Design and Characterization of Liposomes Containing Long-Chain N-AcylPEs for Brain Delivery: Penetration of Liposomes Incorporating GM_1 into the Rat Brain

Margarita Mora,[1] Maria-Luisa Sagristá,[1] Domenico Trombetta,[2] Francesco P. Bonina,[3] Anna De Pasquale,[2] and Antonella Saija[2,4]

Received June 12, 2002; accepted July 1, 2002

Purpose. To develop a suitable liposomal carrier to encapsulate neuroactive compounds that are stable enough to carry them to the brain across the blood-brain barrier with the appropriate surface characteristics for an effective targeting and for an active membrane transport.
Methods. Liposomes containing glycosides and a fusogenic lipid were prepared by extrusion. Photon correlation spectroscopy, fluorescence spectroscopy, and differential scanning calorimetry were used to characterize liposomal preparations. Tissue distribution was determined by using ^{3}H-cholesterylhexadecylether as a marker.
Results. The incorporation of glycoside determinants and N-palmitoylphosphatidylethanolamine gives liposomes with similar initial size, trapped volume, negative surface charge, bilayer fluidity, and melting temperature, except for monosialoganglioside-containing liposomes, which showed less negative surface charge and the highest size, trapped volume and melting temperature. All glycosilated formulations gave liposomes able to retain up to the 95% of encapsulated carboxyfluorescein after 90 min at physiologic temperature even in the presence of serum. Monosialoganglioside liposomes were recovered in the cortex, basal ganglia, and mesencephalon of both brain hemispheres. The liver uptake was higher for sulfatide- and glucose-liposomes, whereas the higher blood levels were observed for glucose- and mannose-liposomes.
Conclusions. These results show the suitability of such liposomal formulations to hold encapsulated drugs. Moreover, the brain uptake of monosialoganglioside liposomes makes them good candidates as drug delivery systems to the brain.

KEY WORDS: liposomes; N-acylPEs; brain delivery; permeability; serum.

INTRODUCTION

The blood-brain barrier (BBB) restricts the brain uptake of many valuable hydrophilic drugs and limits their efficacy in the treatment of brain diseases because of the presence of tight junctions, high metabolic capacity, low pinocytic vesicular traffic, and efficient efflux mechanisms. The lipophilic nature of the BBB permits only small lipid-soluble drugs to pass through this barrier (1) and enter the brain via diffusion. Some essential compounds, such as amino acids (2), glucose (3), and iron transferrin (4), need specific carriers to permeate into the brain, and several saturable transport systems have also been reported for peptides (5). Therefore, different concepts are required to develop systems to facilitate the transport of poorly permeable drugs across the BBB for effective management of brain disorders, and different drug delivery strategies have been proposed to improve drug delivery to the brain (6).

In this way, colloidal carriers, such as nanoparticles and liposomes, which may take advantage of the biochemical transport systems that are also present in the BBB, have been considered (7). However, one of the main problems in the targeted drug delivery is the rapid opsonization and uptake of the injected carrier systems by the reticuloendothelial system, by macrophages in liver and spleen. This problem can be overcome by the use of ideal liposome (or nanoparticle) formulations containing the following features: stability in the blood, controlled circulation lifetime, disease site localization and target cell-specific binding and delivery. Liposomes are versatile drug delivery carriers that have proven to be useful in reducing toxicity and enhancing the activity of a variety of pharmacologically active compounds. High phase-transition lipids, high cholesterol content, and small percentages of components, such as monosialoganglioside (GM_1), phosphatidyl inositol, or polyethyleneglycol, normally comprise long-circulating liposomes. The modification of the liposomal surface with hydrophilic molecules increases the blood circulation time of the particulate by reducing the binding of plasma proteins (8).

Our work has focused on developing a suitable liposomal carrier to encapsulate neuroactive compounds that are stable enough to carry them to the brain (sterically stabilized liposomes) with the appropriate surface characteristics for an effective targeting and for an active membrane transport across the BBB (site-specific delivery carriers). In this way, it has been reported that chemical conjugation of potentially central nervous system (CNS)-active drugs with tyrosine or glucose (9), the addition of sugar moieties to bioactive peptides (10), and the use of saccharide determinants (11) represents a successful means of improving their brain delivery. Mannose-labeled liposomes are efficiently incorporated into the mouse brain, lysosomes, and glial cells, suggesting that mannose can be recognized by the cells of the BBB (12).

To achieve an efficient transfer of the liposomal content into the cells, we have considered the preparation of lipo-

[1] Deparment of Biochemistry and Molecular Biology, Faculty of Chemistry, University of Barcelona, Martí i Franquès 1, 08028-Barcelona, Spain
[2] Department Farmaco-Biologico, School of Pharmacy, University of Messina, Contrada Annunziata, 98168-Messina, Italy
[3] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Catania, Viale A. Doria 6, 95125-Catania, Italy
[4] To whom correspondence should be addressed. (e-mail: saija@pharma.unime.it)

ABBREVIATIONS: BBB, blood brain barrier; CF, carboxyfluorescein; Chol, cholesterol; CNS, central nervous system; DDSs, drug delivery systems; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; GLU, p-aminophenyl-β-D-gluco-pyranoside; GM_1, monosialoganglioside; IUVs, intermediate unilamellar vesicles; MAN, p-aminophenyl-α-D-manno-pyranoside; MLVs, multilamellar vesicles; N-acylPEs, N-acylphosphatidylethanolamines; NPPE, N-palmitoylphosphatidylethanolamine; p, polarization; PBS, phosphate-buffered saline; PCS, photon correlation spectroscopy; RES, reticuloendothelial system; SUL, sulfatide; T_m, main transition temperature; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluensulfonate; ΔH_{cal}, calorimetric enthalpy; ΔS_{cal}, calorimetric entropy; $\Delta T_{1/2}$, width at half peak height.

0724-8741/02/1000-1430/0 © 2002 Plenum Publishing Corporation

somes containing N-acylphosphatidylethanolamines (N-acylPEs). Fusogenic liposomes can be prepared by using fusogenic lipids (13), by conjugation of fusogenic molecules to liposome membranes (14), or by incorporation of viral fusion proteins (15) or fusion peptides (16) to bilayers. We previously showed the capacity of liposomes containing N-acylPEs to fuse in the presence of either monovalent or divalent cations (17) and that the incorporation of N-acylPEs into egg phosphatidylcholine (EPC) liposomes decreases their permeability (18) and stabilizes them against leakage in the presence of serum (19).

This article analyzes the effect of the incorporation of GM_1 or sulfatide (to reduce liposome uptake by the RES) and *p*-aminophenyl-β-D-mannopyranoside or *p*-aminophenyl-β-D-glucopyranoside (as recognition markers), in the efficiency of N-acylPE containing intermediate unilamellar vesicles (IUVs) as a drug delivery system (DDS) to the rat brain.

