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Enumeration of Liposomes by Multinuclear NMR and Photon Correlation Spectroscopy

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Diamagnetic dipalmitoylphosphatidylcholine (DPPC) liposomes dispersed in glucose solution as well as their paramagnetic analogs encapsulating a paramagnetic contrast agent used in magnetic resonance imaging (Gd-HPDO3A, ProHance®) were prepared and characterized. The vesicle diameter was assessed by photon correlation spectroscopy (PCS). ^{31}P NMR spectroscopy was used to measure the phospholipid content and to confirm the highly unilamellar character of the liposome membrane. For both types of liposome preparation, the internal water volume was evaluated below the phase transition temperature (T_m) by natural abundance ^{17}O NMR spectroscopy in the presence of a shift reagent confined to the external compartment. For the paramagnetic vesicles, the internal water content was independently assessed by analysis of the biexponential decay of the proton transverse magnetization below T_m. Knowing the unilamellarity of the vesicles (^{31}P NMR measurements), the number concentration of liposomes was assessed from the liposomal internal volume calculated from PCS data and the total internal water content obtained by ^{17}O NMR spectroscopy or ^{1}H relaxometry. The results obtained are in good agreement and validate these techniques as non invasive methods for the assessment of the number concentration of liposome in suspension.

Keywords: Liposomes; Multinuclear NMR; Number concentration; Drug carriers; MRI contrast agents

INTRODUCTION

Liposomes have "matured" as delivery systems for therapeutic and diagnostic agents [1–5]. Despite their discovery thirty-five years ago [6], it is only during the last decade that the liposome carrier concept has been developed at a "pharmaceutical level". This development is linked to the rational design of liposomal formulations based on physicochemical and biophysical studies of the lipid bilayers. Also, the use of sterically stabilized and/or ligand targeted liposomal delivery has opened the way for more attractive medical applications, such as targeting to tumors and inflammation sites [2,3].

In the early stages of the investigation, a thorough physicochemical characterization of the liposome preparation is required [7]. This is especially important as the liposome behavior *in vitro* and *in vivo* is critically influenced by physicochemical properties such as surface charge, bilayer rigidity, and overall size [8]. Another important parameter is the internal volume of the vesicles, which determines the amount of encapsulated material.

Knowing the number concentration of liposomes has shown to be relevant for the *in vitro* contrast efficacy of liposome encapsulating paramagnetic species [9]. For *in vivo* purposes, this parameter may be even more critical. For example, the tolerance for liposomes depends on the lipid dose and, hence, the number of vesicles injected [10]. More importantly, if the mechanisms of liposome deposition *in vivo* are number or surface-area dependent, knowledge of the number of injected vesicles is mandatory [11]. The latter is also important when considering active targeting; a successful approach requires that a large enough number of liposomes be injected to ensure high occupancy of the cell surface receptors.

Methods for determination of the total liposome internal volume have been described and can be

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classified as invasive and non-invasive [11,12]. For example, a typically invasive procedure is to encapsulate, during liposome manufacture, a water soluble marker which does not interact with the bilayer (i.e. carboxyfluoerescein, ^{3}H inulin, etc). Its quantification is performed after removal or chemical inactivation of extraliposomal material. Non-invasive methods are more attractive; the total entrapped volume can be estimated from densitometry, light scattering or fractionation. The densitometric method is regarded as a robust and simple technique; reliable values of entrapped volume are only obtained if the densities of the internal and external media are known and markedly different.

In the present work, we propose two original and non-invasive methods to determine the total internal volume of liposomes. The first one, based on the natural abundance ^{17}O NMR spectrum, consists of comparing the relative areas of the ^{17}O resonances of internal and external water, the latter one being shifted by a shift agent (dysprosium chloride). The second procedure concerns paramagnetic liposomes for which the internal water content can be obtained by analysis of the biexponential behavior of the proton transverse magnetization decay obtained from a Carr-Purcell-Meiboom-Gill experiment.

