentration, turbidity, size of particles. Initial condition: [PALAmod] = 7.4 mM, HEPES buffer 10 mM, pH = 7, glucose 0.5 M (B7 in Table 2)

Test tube number	Eluted volume/ml	Glucose concentration/ μmole l^{-1}	Turbidity (254 nm)	Particle size/ nm
9	9.9	0	0	No diffusion
10	11	0	0	No diffusion
11	12.1	0	0	No diffusion
12	13.2	0	0	No diffusion
13	14.3	0	0	No diffusion
14	15.4	0	0	No diffusion
15	16.5	19	0.003	No diffusion
16	17.6	67	0.017	65
17	18.7	78	0.032	52
18	19.8	67	0.024	58
19	20.9	57	0.019	62
20	22	41	0.013	58
—	—	—	—	—
24	26.4	23	0.003	50
25	27.5	17	0.002	65
26	28.6	10	0.001	No diffusion
27	29.7	8	0.001	No diffusion
28	30.8	0	0.001	No diffusion
29	31.9	0	0	No diffusion
30	33	0	0	No diffusion

ments, in similar conditions, using lecithin instead of PALAmod. The measured values were 0.25% at concentration 3.6 mM and 0.31% at 7.2 mM. We are closer to the theoretical value, but still far from this value by a factor of 2 to 3. The same kind of gap was also observed by Walde *et al.*[23] in the case of mono-n-alkylphosphate vesicles. Different explanations can be put forward to explain our results: (i) closed vesicles could coexist with fragments of bilayer or with open vesicles presenting some leakage due for instance to the existence of pores across the bilayer; (ii) the calculation is valid only for single-wall vesicles, but in the case of multilayer vesicles, the encapsulated volume could be considerably decreased: experiments are presently in progress to check this point; (iii) we cannot exclude the fact that the closing of lamellar fragments under sonication may be accompanied by some expulsion of the entrapped probe due to volume exclusion or steric effects, resulting in different concentrations of the probe inside and outside the particle; (iv) the volume occupied by the polar heads in the inner water core is not taken into account in the calculation and may also contribute to a reduction of the entrapped volume; (v) finally the light scattering experiments give a hydrodynamic radius so that the particle diameter introduced in eqn. (3) may be slightly overestimated. In addition, it is an average value and it is well known that the largest particles are contributing more than the smallest ones to the scattering intensity. Eqn. (3) strictly applies only to monodisperse systems with a well defined particle diameter. The polydispersity of the system may also contribute to lowering the value of E_V experimentally measured because the particles having diameters much smaller than average offer reduced encapsulation capability.

We should also mention here that we have run some additional gel exclusion chromatographic experiments in which the column was preequilibrated not only with the HEPES buffer, but also with a vesicular dispersion. This was assumed to avoid (or at least reduce) possible interactions between the vesicles under study and the solid phase, which could have destabilized the colloidal particles. The results shown in Table 2 indicate a total absence of effect of this procedure when PALAmod was used in the eluent. A small increase of E_V from 0.17 to 0.22 was however observed when eluting with lecithin, but this would simply indicate a possible exchange of amphiphile molecules during the migration.

We have also checked that the average particle diameter measured is independent of the eluted volume and that the values are thus very similar whatever the fraction collected during the recording of the turbidity peak (see Fig. 2). The results obtained for a PALAmod concentration 7.4 mM, in the presence of encapsulated glucose, are shown in Table 3 for the whole series of collected fractions.

Conclusion

We have shown in this work that the amphiphile derivative of PALA (dioctadecyl *N*-(phosphonoacetyl)-L-aspartate, PALAmod) is able to self-organize in bilayer structures. In addition, the formation of vesicles of either pure PALAmod or of mixtures of PALAmod with lecithin was demonstrated from encapsulation experiments and the average size of the particles was determined. However, we cannot exclude at the present stage of this work the coexistence of other types of objects in the dispersions. These preliminary results encourage us to carry on with research along these lines.

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