MATERIALS AND METHODS

Materials

Synthetic L-α-dimyristoylphosphatidylcholine (DMPC), transphosphatidylated egg phosphatidylethanolamine (EPE), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Bovine brain monosialoganglioside (GM_1) and sulfatide (SUL), p-aminophenyl-α-D-manno-pyranoside (MAN), p-aminophenyl-β-D-gluco-pyranoside (GLU), rat serum, sodium phosphate monobasic and dibasic were from Sigma Chemical Co. (St. Louis, MO, USA). Palmitoyl chloride for synthesis was from Merck (Schuchardt, Germany), Triton X-100 from Merck (Darmstadt, Germany), 6(5)-carboxyfluorescein (CF) from Eastman-Kodak (Rochester, NY, USA) and Sephadex G-50 and Sephadex LH-20 from Pharmacia Biotech (Uppsala, Sweden). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and its ammonium salt (TMA-DPH) were obtained from Molecular Probes (Eugene, OR, USA). ^{3}H-cholesterylhexadecylether (50 Ci/mmol) was from NEN (Boston, MA, USA). All reagents were analytical grade and Milli-Q water (Millipore Bedford, MA, USA, resistivity of 18 MΩ · cm) was used.

Synthesis and Characterization of N-Palmitoylphosphatidylethanolamine

N-palmitoylphosphatidylethanolamine (NPPE) was synthesized by condensing palmitoyl chloride with purified transphosphatidylated EPE as described (18). The product was purified by silicic acid column chromatography and preparative TLC and characterized by proton-NMR and IR-spectroscopy (18). Lipid concentrations were determined by phosphorus analysis (20).

Vesicle Preparation

IUVs were prepared by extrusion following a standard procedure (21). Multilamellar vesicles (MLVs) were prepared by hydration of dried lipid films, containing a fixed molar ratio of DMPC, Chol, and NPPE and a fixed amount of GLU, MAN, GM_1, or SUL. These components were mixed at the following molar ratios: DMPC/Chol/NPPE (8:3:4), DMPC/Chol/NPPE/GLU (8:3:4:1.5), DMPC/Chol/NPPE/MAN (8:3:4:1.5), DMPC/Chol/NPPE/GM_1 (8:3:4:1.5), and DMPC/Chol/NPPE/SUL (8:3:4:1.5), to prepare the lipid films. The dried lipid film was hydrated with 10 mM phosphate buffer (pH 7.4) containing 140 mM NaCl, at a concentration of 1 mg lipid/mL for 10 min at 50°C, and the lipid dispersion was frozen and thawed (five times) and sonicated (bath sonicator, 60 min, 50°C). IUVs were prepared by passing repeatedly (10 times for each membrane) the MLV dispersion under pressure through different pore-sized polycarbonate membranes (Nucleopore; 400-, 200-, and 100-nm pore sizes) in an extrusion device from Lipex Biomembranes Inc. (Vancouver, Canada). When required, liposomes were prepared at a concentration of 20 mg lipid/mL and were labeled with trace amounts of ^{3}H-cholesterylhexadecylether. The final concentration of the marker in the liposomal suspension was 10 μCi/mL.

Vesicle Size Analysis

The size and size distribution of unilamellar vesicles were determined by photon correlation spectroscopy (PCS). A PCS41 size analyzer (Malvern Autosizer IIc, Malvern Instruments, Malvern, Worcestershire, UK) and a 5-mW He-Ne laser (Spectra Physics, Darmstadt, Hessen, Germany), at an excitation wavelength of 633 nm, were used. Data were collected with a Malvern 7032N 72 data channel correlator, and the mean hydrodynamic diameter was calculated from a cumulant analysis of the intensity autocorrelation function. Before measuring, vesicle dispersions were appropriately diluted to avoid multiple scattering. The influence of such dilution on size measurement was previously proved to be nonsignificant (unpublished results).

ζ-Potential Measurement

ζ-potential measurements were performed on MLV suspensions in 10 mM phosphate buffer (pH 7.4) containing 140 mM NaCl, by laser-Doppler anemometzy in a ZetaSizer 4 (Malvern Instruments). The optic unit contained a 5-mW Helium-Neon laser, a ZET5104 electrophoresis cell (4-nm diameter quartz capillary), a sample handling unit, a multi-8 bit correlator with 72 data channels, and 7 monitor channels with variable time expansion. The method uses the autocorrelation function of the light scattered in colloid solution measured by a photon-counting system. The measurements were performed at 25°C on MLV suspensions (0.5 mg/mL). To check the Malvern device, a carboxy-modified polystyrene latex sample, with a ζ-potential of −55 mV at 25°C, was used before each set of measurements.

Determination of the Entrapped Volume

The volume of the aqueous space of IUVs was determined by measuring the fluorescence at 520 nm of an aliquot of the IUVs suspension, free of non-encapsulated CF. The sample (80 μL) was diluted in 2 mL of 10 mM PBS (pH 7.4), in the presence of 150 μL of a 10% Triton X-100 solution. CF concentration was determined by comparison with a standard curve. Lipid concentration was quantified by phosphorus analysis using ammonium ferrothiocyanate (22).

Calorimetric Measurements

Calorimetric measurements were performed by a Mettler DSC-30 differential scanning calorimeter. The temperature scale was calibrated by indium, undecan, and water, and the transitional enthalpies were calibrated with indium. The temperature of the maximum of the transition endotherm (T_m)

and the enthalpy (ΔH_{cal}) were determined with a Mettler TC10A TA processor. The processor analyzes the heat flow curves by integration of the area under the peak over a dynamic baseline type X8. The cooperativity of the transition was evaluated, in an approximate manner, from the widths at half-peak heights (°C) of the main transition endotherms ($\Delta T_{1/2}$) due the heterogeneous chain composition of lipids. The pellet (100 μL) (~7 mg of lipid), obtained by centrifugation of the MLV dispersion, was transferred to a 160-μL aluminum sample pan. Several heating and cooling cycles were performed to equilibrate the samples before to run the definitive calorimetric scans. The heating rate was 2°C min^{-1}, and only the heating scans were analyzed (10–60°C). A 140-μL aluminum pan, filled with 100 μL of 10 mM PBS (pH 7.4), was used as reference. After calorimetric scans, the phospholipid amount was determined by the method of Stewart (22).

