Biochimica et Biophysica Acta, 1022 (1990) 181–186
Elsevier

BBAMEM 74723

The effect of lipid composition on the relaxivity of Gd-DTPA entrapped in lipid vesicles of defined size

Colin Tilcock [1], Peter MacDougall [1], Evan Unger [2], Diego Cardenas [2] and Laurie Fajardo [2]

[1] *Department of Biochemistry, University of British Columbia, Vancouver, B.C. (Canada) and* [2] *Department of Radiology, University of Arizona, Tucson, AZ (U.S.A.)*

(Received 18 July 1989)

Key words: Liposome; Gd-DTPA; Relaxivity; Cholesterol; Water permeability; MR imaging; Magnetic resonance

The effects of lipid composition on the relaxivity of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) entrapped in lipid vesicles has been examined for vesicles of different sizes composed of egg phosphatidylcholine and cholesterol in various molar ratios, as well as the stability of those same vesicles in human serum at 37°C. It is found that the incorporation of cholesterol decreases the apparent relaxivity of the entrapped Gd-DTPA, concomitant with an increase in vesicle stability in serum. Cholesterol has little effect on relaxivity when incorporated at ratios up to 20 mole percent, but has an increasing effect at higher mole percentages. These results correlate with the known effects of cholesterol on the osmotic water permeability coefficients of various model membrane systems and suggest that it is the water flux across the vesicle bilayer that is limiting to the T1 relaxivity of the entrapped Gd-DTPA. The incorporation of up to 20 mole percent cholesterol has little effect on the stability of the vesicles in serum, whereas vesicles containing more than 20 mole percent cholesterol show greater increases in stability. It was also found that the stability of vesicles depends upon the size of the vesicles; smaller vesicles are less stable in human serum at 37°C than larger vesicles.

Introduction

Previous studies have shown that the paramagnetic chelate gadolinium-diethylenetriamine-pentaacetic acid (Gd-DTPA) entrapped in lipid vesicles is an effective agent for contrast-enhanced magnetic resonance (MR) imaging of focal, hypovascular liver metastases [1]. For a given total dose of Gd-DTPA administered, the smaller the vesicle, the greater the enhancement observed using T1-weighted pulse sequences. We have considered that this is related to the observation that the effective T1 relaxivity of Gd-DTPA entrapped in lipid vesicles increases linearly with the surface area to volume ratio of the vesicles [2]. This suggests that the T1 relaxivity reflects the flux of water across the vesicle bilayer, since the flux of water also scales linearly with the surface area to volume ratio of the vesicles.

Contrast enhancement of liver metastases by liposomal Gd-DTPA [1] may involve a combination of both uptake by the phagocytic cells of the reticuloendothelial system (RES) as well as intravascular blood pool effects. In either case, it is desirable that the vesicles remain stable in the serum over the time period during which they will be cleared from the circulation. Methods that have been used to improve the stability of lipid vesicles in the circulation have included the use of sterols such as cholesterol incorporated within the lipid matrix, or else the use of the saturated lipid species that are in the gel state at physiological temperatures [3–7]. In light of the preceding comments, it is significant that the osmotic water permeability coefficient of lipid bilayers containing disaturated lipids in the gel state is approximately two orders of magnitude smaller than in the liquid crystalline state [8]. Cholesterol is also known to decrease water permeability in model membrane systems [8–10]. If the apparent relaxivity of liposomal Gd-DTPA is indeed dependent upon water transport across the vesicle bilayer, it would be expected that the incorporation of cholesterol should decrease the effective relaxivity of entrapped Gd-DTPA by the same factor as the decrease in water permeability coefficient. In this study we present a systematic investigation of the effect of cholesterol on the relaxivity of entrapped

Abbreviations: 6CF, 6-carboxyfluorescein; EPC, egg phosphatidylcholine; Gd-DTPA, gadolinium-diethylenetriaminepentaacetic acid; OGP, *n*-octyl β-D-glucopyranoside.