The number concentration of the liposomes can thus be calculated from: (i) the total internal liposomal volume obtained by ^{17}O and ^{1}H NMR, (ii) the phospholipid content and the lamellarity of the vesicles quantitatively determined by ^{31}P NMR, and (iii) the liposome size obtained by photon correlation spectroscopy (PCS).

MATERIALS AND METHODS

Chemicals

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) (Sygena Facility, Liestal, Switzerland), glucose monohydrate (Fluka, Bornem, Belgium), Gd-HPDO3A (gadolinium (III) complex of 10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (ProHance®, Bracco SpA, Milano, Italy), methylenediphosphonic acid (MPDA) (Aldrich, Bornem, Belgium), manganese(II) chloride tetrahydrate (Aldrich, Bornem, Belgium) and dysprosium(III) chloride (Aldrich, Bornem, Belgium) were used without further purification.

Liposome Preparation

After dissolution of the phospholipids in chloroform, the organic solvent was removed by evaporation to dryness under reduced pressure. The residual phospholipid film was hydrated with a 5% glucose solution above T_m. The resulting multilamellar diamagnetic vesicles were sized-down by repeated extrusions through polycarbonate filters of various pore sizes 200, 400, 600 or 1000 nm at temperatures above T_m (Lipex Biomembranes, Vancouver, Canada), as decribed by Hope *et al.* [13]. The paramagnetic liposomes were prepared analogously by hydration of DPPC with a 5% glucose solution containing 25 mM of Gd-HPDO3A, extruded 10 times and dialyzed (MWCO 12-14000 daltons, Medicell Int. Ltd, London, England) against a 5% glucose solution. The end point of the dialysis was assessed by the absence of extraliposomal Gd-HPDO3A as checked by proton longitudinal relaxometry at 20 MHz.

NMR

^{31}P NMR spectra were recorded at 121.5 MHz on a Bruker AMX-300 spectrometer (Bruker, Karlsruhe, Germany) fitted with a broadband probe (pulse angle: 90°/14 μs, repetition time: 30 s, SW: 62,500 Hz, LB: 50 Hz, NS: 32). No proton decoupling was applied. 10 mm o.d. sample tubes were used. A known amount of MDPA contained in a concentric 5 mm o.d. tube was used as a reference for quantitation. The filling of the 10 and 5 mm tubes was identical and adjusted to the coil height. No field frequency lock was used.

^{17}O NMR spectra were obtained at natural abundance at 40.7 MHz and 298 K on a Bruker AMX-300 spectrometer (Bruker, Karlsruhe, Germany) (pulse angle: 90°/27 μs, repetition time: 110 ms, SW: 3300 Hz, NS $>$ 300). Proton broadband decoupling was applied throughout the experiments. Samples (1.8–2 ml) were contained in 10 mm o.d. sample tubes.

During the ^{17}O and ^{31}P NMR experiments, the temperature was kept constant by an air flow controlled by a Bruker BVT-2000 temperature unit. The area under the peaks was measured using the standard integration routine of the manufacturer.

Proton relaxometry was performed at 20 MHz on a Bruker Minispec PC-20 (Bruker, Karlsruhe, Germany). The temperature regulation was ensured by a perchlorinated liquid flow. Longitudinal relaxation times (T_1) were measured by the inversion recovery method and the data were analyzed by a three parameters fitting procedure. Transverse relaxation times (T_2) were measured using a Carr-Purcell-Meiboom-Gill sequence on at least 50 echoes of the echo train. The amplitudes of the echoes were fed to a personal computer and analyzed by a multiparametric minimization program (Minuit Cern Library program) or by the Scientist program. The data were fitted using Eq. (1) which describes the evolution of the transverse magnetization for nuclei

located in two different sites in slow exchange and where they experience the same chemical shift but different relaxation times.

$$M_t = M_A^0 e^{-tR_2^A} + M_B^0 e^{-tR_2^B} \quad (1)$$

where M_t is the transverse magnetization at time t, M_A^0 and M_B^0 are the initial magnetizations in compartments A and B, respectively, and R_2^A and R_2^B are the corresponding transverse relaxation rates.