Determination of Membrane Fluidity

Membrane fluidity was determined by fluorescence polarization measurements of the fluorescent probes DPH and TMA-DPH. The incorporation of the fluorophores was performed during the annealing process by adding 2 μL of a 2 mM tetrahydrofurane DPH or TMA-DPH solution to 3 mL of the preformed IUVs (0.35 mg/mL) to get a lipid-probe molar ratio of 375:1. The suspension was incubated in the dark (60 min) at $T > T_m$ of the lipids, with gentle stirring and filtered (0.45-μm pore-membrane filter), before the assay. Excitation and emission wavelengths were 360 and 430 nm, respectively. Measures were performed at different temperatures (20–60°C), in a Kontron SFM 25 spectrofluorimeter (optical arrangement in the "L" format). The sample was excited with vertically polarized light, and fluorescence intensities were recorded with the analyzing polarizer oriented parallel (I_{VV}) and perpendicular (I_{VH}) to the excitation polarizer. The polarization, p, was calculated from:

$$p = \frac{I_{vv} - I_{VH}}{I_{vv} + GI_{VH}}$$

where G is an instrumental correction factor. Samples were illuminated by using narrow slits for the shortest time possible and were maintained in the dark between two consecutive measures to prevent decreases in fluorescence intensity due to the formation of dark isomers of the probes.

Permeability Experiments

Liposome permeability was examined by the fluorescence technique using CF described by Weinstein *et al.* (23). The dye was purified by Sephadex LH-20 column chromatography and acid precipitation (pH 4.5) (24). IUVs were prepared as described above, but with a 10 mM phosphate-buffered solution (PBS) (pH 7.4) containing 0.82% NaCl and 50 mM carboxyfluorescein. Non-encapsulated dye was removed by Sephadex G-50 column chromatography (20 × 1 cm). The composition of eluted liposomes was analyzed (20) and found to be the same as that of the film used for their preparation. CF release was monitored on a Kontron SFM25 spectrofluorimeter by using excitation and emission wavelengths of 492 and 520 nm, respectively. Small aliquots of the eluted liposomes (80 μL, 100 μg lipid/mL) were added to each cuvette (total volume 2 mL), and the fluorescence was measured for 90 min. A 0.416 μM CF solution was used to calibrate the 100% fluorescence value. The permeability experiments were performed at 30, 37, 42, and 47°C, in the absence and in the presence of 12.5 or 125 μL rat serum/mL of aqueous volume in the cuvette. The cuvettes were maintained at the assay temperature by a circulating water bath. The rates of CF leakage are expressed as the percentage of total CF released:

$$\%CF_{released} = \left(\frac{F_t - F_0}{F_T - F_0}\right) \times 100$$

where F_t is the fluorescence intensity at a specified time, F_0 is the fluorescence at zero time, and F_T is the total fluorescence obtained by adding 150 μL of a 10% (v/v) Triton X-100 (Merk) solution. F_T was corrected for the dilution introduced by the addition of Triton. Incubation of liposomes with higher concentration of Triton did not affect the value of F_T, indicating that the release of dye from the liposomes was complete.

Tissue Distribution Studies

The experiments were performed on male adult Sprague-Dawley rats (320–350 g body weight; Charles River, Milan, Italy). The animals were maintained under normal controlled lighting and temperature conditions and allowed free access to food and water until used.

On the day of the experiment, the animals were anaesthetized with urethane (1.2 g/kg intraperitoneally; injection volume: 0.5 mL/100 g body weight). Short polyethylene catheters were inserted in the left common carotid artery for retrograde injection of liposome suspension and in the left femoral artery for blood sampling (25). Ten minutes after completion of surgical procedures, an aliquot of liposome suspension (20 mg lipid/mL) containing ^{3}H-cholesterylhexadecylether (2 μCi/200 μL/rat) was injected through the carotid artery. At the end of the experimental period (10 min), a large blood volume was collected in polyethylene tubes, to measure the whole-blood isotope concentration, and, immediately after, the animals were killed by rapid i.v. injection of 1 mL of saturated KCl solution. The brain and the liver were rapidly removed; cerebral tissue specimens (cortex, mesencephalon, and basal ganglia) were dissected from each hemisphere. During the experiment, hematocrit values were monitored.

Tissue samples were placed in preweighed vials that were immediately reweighed. Sample solubilization was accomplished by adding 1 mL of Soluene 300 (Packard) and incubating the vials at 60°C overnight. Each vial was then filled with 5 mL of Hionic-Fluor (Packard). A 20-μL whole-blood sample was digested in 0.5 mL of Soluene 300 and counted in 6 mL of Hionic Fluor acidified with 0.5 mL of 0.5 M HCl. Beta-counting was performed by a Packard TRI-CARB 2100TR liquid scintillation counter. Single sample quenching was monitored by the external standard method. Sample counts were corrected for background and quenching.

Results of *in vivo* experiments are expressed as means ± SD and were analyzed for statistical significance by Student's *t* test for unpaired data. Statistical significance was accepted where $p < 0.05$.

Table I. Characteristic Parameters of the Aqueous Dispersions of Liposomes Containing Glycoside Determinants

Sample composition (molar ratio)	D_H[a] (nm)	Polydispersity index[b]	ζ-Potential[c] (mV)	Trapped volume[d] (μl/μmol)
DMPC/Chol/NPPE (8:3:4)	106.3 ± 1.5	0.173 ± 0.015	−27.5 ± 0.9	1.40 ± 0.08
DMPC/Chol/NPPE/GLU (8:3:4:1.5)	105.4 ± 12.3	0.102 ± 0.098	−27.8 ± 0.8	1.49 ± 0.15
DMPC/Chol/NPPE/MAN (8:3:4:1.5)	113.3 ± 9.1	0.059 ± 0.003	−28.2 ± 1.3	1.40 ± 0.13
DMPC/Chol/NPPE/GM_1 (8:3:4:1.5)	121.5 ± 10.9	0.144 ± 0.010	−15.5 ± 0.6	2.17 ± 0.29
DMPC/Chol/NPPE/SUL (8:3:4:1.5)	102.8 ± 9.7	0.119 ± 0.010	−27.4 ± 0.7	1.48 ± 0.09

[a] Diameter calculated from dynamic light-scattering data expressed as Z average mean.
[b] Indicates the homogeneity of liposomal suspensions.
[c] Determined by laser-Doppler anemometry.
[d] Determined by carboxyfluorescein dye retention and expressed as μL CF 50 mM/μmol lipid. Data are expressed as means ± SD of three independent experiments.

RESULTS AND DISCUSSION

Characterization of Liposomes Containing Glycoside Determinants

Physical size and surface potential of liposomes, quantity of entrapped solutes, thermodynamic parameters, and bilayer fluidity were determined to characterize the liposomal systems. The size and fluidity effects of liposome on their biodistribution and accumulation in cancer tissues after an i.v. administration has been shown (26), and the knowledge of these parameters is necessary for evaluating the results of experiments using phospholipid vesicles.

Liposomes, prepared as for permeability measurements, were analyzed for size and size distribution by PCS. The mean hydrodynamic diameter (Table I) of control liposomes did not change with the incorporation of p-aminophenyl-β-D-gluco-pyranoside and sulfatide, whereas 7 and 15% size increases were observed with p-aminophenyl-α-D-manno-pyranoside and monosialoganglioside, respectively. The polydispersity index was always < 0.2, being the most homogeneous suspension that contains aminophenyl-α-D-manno-pyranoside.