Correspondence: C. Tilcock, Department of Biochemistry, University of British Columbia, Vancouver, B.C., Canada, V6T 1W5.

Gd-DTPA in vesicles of various sizes in order to test this hypothesis. In addition, we examine the effect of cholesterol upon the stability of these same vesicles in human serum at 37° C.

Materials and Methods

Vesicle preparation

Egg phosphatidylcholine (EPC) was obtained from Avanti Polar Lipids (Birmingham, AL), cholesterol and *n*-octyl β-D-glucopyranoside from Sigma (St. Louis, MO), radiolabels from Du Pont Canada (Mississauga, ON) and 6-carboxyfluorescein from Molecular Probes (Junction City, OR). Gd-DTPA was a generous gift of Mallinckrodt Inc. (St. Louis, MO). Unilamellar lipid vesicles composed of EPC and cholesterol were prepared by extrusion of freeze-thawed multilamellar vesicles through polycarbonate filters of defined pore size under nitrogen pressure as previously described [2]. For serum stability measurements, the lipid was dispersed in 25 mM 6-carboxyfluorescein (6CF) 100 mM NaCl, at pH 7.4. External 6CF was removed by column chromatography on Sepharose 4B using 10 mM Hepes, 6 mM KCl, 139 mM NaCl (pH 7.4) as eluant. Vesicles were stored at 4° C until used.

Relaxivity measurements

To characterize the effects of lipid composition on the relaxivity of the encapsulated Gd-DTPA, six different liposome compositions were tested. Frozen and thawed multilamellar vesicles composed of EPC with 0, 10, 20, 30, 40 and 50 mole percent cholesterol were prepared, then each sized through 200 nm, 100 nm and 50 nm diameter pore size filters [2]. Each of the eighteen preparations was then diluted in saline buffer (10 mM Hepes, 6 mM KCl, 139 mM NaCl (pH 7.4)) to give four samples with effective gadolinium concentrations of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM. The effective gadolinium concentrations were calculated as previously described [2], given the known trap volume of the vesicles, the lipid concentration and assuming that the concentration of the entrapped Gd-DTPA was 0.67 M. Samples (10–12 ml) of each of these dilutions were drawn into 20-ml plastic syringes which were then sealed. The syringes were then placed in a custom phantom holder and imaged with either a Siemens 1.5T Magnetom whole-body scanner or else a Toshiba 0.5T MRT-50A whole body scanner. For T1 measurements at 1.5 T, images were acquired using spin-echo sequences with a fixed TE of 17 ms and TR times of 100, 300, 450, 600, 900, 1200, 1800, 2500 and 3500 ms. Each image was composed of a 256×256 matrix of a 10 mm slice, 25 cm field of view, with four acquisitions. The same parameters were used at 0.5 T except that the TE value was 15 ms. For T2 measurements at 0.5 T, a fixed TR of 3500 ms was used and TE varied between 30, 50, 90, 120, 180, 210 and 250 ms. Several samples of phosphate-buffered saline were included in different position in the sample holder as monitors of field inhomogeneities that would cause variations in the signal intensities of the samples. From these data, T1 values were calculated using a Marquardt least-squares fit to the relation $M = A \cdot \exp(-Bx) + C$, where M is the signal intensity for successive times $x = \mathrm{TR}$ and $B = 1/\mathrm{T1}$. T2 values were similarly calculated assuming a fit to the relation $M = A \cdot \exp(-Bx)$ where M is the signal intensity for successive $x = \mathrm{TE}$ and $B = 1/\mathrm{T2}$. Measurement of T1 and T2 relaxation rates for standard solutions of Gd-DTPA indicated that the instrumental accuracy of the imagers used was of the order of $\pm 20\%$ of nominal. In both instances, the data was weighted according to the inverse of the square of the standard deviation of the measured signal intensities. Data analyzed with no imposed error weighting yielded calculated relaxation rates that differed by no more than 5% from the weighted analyses. Relaxivities were calculated from linear regression fits of 1/T1 versus the effective Gd-DTPA concentration.