Photon Correlation Spectroscopy

PCS was performed on a BIC multiangle laser light scattering system at room temperature and with a 90° scattering angle (Brookhaven Instruments Corporation, Holtsville, USA). The intensity weighted liposome diameter was measured on diluted suspensions and calculated by a non-negatively constrained least squares (multiple pass) routine. The liposome preparations were diluted with isoosmotic glucose solutions. The osmolality of the solutions was measured with a Microosmometer (model 3M0, Advanced Instruments, Massachussets, USA).

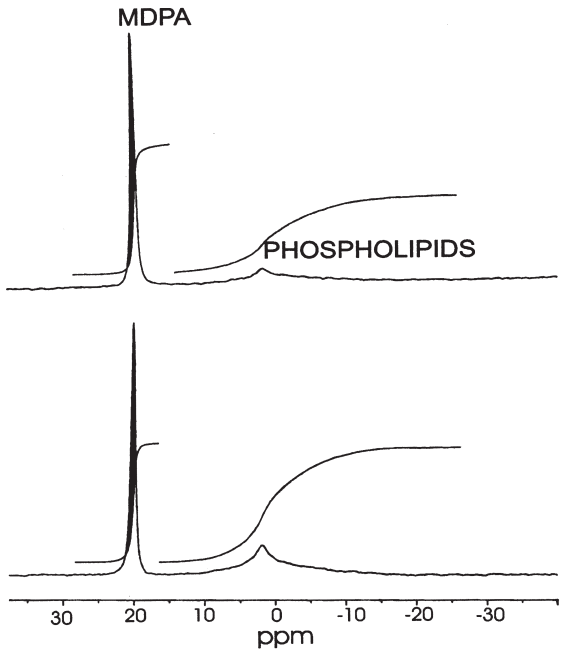

FIGURE 1 ^{31}P NMR spectra of paramagnetic DPPC based liposomes (25 mM of Gd-HPDO3A encapsulated) before (bottom trace) and after (top trace) addition of 4.26 mM of Mn^{++} (T = 335 K).

RESULTS AND DISCUSSION

Phospholipid Content

The phospholipid concentration in the liposome dispersions was obtained by measurement of the area of the ^{31}P NMR resonances of the phospholipids in intact vesicles relative to the reference contained in a coaxial tube (methylene diphosphonic acid: MDPA) at 335 K. Temperature higher than the phase transition (T_m = 314.5 K) was chosen because narrower resonances are observed and consequently, quantification is more accurate. A long repetition time (TR = 30 s) was used to guarantee the complete relaxation of the nuclei. Although the resonance of the phospholipids is quite broad even at this temperature, it does not overlap the reference signal and the integration of the peaks gives reliable results (see Fig. 1, lower trace).

The results obtained on several samples agree very well with the values expected from the amount of phospholipids initially used for the liposome preparation, indicating a negligible loss of phospholipid material during the various processes (Table I). However, phospholipid degradation (i.e. hydrolysis) cannot be discarded. It is to be noted that this analytical technique is purely non destructive since it does not require the destruction of the liposomal structure by a detergent.

Lamellarity

Numerous ^{31}P NMR studies have been used to assess the lamellarity of the liposomes. If they do not cross the bilayer, paramagnetic shift (Pr^{3+}, Nd^{3+}, Eu^{3+}) or relaxation (Mn^{2+}, Co^{2+}) agents influence only the external phospholipidic heads and cause a chemical shift and/or a marked broadening of the resonance of the outer phospholipids whereas the resonance of the inner phospholipids is unaffected [13–17].