To assess the stability of the liposomal suspensions, their size and size distribution were also measured after 3 weeks of storage. The results (Fig. 1) show a small size increase for all the dispersions during the first week, but an important size increase for liposomes containing aminophenyl-β-D-gluco-pyranoside, aminophenyl-α-D-manno-pyranoside, and monosialoganglioside after 3 weeks of storage. The instability of glucose- and mannose-containing liposomes can be attributed to some undesirable interaction between the aminophenyl-carbohydrate and other bilayer components because DPPC/Chol/NPPE liposomes are more stable. Note that Umezawa and Eto (12) described the preparation of lecithin/cholesterol/aminophenyl-α-D-manno-pyranoside (7:2:1) liposomes but did not indicate the stability of the liposomal systems. The instability of the liposomes containing monosialoganglioside can be explained by the fact that its incorporation into NPPE-containing bilayers may introduce significant structural changes, as revealed in our previous studies on bilayer arrangement by ^{31}P-NMR (27), showing the decrease in the fluidity of the bilayers after GM_1 incorporation.

The ζ-potential values for MLVs are summarized in Table I. The negative value of the ζ- potential obtained for DPPC/Chol/NPPE liposomes is in agreement with the anionic nature of NPPE, due to the substitution of the amine group of EPE by an amide bond in NPPE. The incorporation of the aminophenyl-carbohydrates into the bilayer did not modify the ζ-potential, as could be expected for the uncharged nature of the carbohydrates. Despite the presence of a negative sulfate group in sulfatide, at the pH of the medium, their incorporation into the bilayer did not modify the ζ-potential. The decrease in the negative charge of liposomes with the addition of monosialoganglioside (with a negative sialic acid group) could be explained by the shielding of the negative charge of the polar head of NPPE by the voluminous polar head of the ganglioside.

The aqueous space volume (Table I) increased with the addition of monosialoganglioside to the liposomal bilayers, but this parameter was not modified with the incorporation of

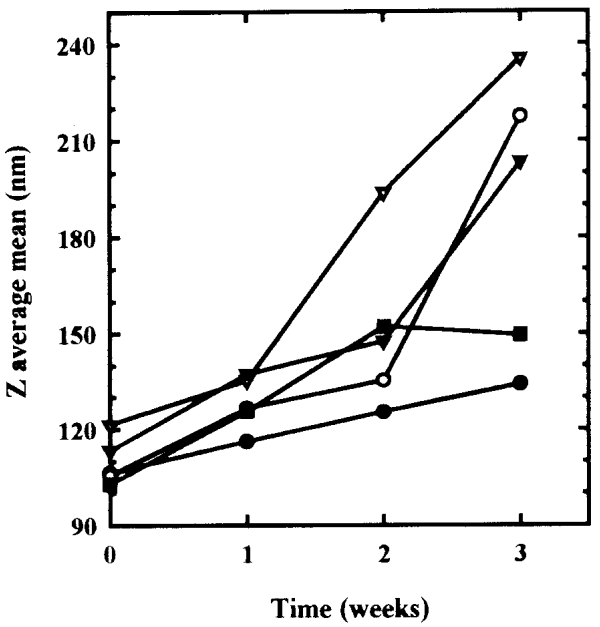

Fig. 1. Effect of time storage in liposome size. The vesicle diameter was measured by dynamic light scattering immediately after liposomes preparation and during the first 3 weeks of storage. Liposomes were prepared by using: (●) DMPC/Chol/NPPE (8:3:4), (○) DMPC/Chol/NPPE/GLU (8:3:4:1.5), (▼) DMPC/Chol/NPPE/MAN (8:3:4:1.5), (▽) DMPC/Chol/NPPE/G_{M1} (8:3:4:1.5), and (■) DMPC/Chol/NPPE/SUL (8:3:4:1.5). Total lipid concentration was in all cases 0.1 mg/mL. The values in the plots correspond to the mean values of three independent experiments. The coefficients of variation ranged from 2 to 15%.

the other glycoside determinants. This result is in agreement with the greater size for DPPC/Chol/NPPE/GM_1 liposomes in comparison with DPPC/Chol/NPPE, DPPC/Chol/NPPE/GLU, and DPPC/Chol/NPPE/SUL liposomes, which showed a similar size by photon correlation spectroscopy. The greatest size and aqueous space volume of DPPC/Chol/NPPE/GM_1 liposomes could be the result of the increased bilayer rigidity due to the incorporation of GM_1 into NPPE-containing bilayers and the resulting steric hindrance to give liposomes with high curvature radium. The same entrapped volume for DPPC/Chol/NPPE/MAN and control liposomes could be explained by the lesser degree of heterogeneity of the liposomes containing aminophenyl-mannose.

Differential scanning calorimetry was used to determine the miscibility of the different components present in the liposomal bilayers and to study their thermotropic behavior. The endothermic transition profiles showed, in all cases, a broad single endotherm, as expected from the complex acyl chain composition of the samples, and pointed out the miscibility of the components used. The thermodynamic parameters are given in Table II. The transition temperatures did not show significant changes after the incorporation of the glycoside compounds, except in the case of the DPPC/Chol/NPPE/GM_1 mixture. However, ΔH_{cal}, ΔS_{cal}, and $T_{1/2}$ values increased always in the presence of the glycoside determinants, indicating that the incorporation of carbohydrates gives bilayers with transitions more energetic and less cooperative.

DPH and TMA-DPH were used to determine membrane fluidity (Fig. 2). Fluorescence studies with DPH (Fig. 2a) revealed that the packing of the hydrocarbon chains was only slightly modified at temperatures below the T_m determined by DSC. The variation of DPH polarization with the temperature is almost lineal and does not show the characteristic inflexion for the gel to liquid-crystal transitions. Nevertheless, a little change in the slope at 30°C can be related to the DSC results, except for the bilayers containing monosialoganglioside. This behavior pattern was also observed for other NPPE and cholesterol-containing bilayers. The variation of TMA-DPH polarization with the temperature also indicates that the incorporation of the glycosides does not significantly modify the fluidity at the lipid-water interface (Fig. 2b). However, the change in the slope at 35°C and 30°C for the ganglioside containing bilayers and for the other bilayers, respectively, accounts for the DSC results.

