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Liposomal Gd-DTPA: Preparation and Characterization of Relaxivity[1]

Gadolinium diethylenetriaminepentaacetic acid (DTPA) has not proved particularly useful for hepatosplenic magnetic resonance imaging. However, by entrapping the Gd-DTPA within lipid vesicles, one may exploit the ability of the reticuloendothelial system to endocytose particulates, permitting passive delivery of contrast agent to the liver and spleen while decreasing the rate of clearance of Cd-DTI'A from the vasculature. Liposomes of 70-400 nm diameter containing Cd-DTPA were prepared by a freeze-thaw extrusion process. These exhibited high trapping efficiencies and excellent stability during storage. For all sizes of liposomes, the relaxivity of the entrapped Gd-DTPA was less than that of free Gd-DTPA. Relaxivity varied linearly with the surface-area-to-volume ratio of the liposomes; the smaller the liposomes, the greater the relaxivity. Liposomcs containing cholesterol had a lower relaxivity than liposomes without cholesterol. The authors suggest this reflects the decrease in the water permeability coefficient caused by the presence of cholesterol in the liposome membrane.

index terms: Cadolinium • Liposomes • Magnetic resonance (MR), contrast media

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THE paramagnetic contrast agent gadolinium diethylenetriaminepentaacetic acid (DTPA) functions as an effective extracellular fluid contrast agent for magnetic resonance (MR) imaging (1,2), but early experience with Gd-DTPA as a contrast agent for imaging the liver has been disappointing (3). Its utility as a liver and spleen contrast agent is diminished by rapid equilibration between intra- and extravascular fluid compartments. If Gd-DTPA could be confined to the intravascular space and targeted to liver and splenic tissue, its efficacy as a hepatosplenic and blood pool contrast agent should be improved. In our previous work we showed that Gd-DTPA entrapped in liposomes caused increased enhancement of the liver and blood pool (4). The enhancement caused by liposomal Cd-DTPA in 400-nm-diameter vesicles in vitro was less than that caused by free Gd-DTPA. This observation presumably is explained by shielding of the paramagnetic ccnters from adjacent water by the liposomal membrane. In this study we detail the preparation of liposomal Gd-DTPA and characterize the preparations with regard to in vitro stability and relaxivity.

MATERIALS AND METHODS

Preparation of Lipid Vesicles with Entrapped Cd-DTPA

Vesicles were prepared by extrusion through polycarbonate filters under moderate pressure (5). Vesicles were composed of egg phosphatidylcholine (PC), either alone or with 40 mole percent cholesterol. Egg PC was obtained from Avanti Polar Lipids (Birmingham, Ala) and cholesterol from Sigma (St Louis); both were used without further purification. Typically, 3.6 mmol (2.83 g) of egg PC and 2.4 mmol (0.93 g) of cholesterol were dissolved together, in a minimum volume of chloroform, in a 250-mL round-bottom flask to which was added 50 μL hydrogen-3 dipalmitoyl phosphatidylcholine (DPPC) stock solution (250 μCi [9.25 MBq]/2.5 mL toluene; New England Nuclear, Mississauga, Ontario, Canada). The H-3 DPPC provides a means of quantifying the lipid concentration. The chloroform was removed by rotary evaporation under reduced pressure to leave a thin film on the walls of the flask; the contents were then held under reduced pressure (<0.1 mm Hg) for at least 2 hours to remove residual solvent. The lipid was dispersed by vigorous shaking at room temperature in 20 mL of 0.67 mol/L Cd-DTPA, pH 7 (sodium salt), to which was added 5 μL of carbon-14 inulin (250 μCi [9.25 MBq]/2.5 mL water, NEN) or 2 μCi [0.074 MBq] of Cd-153 DTPA. The C-14 inulin (or Cd-153 DTPA) serves as an aqueous marker and permits facile determination of the trap volumes of the vesicles. The multilamellar vesicles formed upon dispersion were transferred to cryovials and then quench frozen in liquid nitrogen. The cryovials were then placed in warm water until the lipid suspension had completely thawed. This cycle of freezing and thawing was repealed four more times. The frozen and thawed vesicles were then sized by ten passes under nitrogen pressure (<100 psi) through two stacked 0.4-μm polycarbonate filters (Nuclepore, Pleasanton, Calif) with use of the extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada). A portion of this sized preparation was then passed ten times through two stacked 0.2-μm filters. A portion of this latter preparation was then passed an additional ten times through either two stacked O.l-.or 0.05-μm filters. An aliquot of each of the sized preparations was removed for scintillation counting to determine the content of H-3 DPPC and C-14 inulin.

For each of the sized vesicles, external untrapped Cd-DTPA was removed by exhaustive dialysis at 4°C against 4 L of saline buffer (10-mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 6-mmol/L KCI, 139-mmol/L NaCl; pH

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See also the article by Unger et al (pp RI -85) and the editorial by Seltzer (pp 19-21) in this issue.

Abbreviations: dpm = disintegrations per minute, DPI'C = dipalmitoyl phosphatidylcholine, DTPA = diethylenetriaminepentaacetic acid, PC = phosphatidylcholine, PBS = phosphate buffrrrd saline, TE = echo lime, TR = repetilion time.