Stability of vesicles in serum

The extent of efflux of 6CF from vesicles was used as a measure of their stability in serum according to Senior and Gregoriadis [11]. Freshly rehydrated lyophilized human serum (Monitrol-ES, Dade Diagnostic) of one lot number was used in order to reduce variation caused by the use of different sera. A 40-μl aliquot of the chromatographed vesicles with entrapped 6CF was added to 0.5 ml of the serum and incubated at 37° C. The lipid concentrations of the chromatographed vesicles were between 10 and 20 μmol/ml; therefore the final lipid concentration was approximately 1 mg/ml. This lipid concentration was chosen as it corresponded to the maximum serum concentration expected on the basis of previous in vivo imaging studies [1]. At various times, two 10-μl aliquots were taken from the serum-vesicle mixture. One aliquot was added to 4 ml of the standard buffer containing the detergent *n*-octyl β-D-glucopyranoside (OGP). The tubes containing only buffer were kept on ice until they were measured for 6CF fluorescence. The tubes containing buffer plus detergent contained either 25 mM OGP and were left at room temperature for 30 min, or else contained 10 mM OGP and were incubated at 37° C for 30 min to ensure complete disruption of the intact vesicles. To be as consistent as possible, several experiments were run simultaneously and in duplicate so that variation either due to size or vesicle composition would not be obscured by variation in the experimental procedure. Fluorescence was measured with a 490 nm excitation wavelength with emission measured at 520 nm. The percentage 6CF latency was taken as a measure of the leakiness of the vesicles in serum. The 6CF latency was

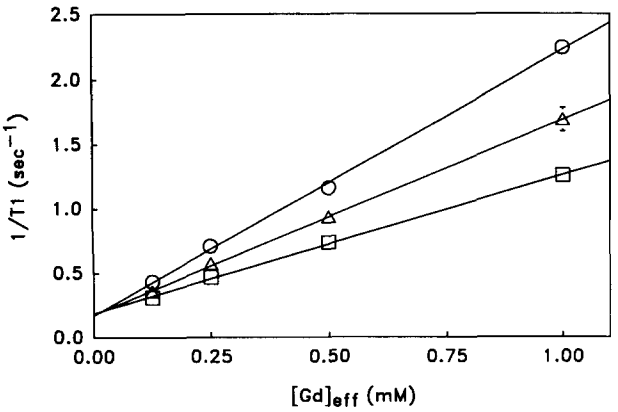

Fig. 1. 1/T1 (s^{-1}) at 1.5 T versus effective concentration of Gd-DTPA ($[Gd]_{eff}$) for egg phosphatidylcholine/cholesterol (9:1) vesicles of (○) 70, (△) 100 and (□) 170 nm average diameter.

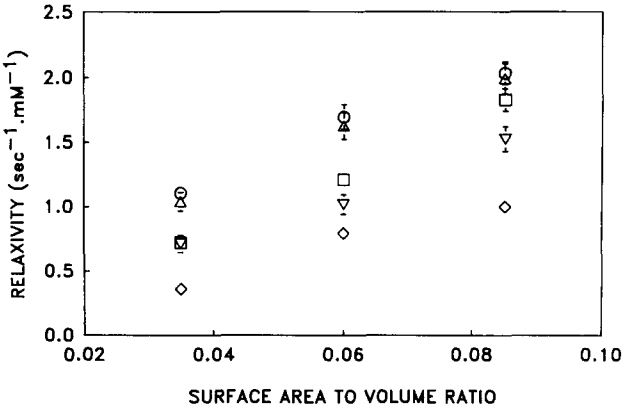

Fig. 2. Relaxivity ($s^{-1} \cdot mM^{-1}$) at 1.5 T versus surface area to volume ratio for Gd-DTPA trapped in 100 nm vesicles composed of egg phosphatidylcholine and (○) 10, (△) 20, (□) 30, (▽) 40 and (◇) 50 mole percent cholesterol.

calculated at (dye total − dye released) · 100/dye total, where dye total was the fluorescence of the samples with detergent and dye released was the fluorescence of the sample without detergent. The zero time point was normalized to 100% 6CF latency. Neither the buffer nor the buffer plus OGP, by themselves or with added serum or the dispersed lipid, showed any fluorescence at the same wavelengths at which 6CF was measured.