Permeability of Liposomes Containing Glycoside Determinants

Permeability studies were performed to assess the stability of liposomes in serum. The rate of CF leakage was measured in PBS and in the presence of rat serum at 30, 37, 42, and 47°C, at a lipid-serum ratio of 0.38 and 0.038 μmol lipid/mL serum. IUVs of DPPC/Chol/NPPE (8:3:4) were used as the control system. The efflux of the dye was expressed as the percentage of the initial entrapped dye lost vs. time. The initial entrapped dye (initial latency) is the initial carboxyfluorescein self-quenching, which is related to the concentration into vesicles, and is defined by $100(1 - (F_0/F_\infty))$, where F_∞ and F_0 correspond to the fluorescence values obtained by adding Triton X-100 and that found at zero time, respectively. Fig. 3 a and b shows the time course for carboxyfluorescein release at 37°C for control liposomes and the percentage of carboxyfluorescein leakage at 90 min and different temperatures, respectively. The leakage of CF from liposomes was higher in the presence of serum than in buffer at physiologic temperature, but in all cases the CF leakage is lower than the 9% of the total entrapped solute, after 90 min. This result indicates that the lipid composition assayed gives liposomes stable enough, at physiologic temperature, in the presence of serum to be used for the incorporation of the glycoside determinants. In the presence of serum, CF leakage increases with the temperature up to 42°C, but at 47°C a decreased leakage was observed. The addition of NPPE to liposomes composed by phosphatidylcholine or by phosphatidylcholine-cholesterol mixtures stabilizes the liposomes in PBS and in the presence of serum (19,21). Nevertheless, the higher stabilization in serum than in buffer depends on the phosphatidylcholine nature and on the molar ratio of the three lipid components (19,21).

The data corresponding to liposomes containing a 9.09 mol% of all glycoside determinants (Fig. 4a–c) showed a decrease in carboxyfluorescein release with respect to control liposomes, at physiologic temperature. The incorporation of aminophenyl-mannose into the bilayer gave liposomes with a lower permeability in the presence of serum, but in the other cases carboxyfluorescein leakage was a bit higher when serum was added to the medium. At physiologic temperature, liposomes containing monosialoganglioside exhibit the lower CF release, both in PBS and in the presence of serum, which agrees with the greater molecular order at the head group

Table II. Effect of Incorporation of the Glycoside Determinants into DMPC/Chol/NPPE Bilayers on the Thermodynamic Parameters of the Aqueous Dispersions

Sample composition (molar ratio)	T_m[a] (°C)	ΔH_{cal}[b] (Kcal mol^{-1})	ΔS_{cal} (cal mol^{-1} K^{-1})	$\Delta T_{1/2}$[c] (°C)
DMPC/Chol/NPPE (8:3:4)	30.1 ± 0.8	1.9 ± 0.1	6.4 ± 0.3	9.1 ± 0.3
DMPC/Chol/NPPE/GLU (8:3:4:1.5)	30.1 ± 1.1	2.8 ± 0.2	9.1 ± 0.7	12.8 ± 0.5
DMPC/Chol/NPPE/MAN (8:3:4:1.5)	31.3 ± 0.7	3.2 ± 0.2	10.4 ± 0.7	15.3 ± 0.3
DMPC/Chol/NPPE/G_{M1} (8:3:4:1.5)	35.2 ± 0.9	2.9 ± 0.3	9.5 ± 1.0	14.8 ± 0.4
DMPC/Chol/NPPE/SUL (8:3:4:1.5)	31.4 ± 1.0	3.1 ± 0.1	10.2 ± 0.4	16.7 ± 0.5

[a] Temperature at the maximum.
[b] Calorimetric enthalpy calculated from the area under the peak.
[c] Width of the calorimetric peak at half-peak height. Data are expressed as means ± SD of three independent experiments.

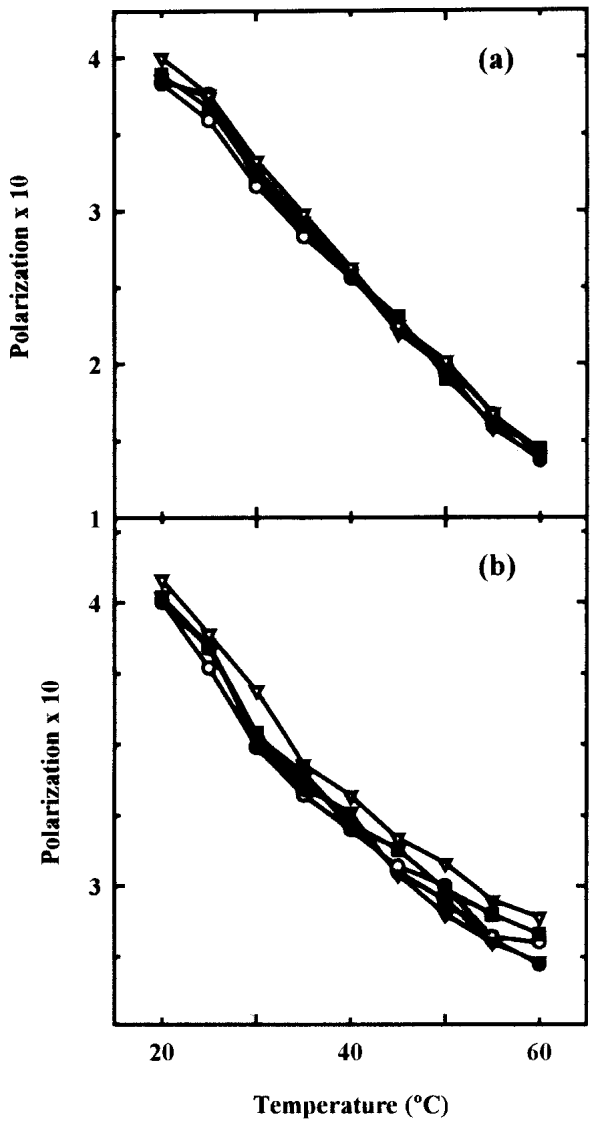

Fig. 2. Influence of liposome composition on the packing degree of the acyl chains in the bilayer. Temperature profiles corresponding to the fluorescence polarisation of DPH (a) and TMA-DPH (b) incorporated into lipid bilayer. The results indicated in the plots are the means of individual experiments performed in triplicate and the coefficients of variation ranged from 2 to 4%. Lipid concentration was 0.35 mg/mL. (●) DMPC/Chol/NPPE (8:3:4), (○) DMPC/Chol/NPPE/GLU (8:3:4:1.5), (▼) DMPC/Chol/NPPE/MAN (8:3:4:1.5), (▽) DMPC/Chol/NPPE/G_{M1} (8:3:4:1.5), and (■) DMPC/Chol/NPPE/SUL (8:3:4:1.5).

level and with the higher main transition temperature of this system. This result could be the consequence of the rigidifying effect of GM_1. The results showed that all the glycosylated formulations assayed gave liposomes with very little permeability (< 4.5% at 90 min) at physiologic temperature. These results point out the high ability of such liposomal formulations to hold the encapsulated material and their potential use for drug delivery.