The phospholipid ^{31}P resonance areas were measured before and after addition of paramagnetic Mn^{2+}, an ion which markedly broadens the absorption of the phospholipids with which it interacts

TABLE I Phospholipid content of diamagnetic and paramagnetic DPPC-based liposome dispersions determined by ^{31}P NMR

Encapsulated solution	Initial phospholipid content (mg/ml)	Phospholipid content measured by ^{31}P NMR after preparation (mg/ml)	Decrease of phospholipid resonance after addition of Mn^{2+} (%)
Glucose 5%	69.6	69.95	45
Glucose 5%	70	69.56	47
Gd-HPDO3A 25 mM in glucose 5%	70	69.56	44
Gd-HPDO3A 25 mM in glucose 5%	70	66.16	42

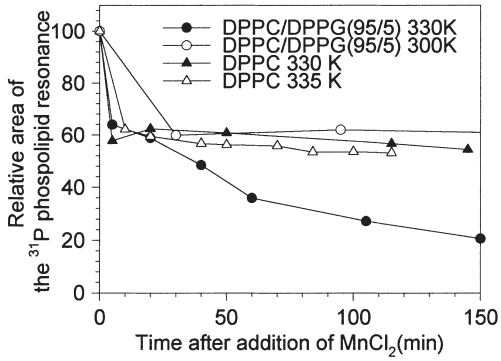

FIGURE 2 Area of the phospholipid ^{31}P resonance as a function of time after addition of $MnCl_2$ (1.5 and 2.1 mM for phospholipid concentrations equal to 50 and 70 mg/ml, respectively). Closed circles: DPPC/DPPG liposomes (95/5 (w/w), 50 mg/ml) extruded ten times through 200 nm filters ($T = 330$ K); open circles: DPPC/DPPG liposomes (95/5 (w/w), 50 mg/ml) extruded four times through 400 nm filters ($T = 300$ K); closed triangles: DPPC liposomes (50 mg/ml) extruded seven times through 200 nm filters ($T = 330$ K); open triangles: DPPC liposomes (70 mg/ml) extruded ten times through 400 nm filters ($T = 335$ K).

[13,17]. These resonances become undetectable at high magnetic fields (Fig. 1). Provided the Mn^{2+} ion does not permeate the bilayer, the decrease in the peak area is directly related to the lamellarity. Recently, Frölich *et al.* [18] reported that the buffer concentration may influence the determination of the lamellarity by ^{31}P NMR because Mn^{2+} internalisation can happen. In our experimental conditions (glucose 5%), the Mn^{2+} ion indeed crosses the membrane of negatively charged DPPC/DDPG liposomes but only at temperatures above T_m (Fig. 2) [19]. On the contrary, "neutral" DPPC liposomes are not permeable to Mn^{2+} ions below and above T_m during at least 2 h. This second type of liposomes was therefore preferred and the ^{31}P NMR measurements were performed at $T > T_m$ in order to observe narrower resonance. All the liposome preparations extruded several times show a decrease of 42–47% of the phospholipid resonance after addition of Mn^{2+} ions. These results therefore demonstrate the highly unilamellar character of the vesicles (Table I).

Photon Correlation Spectroscopy

PCS showed narrow and monomodal size distribution for all the liposome samples. Repeated extrusion through membranes filters with a 200 nm pore size resulted in liposomes with a mean diameter of 179–209 nm. With the 400 nm pore size membranes, the mean diameter ranged from 260 to 306 nm and with larger pore size (1000 nm), the diameter was 409–454 nm.

^{17}O NMR

In the absence of shift agent, the ^{17}O spectrum of a liposome suspension shows only one detectable