Results

A typical result for the variation in 1/T1 with effective Gd-DTPA concentration is shown in Fig. 1. The following tends are evident. First, as expected, there is a linear relationship between the T1 relaxation rate and the effective Gd-DTPA concentration. Second, the smaller the vesicle, the greater the relaxivity. Third, the relaxivity of the Gd-DTPA entrapped in the lipid vesicle is always less than that for free Gd-DTPA in solution [2]. There is an approximately linear relationship between the relaxivity and the surface area to volume ratio of the vesicles as indicated in Fig. 2. The T1 relaxivities for each of the different sizes and compositions of the vesicles examined, at both 1.5 T and 0.5 T, are presented in Table I and portrayed graphically in Fig. 3.

There are several points to note from these results. For any given vesicle size and composition, the relaxivity is greater at 0.5 T than at 1.5 T. The addition of cholesterol results in a decrease in the T1 relaxivity of the entrapped Gd-DTPA. For vesicles containing 50 mole percent cholesterol, the relaxivity is decreased by a factor of 2–3, depending on the size of the vesicle. The smaller the vesicle, the greater the relaxivity at any given cholesterol content and the larger the absolute decrease in relaxivity upon addition of cholesterol. T2 rates at 0.5T are listed in Table II. It is evident by comparison to Table I, that in all instances T2 is either approximately equal to or less than T1. For samples

TABLE I

T1 relaxivities ($s^{-1} \cdot mM^{-1}$) at 0.5 T and 1.5 T for three sizes of vesicles composed of EPC and cholesterol

Relaxivity values are expressed as the means ± S.D. based on a linear regression to four values of the effective Gd-DTPA concentration.

Cholesterol (mol%)	T1 relaxivity; average vesicle diameter (nm)		
	170	100	70
0.5 T			
0	1.40 ± 0.18	1.73 ± 0.19	2.85 ± 0.43
10	1.29 ± 0.17	1.79 ± 0.22	2.62 ± 0.33
20	1.40 ± 0.22	1.87 ± 0.22	2.56 ± 0.29
30	0.88 ± 0.16	1.56 ± 0.23	2.24 ± 0.26
40	0.81 ± 0.17	1.30 ± 0.19	1.86 ± 0.21
50	0.55 ± 0.18	0.88 ± 0.22	1.35 ± 0.21
1.5 T			
10	1.10 ± 0.06	1.70 ± 0.10	2.03 ± 0.09
20	1.04 ± 0.07	1.63 ± 0.10	1.99 ± 0.12
30	0.72 ± 0.05	1.21 ± 0.06	1.83 ± 0.9
40	0.71 ± 0.06	1.02 ± 0.08	1.53 ± 0.10
50	0.36 ± 0.06	0.79 ± 0.04	1.00 ± 0.05

TABLE II

T2 relaxation rate (s^{-1}) at 0.5 T for samples with an effective Gd-DTPA concentration of 1 mM

T2 values are expressed as the means ± S.D. based on a monoexponential fit using eight TE values.

Cholesterol (mol%)	T2 relaxation; average vesicle diameter (nm)		
	170	100	70
0.5 T			
10	1.66 ± 0.25	2.52 ± 0.21	2.83 ± 0.23
20	1.97 ± 0.19	2.40 ± 0.21	2.71 ± 0.21
30	1.83 ± 0.19	2.35 ± 0.17	2.98 ± 0.20
40	1.97 ± 0.19	2.58 ± 0.17	3.05 ± 0.20
50	1.15 ± 0.20	1.42 ± 0.19	2.17 ± 0.31