The effect of the temperature on the behavior of glycosylated liposomes was also shown by changes in their permeability properties (Fig. 5). In general, there was a progressive increase in carboxyfluorescein release when temperature increased, both in PBS and in the presence of rat serum. Nevertheless, the temperature-leakage relationship depends on the glycoside nature and on the medium. Thus, at 30°C, the percentage of leakage after 90 min, was always lower than 3%, and the CF released was slightly higher in the presence of

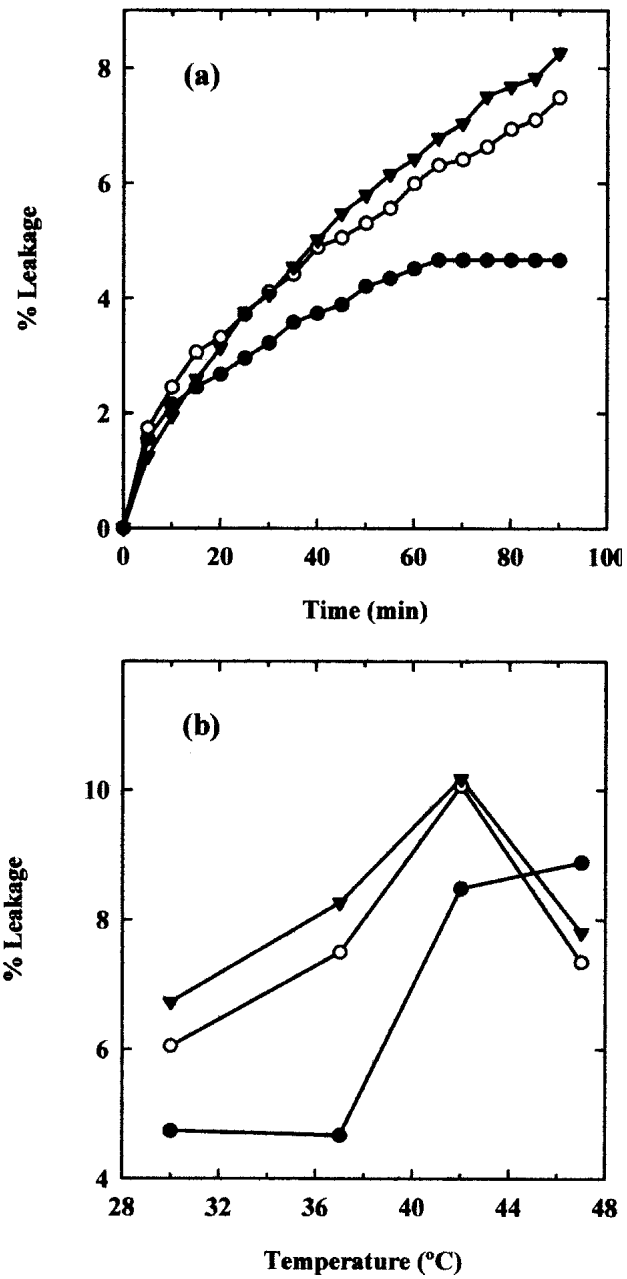

Fig. 3. Effect of serum and temperature in carboxyfluorescein efflux from DMPC/Chol/NPPE (8:3:4) liposomes. Efflux was measured during 1.5-h periods in the absence (●) and in the presence of rat serum at lipid serum ratio of 0.38 mmols lipid/mL serum (○) and 0.038 mmols lipid/mL serum (▼). (a) Time curves of carboxyfluorescein efflux at 37°C expressed as the percentage of initial trapped solute lost over a given time. (b) Carboxyfluorescein efflux at 90 min expressed as the percentage of initial trapped solute at different temperatures. Lipid concentration in the spectrofluorimeter cuvettes was 4 μg/mL. The results shown are the means of individual experiments performed in triplicate. The variation coefficients ranged between 3 and 12% for (a) and between 4 and 9% for (b).

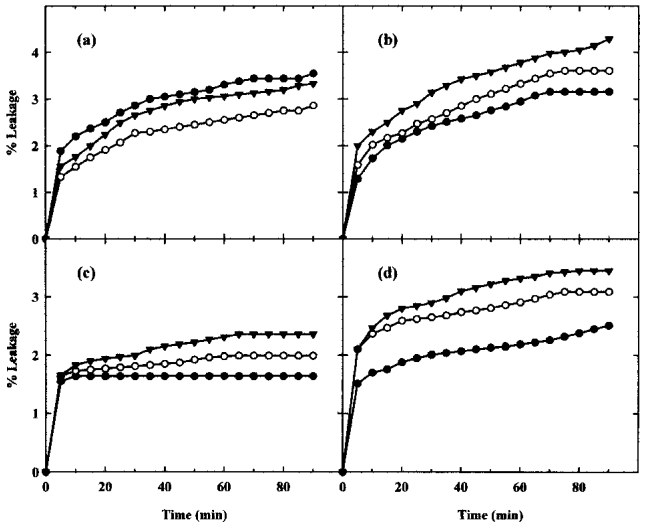

Fig. 4. Time curves of carboxyfluorescein efflux from liposomes containing glycoside determinants. Efflux was measured during 1.5-h periods at 37°C in the absence (●) and in the presence of rat serum at lipid serum ratio of 0.38 mmols lipid/mL serum (○) and 0.038 mmols lipid/mL serum (▼). Efflux is expressed as the percentage of initial trapped solute lost over a given time. Molar ratio of bilayer components was as follows: (a) DMPC/Chol/NPPE/MAN (8:3:4:1.5), (b) DMPC/Chol/NPPE/GLU (8:3:4:1.5), (c) DMPC/Chol/NPPE/G_{M1} (8:3:4:1.5), and (d) DMPC/Chol/NPPE/SUL (8:3:4:1.5). Lipid concentration in the spectrofluorimeter cuvettes was 4 μg/mL. The results shown are the means of individual experiments performed in triplicate. The coefficients of variation ranged from 2 to 11%.

serum when liposomes contained GM_1. At 37°C, CF efflux was higher in the presence of serum, except for liposomes containing mannose. At 42°C, a decrease in the permeability in the presence of serum was observed for glucose-containing liposomes. And finally, at 47°C, a stabilization of liposomes in the presence of serum was observed in all cases except for liposomes containing GM_1 at a lipid-serum ratio of 0.038 mmol lipid/mL serum. Figure 5 also shows that, after 90 min, CF leakage in the presence of serum, at temperature up to 47°C, never surpasses the 8% of the total dye entrapped into liposomes.

Targeting of Liposomes Containing Glycoside Determinants to the Brain

The more interesting results were observed after injection of GM_1 liposomes (Table III). A brain tracer uptake higher for GM_1 liposomes than for control liposomes was recovered in the cortex, basal ganglia, and mesencephalon of both hemispheres; conversely, no significant changes were observed in liver uptake and blood concentration of the tracer contained in GM_1 vesicles. Thus, GM_1 liposomes appear good candidates as a DDS to the brain. GM_1 is endowed with neurotrophic and neuroprotective properties (28) and the use of GM_1 liposomes might be particularly favorable in certain cerebral pathologic conditions, acting this component of the liposomal system synergically together with the liposome-encapsulated drug.