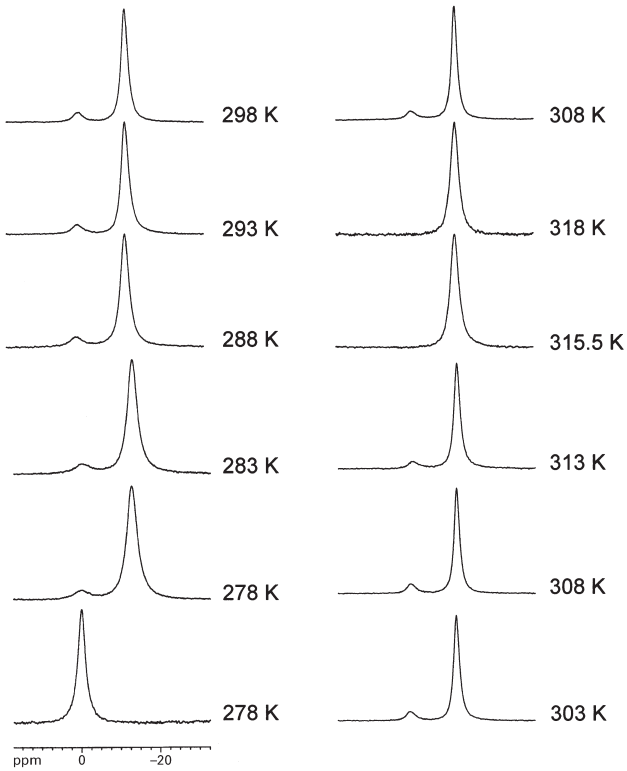

FIGURE 3 ^{17}O NMR spectra of diamagnetic DPPC liposome dispersions (70 mg/ml) extruded ten times through 400 mm filters at various temperatures before addition of $DyCl_3$ (bottom left spectrum, mean diameter 286 nm) and after addition of 21.7 mM of $DyCl_3$ (mean diameter: 236 nm). The temperature was raised from 278 to 318 K, the last spectrum (right upper spectrum) was obtained after cooling the dispersion from 318 to 308 K and confirms the perfect reversibility of the process, excluding any internalization of the shift agent.

resonance corresponding to the water oxygen nuclei irrespective of their intra- or extra-vesicular location (Fig. 3). At temperatures below T_m and after addition of the paramagnetic shift reagent ($DyCl_3$) which does not enter the liposome, this resonance splits into two peaks. The small unshifted resonance corresponds to the internal water whereas the shifted one arises from the external water in contact with the paramagnetic shift agent. These two peaks are observed until T_m is reached where a coalescence is observed. The phenomenon is reversible as shown by the similarity of the spectra recorded at 308 K before and after heating to 318 K. These results thus show that (i) as previously observed for Mn^{2+} ions, Dy^{3+} ions are confined to the external compartment below and above T_m of DPPC liposomes, (ii) below T_m, the exchange rate between internal and external water is low as compared to the chemical shift difference of the internal and external water signals, and (iii) above T_m, the increase of the water exchange rate leads to the coalescence of the peaks. From the chemical shift difference between the peaks (0.50–0.55 ppm per millimole of Dy^{3+}), an upper limit of approx. 2500 s^{-1} can be calculated for the water exchange rate

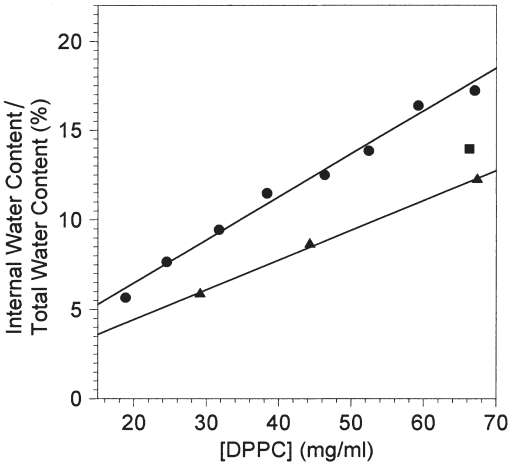

FIGURE 4 Internal water content/total water content measured by ^{17}O NMR of diamagnetic DPPC liposome dispersions of different sizes and at various osmolalities ($T = 298\,K$; closed circles: mean diameter 242 nm, osmolality 340 mOsm/kg; closed triangles: mean diameter 169 nm, osmolality 324 mOsm/kg; closed square: mean diameter 236 nm, osmolality 384 mOsm/kg).