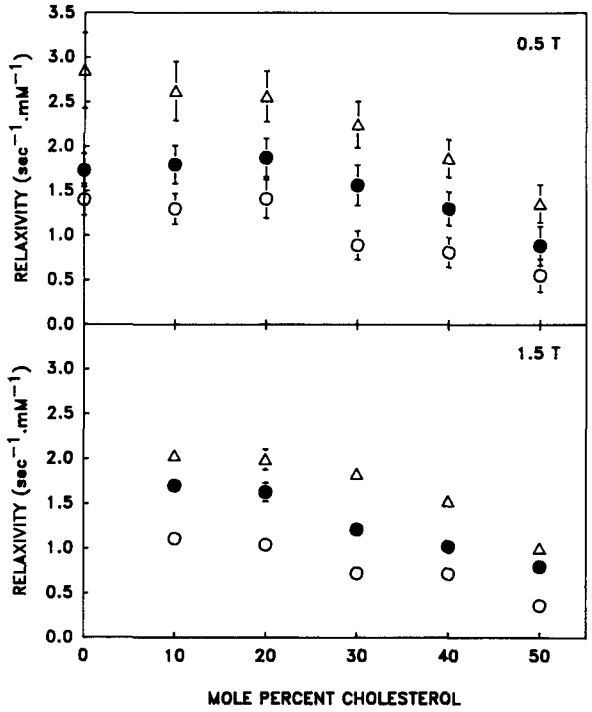

Fig. 3. Relaxivity ($s^{-1} \cdot mM^{-1}$) at 0.5 T and 1.5 T versus cholesterol content for vesicles of (△) 70, (●) 100 and (○) 170 nm average diameter.

with 0, 10 and 20 mole percent cholesterol, T1 and T2 rates are approximately equal for 70 nm vesicles, whereas T2 becomes increasingly smaller compared to T1 for larger vesicles.

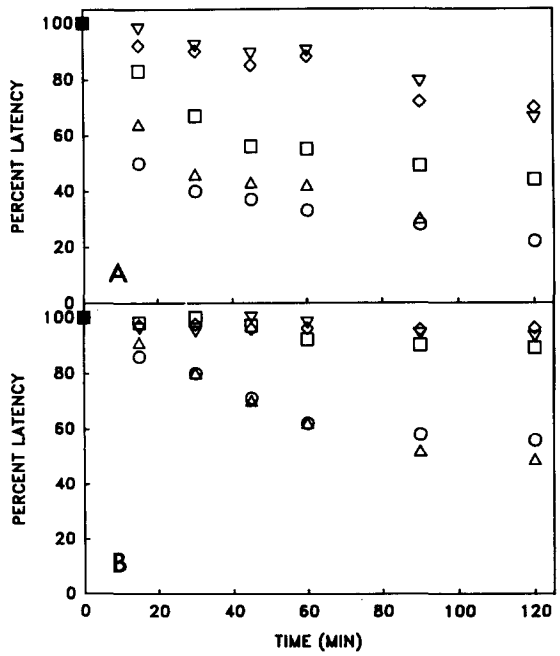

Fig. 4. Kinetics of the release of 6CF from (A) 50 nm and (B) 400 nm lipid vesicles composed of egg phosphatidylcholine and (○) 10, (△) 20, (□) 30, (◇) 40 and (▽) 50 mole percent cholesterol in human serum at 37 °C. For experimental details see Methods.

The effect of cholesterol on the latency of two sizes of vesicles in serum is presented in Fig. 4. Two trends are evident. First, cholesterol serves to stabilize the vesicles in serum and second, smaller vesicles are apparently less stable than larger vesicles. These data also show that the incorporation of up to 20 mole percent cholesterol does not markedly improve the stability of the vesicles, but that higher concentrations of cholesterol have a proportionately greater effect on vesicle stability in serum.