Furthermore, our results seem to exclude that the observed best brain uptake of [H^3] is due to repeated liposome passages through the cerebral districts, given that no increase in blood tracer levels was observed. Of course, further experiments are needed to clarify if GM_1 liposomes might be sequestered in the brain vessel endothelium.

Concerning MAN, GLU and SUL liposomes, no significant increase in brain uptake of tracer was found in comparison with control liposomes. The liver uptake of tracer was higher in rats treated with SUL and GLU liposomes than in those receiving control liposomes, but the difference was only statistically significant in the first case. Moreover, higher blood levels of tracer were observed in GLU- and MAN-liposomes injected rats than in control animals.

These results could mean that glucose and, particularly, mannose incorporation might improve the survival of liposomes in blood circulation (so functioning, perhaps, as circulating reservoirs), but not their selective delivery to the brain, also because of an unfavorable competition with endogenous substrates. Conversely, a better proclivity to liver sequestration may be supposed for GLU and, especially, SUL liposomes. Consistent with this finding, glycosylated (particularly galactosylated and mannosylated) liposomes recently have proved to be a promising approach to obtain selective hepatic drug delivery (11,29), and sulfatide-containing reverse-phase evaporation vesicles seemed able to target the encapsulated drug into the liver (30).

CONCLUSIONS

This work describes the preparation and characterization of liposomal carriers to encapsulate neuroactive compounds and to carry them to the brain across the blood-brain barrier. The incorporation of glycoside determinants, for an effective

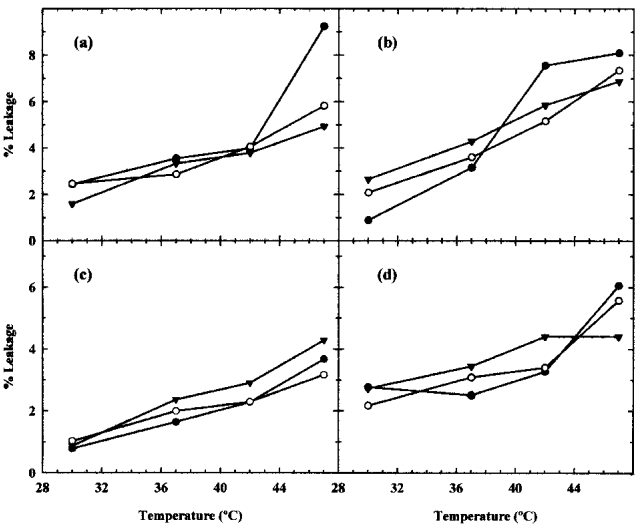

Fig. 5. Carboxyfluorescein efflux of liposomes containing glycoside determinants at 90 min expressed as the percentage of initial trapped solute at different temperatures. Efflux was measured after a 90-min liposome incubation in the absence (●) and in the presence of rat serum at lipid serum ratio of 0.38 mmols lipid/mL serum (○) and 0.038 mmols lipid/mL serum (▼). Efflux is expressed as the percentage of initial trapped solute lost at different temperatures. Molar ratio of bilayer components was as follows: (a) DMPC/Chol/NPPE/MAN (8:3:4:1.5), (b) DMPC/Chol/NPPE/GLU (8:3:4:1.5), (c) DMPC/Chol/NPPE/G_{M1} (8:3:4:1.5), and (d) DMPC/Chol/NPPE/SUL (8:3:4:1.5). Lipid concentration in the spectrofluorimeter cuvettes was 4 μg/mL. The results shown are the means of individual experiments performed in triplicate. The coefficients of variation ranged from 2 to 8%.

Table III. Biodistribution of DMPC/Chol/NPPE Liposomes Containing Different Glycoside Determinants in Healthy Rats

Tissue	[H^3] Tissue uptake[a]				
	Control liposomes	Glucose liposomes	Mannose liposomes	GM_1 liposomes	Sulfatide liposomes
Cortex	Right 0.11 ± 0.04	Right 0.14 ± 0.03	Right 0.15 ± 0.01	Right 0.48 ± 0.11[b]	Right 0.08 ± 0.02
	Left 0.16 ± 0.05	Left 0.33 ± 0.07[b]	Left 0.16 ± 0.02	Left 3.25 ± 0.82[b]	Left 0.10 ± 0.02
Basal ganglia + mesencephalon	Right 0.08 ± 0.03	Right 0.12 ± 0.03	Right 0.14 ± 0.01	Right 0.26 ± 0.06[b]	Right 0.08 ± 0.01
	Left 0.10 ± 0.06	Left 0.24 ± 0.10	Left 0.14 ± 0.01	Left 2.48 ± 0.70[b]	Left 0.09 ± 0.02
Liver	7.16 ± 1.42	10.88 ± 2.56	7.39 ± 1.45	4.66 ± 0.76	18.66 ± 4.25[c]
Whole blood	17.32 ± 2.50	26.45 ± 3.89[c]	101.97 ± 28.9[c]	20.66 ± 2.78	19.32 ± 2.86

[a] Tissue uptake was calculated by the following equation: (tissue dpm/g tissue) × (g rat body weight/total injected dpm). Data are expressed as means ± SD of four experiments at least.
[b] $p < 0.05$ vs. the respective.
[c] $p < 0.05$ vs. control, of control.

targeting, and a fusogenic lipid, to facilitate the transfer of the encapsulated material into the cells, has been considered. The results show that all glycosylated formulations made liposomes able to retain up to 95% of the encapsulated carboxyfluorescein after 90 min at physiologic temperature, even in the presence of serum.

Biodistribution studies show that GM_1 liposomes are good candidates as a DDS to the brain. Nevertheless, glucose and, particularly, mannose incorporation might improve the survival in blood circulation of liposomes acting as circulating reservoirs, but not their selective delivery to the brain. Conversely, a better proclivity to liver sequestration may be supposed for GLU and, especially, SUL liposomes.

Both the potential fusogenic characteristics and the long-circulating behavior of these NPPE- and glycoside-containing liposomes are now being considered on the basis of our previous studies in this regard (21) and of the results of the permeability and biodistribution experiments.

ACKNOWLEDGMENTS

This work was supported by a grant from DGICYT (Spain) (PB94-0911-A). The authors thank the University of Messina for the postdoctoral fellowship given to Dr. Domenico Trombetta.