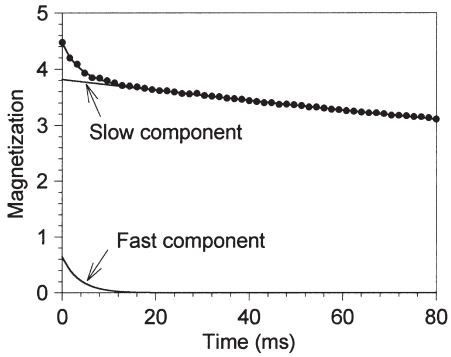

FIGURE 5 Decay of the proton transverse magnetization (CPMG sequence) of a dispersion of paramagnetic DPPC based liposomes (70 mg/ml, mean diameter: 282 nm) encapsulating 25 mM of Gd-HPDO3A at 278 K. The fitted values of M_A^0, M_B^0, R_2^A, R_2^B are 14.5, 85.5%, 270 and 2.6 s^{-1}, respectively.

below T_m. This corresponds to a water mean residence time longer than 0.4 ms.

From the relative area of the resonances, the respective volumes of the internal and the external water compartments can be calculated. The method was applied to solutions containing liposomes of different sizes and at various phospholipid concentrations (Fig. 4). As expected, the results show that for similar phosholipid content, the larger the mean diameter, the larger the internal volume of unilamellar vesicles. It is however noteworthy that the error increases when the internal water content decreases and that accurate measurements of water content smaller than 5% are difficult to carry out.

The Dy^{3+} content needed to obtain well resolved peaks was about 15–22 mM for diamagnetic liposomes. For paramagnetic liposomes, the presence of intraliposomal Gd-HPDO3A induces a broadening of the internal water resonance ($\Delta\nu_{1/2}$ increases by $\sim$120 Hz at 298 K in the presence of 25 mM of Gd-HPDO3A). Addition of larger concentrations of Dy^{3+} (30–32 mM) are therefore necessary to avoid an overlap of the resonances.

It should be pointed out that the addition of dysprosium salt increases the extraliposomal osmolality which in turn leads to a decrease of the mean diameter of the liposomes (Table II). The samples prepared for PCS measurements were thus diluted with glucose solutions whose concentration was adjusted to the osmolality of the original Dy^{3+} doped samples submitted to the ^{17}O analysis.

^1H NMR

Below T_m, the water exchange rate between the interior and exterior of the currently investigated DPPC liposomes is slow (50–100 s^{-1}) [20] and limits the efficacy of the entrapped paramagnetic complex (Gd-HPDO3A) as an MRI T_1 contrast agent. A biexponential decay of the proton transverse magnetization during a CPMG sequence at a temperature lower than T_m is thus expected, its first part corresponds to the fast relaxing water molecules in contact with the paramagnetic contrast agent and its second part results from the slowly relaxing external water (Fig. 5). This situation can thus be exploited to assess the internal and the external volumes by analyzing the experimental data. As shown in Fig. 5 for a suspension of liposomes encapsulating 25 mM of Gd-HPDO3A at 278 K, R_2 of the fast relaxing component is 270 s^{-1} for a relative population of 14.5%, and R_2 of the slow relaxing component is 2.57 s^{-1}. These R_2 values are in rather good agreement with those measured for a paramagnetic 5% glucose solution of 25 mM of Gd-HPDO3A ($\sim$210 s^{-1}) and the corresponding glucose solution

TABLE II Osmolality and mean diameter of various diamagnetic and paramagnetic DPPC-based liposomal dispersions (phospholipid concentration: 70 mg/ml)

Encapsulated solution	Osmolality before addition of Dy^{3+} (mOsm/kg)	Mean diameter before addition of Dy^{3+} (nm)	Osmolality after addition of Dy^{3+} (mOsm/kg)	Mean diameter after addition of Dy^{3+} (nm)
Glucose 5%	288	179	324	169
Gd-HPDO3A 25 mM in glucose 5%	306	260	378	231
Glucose 5%	301	286	384	236
Gd-HPDO3A 25 mM in glucose 5%	323	240	428	210

TABLE III Number concentration of various diamagnetic and paramagnetic DPPC-based lipsosomes dispersed in glucose 5% (DPPC 70 mg/ml)