Discussion

The results presented in Fig. 2 indicate that the T1 relaxivity of Gd-DTPA entrapped within lipid vesicles exhibits a linear dependence upon the surface area to volume ratio of the vesicles of various compositions and is always less than that for free Gd-DTPA in solution. We have suggested [2] that this is a reflection of the water flux across the bilayers of the lipid vesicles since the flux of water is itself a linear function of the surface area to volume ratio. Thus if the water flux is limiting to the relaxation, it would be expected that the T1 relaxivity would scale similarly. Clearly, for two populations of vesicles of different radius R_1 and R_2 with equivalent internal concentration of Gd-DTPA, at the same effective Gd-DTPA concentration, their internal volumes must be equal. Given this condition, the ratio of the total surface area of the two vesicle populations is proportional to R_2/R_1. Thus a dispersion of small vesicles presents a larger surface area for water transport per unit time, i.e. the residence time for water inside the vesicle is decreased, compared to a population of larger vesicles and hence the relaxivity is greater [12–14].

Other authors have shown that the presence of 50 mole percent cholesterol in egg phosphatidylcholine membranes decrease the osmotic permeability of model membrane systems by a factor of approximately two [9,10]; the effects of lower amounts of cholesterol on water permeability have not been previously investigated. This approximately 2-fold decrease in water permeability coefficient is consistent with our observation (Fig. 3) that the T1 relaxivity of the entrapped Gd-DTPA is also decreased by a factor of 2–3 in vesicles containing 50 mole percent cholesterol and supports the hypothesis that water flux across the vesicle bilayers is limiting to the T1 relaxation.

We note that the T2 relaxation rates (Table II) scale according to T1 except for larger vesicles. It is probable that there is a bulk magnetic susceptibility effect that contributes to T2 for these vesicles [15] and this may be part of the explanation why larger vesicles are less effective at providing contrast enhancement in vivo [1]. Given the errors associated with the T1 and T2 measurements, it is difficult to determine whether there are

consistent trends in the ratio of the T1 and T2 relaxivities as a function of vesicle composition, although the data suggests that the T2/T1 ratio increases with sterol content. This may reflect composition-dependent changes in the diamagnetic susceptibility of the membranes and is clearly an area where further experimentation is warranted.

There was relatively little change in the relaxivity for vesicles containing up to 20 mole percent cholesterol, but at higher cholesterol contents there were proportionately greater decreases in relaxivity. It is of interest to view these results in terms of current models for water permeation across lipid bilayers and the known effects of cholesterol upon the packing properties of lipids.

There are at least two models of water permeation across lipid bilayers. These include the solubility-diffusion model whereby individual water molecules or clusters of water molecules dissolve in the hydrocarbon region of the bilayer and cross by simple diffusion and models involving the formation of transient pores arising through thermal fluctuations in the membrane [10]. Cholesterol serves to increase the viscosity of the hydrocarbon region of a lipid matrix in which it is embedded [9] and may thus cause a decrease in the diffusion coefficient of water within the phase. Other studies have indicated that cholesterol does not greatly influence the interlipid spacing within unsaturated dioleoyl phosphatidylcholine bilayers at below 20 mole percent cholesterol but has a much greater effect on lipid packing between 20 and 50 mole percent cholesterol [16,17]. It is tempting to suggest that there is a causal relation between such changes in the close packing of the acyl chains above 20 mole percent cholesterol which, either by restricting diffusion or the formation of transient defects, serves to decrease water permeability, and our observation of relatively little effects upon relaxivity except above 20 mole percent cholesterol.

Our results upon the serum stability of vesicles extend previous studies in this area [3–7]. Our results are broadly consistent with literature; namely the addition of cholesterol improves serum stability and smaller vesicles are less stable. It is of interest to note that while cholesterol contents of less than approximately 20 mole percent do not markedly decrease the apparent relaxivity of entrapped Gd-DTPA, neither do they markedly increase serum stability. The mechanism(s) by which vesicles are degraded in the circulation has been extensively studied and reviewed [6]. It is conceivable that cholesterol, above 20 mole percent, induces a tighter packing of the lipid acyl chains [17] and prevents penetration and subsequent solubilization by serum lipoproteins.