REFERENCES

1. W. M. Pardridge. New approaches to drug delivery through the blood brain barrier. *Trends Biotechnol.* **12**:239–245 (1994).
2. W. H. Oldendorf and J. Szabo. Amino acid assignment to one of three blood-brain barrier amino acid carriers. *Am. J. Physiol.* **230**:94–98 (1976).
3. A. Gjedde. High- and low-affinity transport of D-glucose from blood to brain. *J. Neurochem.* **36**:1463–1471 (1981).
4. W. A. Jefferies, M. R. Brandon, S. V. Hunt, A. F. Williams, K. C. Gatter, and D. Y. Mason. Transferrin receptors on endothelium of brain capillaries. *Nature* **312**:162–163 (1984).
5. A. J. Kastin, W. Pan, L. M. Maness, and W. A. Banks. Peptides crossing the blood-brain barrier: some unusual observations. *Brain Res.* **848**:96–100 (1999).
6. R. A. Kroll and A. A. Neuwelt. Outwitting the blood-brain barrier for therapeutic purposes: osmotic opening and other means. *Neurosurgery* **42**:1083–1100 (1998).
7. A. Prokop. Bioartificial organs in the twenty-first century: Nanobiological devices. *Ann. NY Acad. Sci.* **944**:472–490 (2001).
8. D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K. D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* **88**:11460–11464 (1991).
9. F. P. Bonina, L. Arenare, F. Palagiano, A. Saija, F. Nava, D. Trombetta, and P. Caprariis. Synthesis, stability and pharmacological evaluation of nipecotic acid prodrugs. *J. Pharm. Sci.* **88**: 561–567 (1999).
10. R. D. Egleton, S. A. Mitchell, J. D. Huber, J. Janders, D. Stropova, R. Polt, H. I. Yamamura, V. J. Hruby, and T. P. Davis. Improved bioavailability to the brain of glycosylated Met-enkephalin analogs. *Brain Res.* **881**:37–46 (2000).
11. S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, and M. Hashida. Biodistribution characteristics of mannosylated, fucosylated and galactosylated liposomes in mice. *Biochim. Biophys. Acta* **1524**:258–265 (2000).
12. F. Umezawa and Y. Eto. Liposome targeting to mouse brain: mannose as a recognition marker. *Biochim. Biophys. Res. Commun.* **153**:1038–1044 (1988).
13. T. Shangguan, C. C. Pak, S. Ali, A. S. Janoff, and P. Meers. Cation-dependent fusogenicity of an N-acyl phosphatidylethanolamine. *Biochim. Biophys. Acta* **1368**:171–183 (1998).
14. K. Kono, M. Iwamoto, R. Nishikawa, H. Yanagie, and T. Takagishi. Design of fusogenic liposomes using a poly(ethylene glycol) derivative having amino grups. *J. Control. Release* **68**:225–235 (2000).
15. H. Haller, C. Maasch, D. Dragun, M. Wellner, M. Vonjantalipinski, and F. C. Luft. Antisense oligodesoxynucleotide strategies in renal and cardiovascular disease. *Kidney Int.* **53**:1550–1558 (1998).
16. C. Puyal, L. Maurin, G. Miruel, A. Bienvenüe, and J. Philippot. Design of a short membrane-destabilizing peptide covalently bound to liposomes. *Biochim. Biophys. Acta* **1195**:259–266 (1994).
17. M. Mora, F. Mir, M. A. De Madariaga, and M. L. Sagristá. Aggregation and fusion of vesicles composed of N-palmitoyl derivatives of membrane phospholipids. *Lipids* **35**:513–524 (2000).
18. J. C. Domingo, M. Mora, and M. A. De Madariaga. Incorporation of N-acylethanolamine pohospholipids into egg phosphatidylcholine vesicles: characterization and permeability properties of the binary systems. *Biochim. Biophys. Acta* **1148**:308–316 (1993).
19. M. Mercadal, J. C. Domingo, M. Bermúdez, M. Mora, and M. A. De Madariaga. N-Palmitoylphosphatidylethanolamine stabilizes liposomes in the presence of human serum: Effect of lipidic composition and system characterization. *Biochim. Biophys. Acta* **1235**:281–288 (1995).
20. G. Rouser, A. N. Siakotos, and S. Fleischer. Quantitative analysis of phospholipids by thin layer chromatography and phosphorus analysis of spots. *Lipids* **1**:85–86 (1966).
21. M. L. Sagristá, M. Bermúdez, M. A. De Madariaga, and M. Mora. N-acylaminophospholipids give negative charge and fusogenic properties to lipid bilayers. Use of N-palmitoylphosphatidylethanolamine to obtain long-circulating and fusogenic liposomes to encapsulate tuberculostatic drugs. In: S. G. Pandalai (ed.), *Recent Research Development in Lipids Research*, Vol. 3. Transworld Research Network, Trivandrum, India, 1999, pp. 127–158.

22. J. C. M. Stewart. Colorimetric determination of phospholipids with ammonium ferrocyanate. *Anal. Biochem.* **104**:10–14 (1980).
23. J. N. Weinstein, E. Ralston, L. D. Leserman, R. D. Klausner, P. Dragsten, P. Henkart, and R. Blumental. Self-quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome cell interaction. In: G. Gregoriadis (ed.), *Liposome Technology*, Vol. 3, CRC Press, Boca Raton, Florida 1984, pp. 183–204.
24. J. C. Domingo, F. Rosell, M. Mora, and M. A. De Madariaga. Importance of the purification grade of 5(6)-carboxyfluorescein on the stability and permeability properties of N-acylphosphatidylethanolamine liposomes. *Biochem. Soc. Trans.* **17**:997–999 (1989).
25. A. Saija, P. Princi, M. Lanza, M. Scalese, E. Aramnejad, and A. De Sarro. Systemic cytokine administration can affect blood-brain barrier permeability in the rat. *Life Sci.* **56**:775–784 (1995).
26. K. Hoshiyama, A. Nagayasa, Y. Yamagiwa, T. Nishida, H. Harashima, and H. Kiwada. Effects of the size and fluidity of liposomes on their accumulation in tumors: A presumption of their interaction with tumors. *Int. J. Pharmaceutics* **121**:195–203 (1995).
27. M. Bermúdez, E. Martínez, M. Mora, M. L. Sagristá, and M. A. De Madariaga. Molecular and physicochemical aspects of the interactions of the tuberculostatics ofloxacin and rifampicin with liposomal bilayers: A ^{31}P-NMR and DSC study. *Colloids Surf.* **158**:59–66 (1999).
28. J. M. Wells, R. F. Ventura, P. B. Eisenhauer, D. C. McKenna, R. E. Fine, and M. D. Ullman. Transport of GM1 and GM1 inner ester across an in vitro model of the blood-brain barrier. *Neurosci. Lett.* **217**:121–124 (1996).
29. Y. Hattori, S. Kawakami, F. Yamashita, and M. Hashida. Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting. *J. Control. Release* **69**:369–377 (2000).
30. D. Chen and K. H. Lee. Biodistribution of calcitonin encapsulated in liposomes in mice with particular reference to the central nervous system. *Biochim. Biophys. Acta* **1158**:244–250 (1993).