Initial diameter (nm)	Diameter after addition of Dy^{3+} (nm)	Internal water content (% per mg of phospholipid)	Number concentration per ml and per mg of phospholipids	Average number of phospholipid molecules per vesicle
Diamagnetic liposomes*				
179	169	0.18†	8.3×10^{11}	9.9×10^{5}
286	236	0.21†	3.4×10^{11}	2.4×10^{6}
306	242	0.26†	3.9×10^{11}	2.1×10^{6}
Paramagnetic liposomes*				
282	241	0.13†	1.9×10^{11}	4.3×10^{6}
282		0.21‡	1.9×10^{11}	4.3×10^{6}
260	231	0.17†	3.0×10^{11}	2.7×10^{6}
260		0.24‡	2.9×10^{11}	2.8×10^{6}

* The osmolality ranged from 322 to 384 mOsm/kg. † Internal volume obtained by ^{17}O NMR. ‡ Internal volume obtained by ^{1}H NMR.

of diamagnetic liposomes ($\sim 2\,s^{-1}$). The internal water volume can be correlated with the results obtained by ^{17}O NMR on the same stock suspension of liposomes with Dy^{3+} added. After addition of Dy^{3+}, the mean diameter decreased from 282 to 241 nm, due to the osmolality change, and the internal water content was 8.05%. Assuming that the membrane thickness of unilamellar spherical liposomes is 4 nm, and that the addition of Dy^{3+} solution to the liposome sample used for ^{17}O NMR induced a dilution by a factor of 1.08, an internal volume of 14.2% is calculated for the initial suspension, a value which is in excellent agreement with the value calculated from proton relaxometry.

Number Concentration of Liposomes

Assuming a spherical shape and a unilamellar membrane thickness of 4 nm, the internal volume of a vesicle was calculated from the mean diameter obtained by PCS meaurements. The total internal volume estimated by ^{17}O NMR or ^{1}H relaxometry was then used to calculate the number concentration of the vesicles (Table III). As expected, for a similar phospholipid content, the number concentration of diamagnetic liposomes increases when the vesicle size decreases. For paramagnetic liposomes, the number concentration values obtained from ^{17}O spectroscopy and ^{1}H relaxometry are in very good agreement but seem smaller than for the diamagnetic ones of similar size (1.9×10^{11} vs. $3.4 \times 10^{11}\,ml^{-1}$ (mg of phospholipids)$^{-1}$ for a diameter of 282–285 nm). This apparent discrepancy could be related to a loss of some signal resulting from a very fast relaxing fraction or to closer packing of the phospholipids in paramagnetic liposomes.

In summary, the ^{31}P NMR data indicate that the phospholipid content after the liposome preparation and down sizing is almost identical to its initial value, confirming thus a quasi quantitative process. On the other hand, the vesicles obtained after 10 extrusions are mainly unilamellar as shown by ^{31}P NMR after addition of Mn^{++} to the outer compartment. ^{31}P NMR also demonstrates that the membrane of "neutral" liposomes is not permeable to Mn^{2+} neither below, nor above the phase transition temperature. Natural abundance ^{17}O NMR spectroscopy in the presence of a shift reagent in the external compartment allows the evaluation of the overall internal volume of the liposomal suspension. This method is applicable to diamagnetic vesicles as well as to liposomes encapsulating paramagnetic agents characterized by a slow transmembrane water exchange. For paramagnetic liposomes, a second method for the determination of the overall internal volume was used. It is based on the analysis of the biexponential decay of the proton transverse magnetization observed when the exchange rate between the internal and external water compartments is slow as compared to their transverse relaxation rates. Finally, the number concentration of liposomes can be calculated from the liposome diameter obtained by PCS, the unilamellarity assessed by ^{31}P NMR spectroscopy, the overall internal volume estimated either by ^{17}O NMR spectroscopy or proton relaxometry, and the assumption of a membrane thickness of 4 nm. Multinuclear NMR combined with PCS can thus be successfully used to characterize diamagnetic and paramagnetic liposomes formulations intended to be used as carriers of diagnostic or therapeutic agents.

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