We noted that upon addition to the serum there was a rapid initial disruption of the vesicles (see Fig. 4), followed by a longer slower release. The data could not be fitted to a simple single exponential. We have considered that this may in part be due to osmotic lysis of the vesicles and that the addition of cholesterol serves to decrease the lysis. We cannot discount this possibility on the basis of our data, but would point out that the phantoms used to study the relaxivity were prepared by dilution of vesicles containing 0.67 M Gd-DTPA in a saline buffer with an osmolarity close to that of a physiological milieu. If the vesicles had been ruptured so as to release all their entrapped Gd-DTPA, it would have been expected that all samples with equivalent effective Gd-DTPA concentrations would have exhibited the same relaxivity. This result was never observed and in fact dialysis studies of ^{153}Gd-DTPA vesicles have shown that the vesicles, after dilution, retain their contents. For these reasons we feel that the differences observed in the latency of the vesicles, either by composition or size, are not primarily due to lysis.

The studies presented here allow us to make the following general conclusions concerning T1 contrast agents such as liposomal Gd-DTPA, in which paramagnetic chelates are trapped in the interior aqueous space of lipid vesicles.

(1) In order to maximize relaxivity, the vesicles should be as small as possible, should be unilamellar, and the lipids comprising the vesicle bilayer should be in the liquid-crystal state at physiological temperature. The use of lipid vesicles composed of saturated lipid species that are gel state at physiological temperature will result in a lower relaxivity for the entrapped paramagnetic agent due to reduced water permeation across the vesicle bilayer.

(2) In order to increase serum stability cholesterol may be incorporated within the lipid matrix. There will be a trade-off between increased serum stability and decreased apparently relaxivity. The present studies do not permit us to determine which of these two parameters is the more important, but do suggest that cholesterol compositions between 20 and 40 mole percent would prove optimal in this application.

(3) For a given dose of liposomal Gd-DTPA, contrast enhancement will be greater at lower magnetic field strengths, although the lower signal to noise inherent at lower field may outweigh the advantages of the greater relaxivity.

Acknowledgements

This research was supported by BCHCRF grant No. 5-52667 and MRC grant No. 5-90690 to C.T.

References

1 Unger, E., Winokur, T., MacDougall, P. and Tilcock, C. (1989) Radiology 171, 81–85.

2 Tilcock, C., Unger, E., Cullis, P. and MacDougall, P. (1989) Radiology 171, 77–80.
3 Finkelstein, M.C. and Weismann, G. (1979) Biochim. Biophys. Acta 587, 202.
4 Allen, T.M. and Cleland, L.G. (1980) Biochim. Biophys. Acta 597, 418.
5 Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591.
6 Bonte, F., Juliano, F. and Juliano, R.L. (1986) Chem. Phys. Lipids 40, 259 (1986).
7 Scherphof, G.L., Damen, J. and Wilschut, J. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 3, p. 205, CRC Press, Boca Raton.
8 Carruthers, A. and Melchior, D.L. (1983) Biochemistry 212, 5797.
9 Finkelstein, A. and Cass, A. (1967) Nature 216, 717.
10 Deamer, D. and Bramhall, J. (1986) Chem. Phys. Lipids 40, 167.
11 Senior, J. and Gregoriadis, G. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 3, p. 263, CRC Press, Boca Raton.
12 Hazelwood, C.F., Chang, D.C., Nichols, B.L. and Woessner, D.E. (1974) Biophys. J. 14, 583.
13 Belton, P.S. and Ratcliffe, R.G. (1985) Prog. NMR Spectrosc. 17, 241.
14 Bacic, C., Niesman, M.R., Bennett, H.F., Magin, R.L. and Swartz, H.M. (1988) Magn. Res. Med. 6, 445.
15 Villringer, A., Rosen, B.R., Belliveau, J.W., Ackerman, J.L., Lauffer, R.B., Buxton, R.B., Chao, Y.S., Wedeen, V.J. and Brady, T.J. (1988) Magn. Reson. Med. 6, 164–174.
16 Lecuyer, H. and Dervichian, D.G. (1969) J. Mol. Biol. 45, 39.
17 Finean, J.B. and Hutchinson, A.L. (1987) Chem. Phys. Lipids 46, 63.