Table 1
Characteristics of Egg PC/Cholesterol (6:4) Vesicles

Diameter (nm)* (Mean ± Standard Deviation)	Trap Volume (μL/μmol)	Lipid Concentration† (μmol/mL)	$[Gd]_{eff}$ (μmol/mL)	Trapping Efficiency‡ (%)	Lipid Dose§ (mg/kg)	Percentage‖ Retention	Relaxivity (sec^{-1} · $mmol/L^{-1}$)
Free Cd-DTPA	NA	NA	NA	NA	NA	NA	2.79
400 ± 40	1.95	181	236	35	95	100	0.42
200 ± 30	1.17	175	137	20	140	94	0.51
100 ± 20	0.87	185	108	16	214	100	1.05
70 ± 20	0.71	169	79	12	267	100	1.60

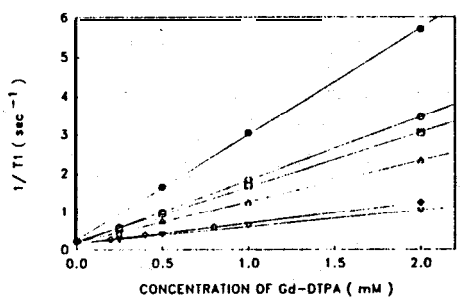
* Based on quasielastic light scattering.
† Lipid concentrations are typical values for preparations used in this study.
‡ Trapping efficiency = $([Gd]_{eff} \cdot 100)/[Gd]_{int}$. In this study, $[Gd]_{int}$ was 670 μmol/mL.
§ This is the lipid dose required to give a $[Gd]_{eff}$ of 0.2 mmol/kg. For egg PC/cholesterol (6:4) mixtures, the average molecular weight was taken as 626.
‖ Percentage retention of internal contents after 4 months storage in saline buffer at 4°C.

7.4). The external dialysis medium was changed a total of eight times (8 X 41) over 2-3 days. The sizes of the dialyzed particles were determined by quasielastic light scattering with a Nicomp (Goleta, Calif) model 270 particle sizer operating at 634.2 nm by standard cumulants analysis. The entrapment efficiencies of the vesicles were determined by means of chromatography of an aliquot of the dialyzed vesicles on a Scphadex G50 F (Pharmacia, Uppsala, Sweden) equilibrated with saline buffer. Vesicles with entrapped C-14 inulin eluted in the void volume, whereas nonentrapped C-14 inulin was retained on the column. A sample of the eluted vesicles was taken for dual-label scintillation counting as before. Vesicles were stored at 4°C until further use. Trap volumes were calculated as follows: The amount (in μmol) of lipid eluted from the column L is given by the equation $L = V/A$, the volume (in μL) trapped in the vesicles eluted from column by $V = (C/B) X 100$, and the trapped volume T(in μL/μmol) by $T = V/L$, where A is the specific activity of lipid suspension before gel filtration (H-3 DPPC disintegrations per minute (dpm)/μmol), B is the C-14 inulin dpm in 100 μL of initial lipid suspension before gel filtration, C is the C-14 inulin dpm in 100 μL of vesicles eluted from column, and V is the H-3 DPPC dpm in 100 μL of vesicles eluted from column.

An implicit assumption in this calculation is that the entrapment of C-14 inulin is representative of the entrapment of Gd-DTPA. We have prepared other batches of vesicles with Cd-153 DTPA as an aqueous marker and have obtained similar entrapment values, indicating that this assumption is reasonable.

Preparation of Phantoms

We define the effective concentration of Gd-DTPA in the vesicle preparations to be the aqueous concentration of Gd-DTPA that would result if all the vesicles in suspension were to be disrupted and their contents released. On this basis the effective concentration of Cd-DTPA ($[Gd]_{eff}$) may be calculated from the relationship $[Gd]_{eff} = (T X [L] X [Gd]_{int})/1,000$,



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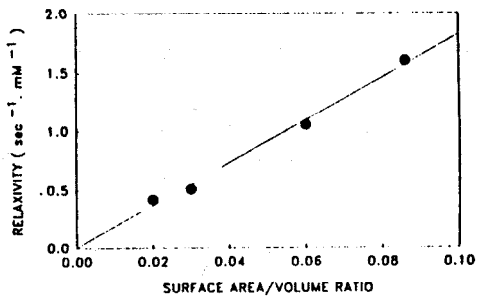

2.

Figures 1,2. (1) l/T1 (sec^{-1}) versus concentration of Cd-DTPA (mmol/L) for free Gd-DTPA (●) and Gd-DTPA entrapped in egg PC/cholesterol (6:4) vesicles with average diameters of 70 (0), 100 (A), 200 (◊), and 400 (▽) nm. □ = Cd-DTPA in 100-nm egg PC vesicles. (2) Relaxivity ($sec^{-1} \cdot mmol/L^{-1}$) versus surface-area-to-volume ratio for Cd-DTPA entrapped in egg PC/cholesterol (6:4) vesicles.

where T = trapped volume (μL/μmol), [L] = lipid concentration (μmol/mL), and $[Gd]_{int}$ = concentration of Cd-DTPA inside the vesicles.

It is assumed that $[Gd]_{int}$ is equivalent to the initial concentration of GD-DTPA used to disperse the lipid film to form multilamellar vesicles.

Phantoms were prepared by diluting the vesicle preparation with Dulbecco phosphate buffered saline (PBS) (Gibco Laboratories, New York) so as to obtain several samples for each vesicle preparation with $[Gd]_{eff}$ in the range of 0.2-2 mmol/L. Approximately 10 mL of each dilution was placed in 20-mL plastic syringes (Becton Dickinson, Rutherford, NJ), which were sealed with Parafilm (American Can, Greenwich, Conn) to prevent leakage and placed in a custom-built acrylic phantom holder that positioned the syringes with their long axes horizontal within the bore of the magnet.

Imaging

Phantoms were imaged with a Siemens (Iselin, NJ) 1.5-T Magnetom whole-body imager with use of a body coil. For T1 calculations, images were acquired with spin-echo sequences with a fixed echo time (TE) of 17 msec and repetition times (TRs) of 100,300,450,600, 900, 1,200, 1,800, 2,500, and 3,500 msec. Other parameters were as follows: four acquisitions, 256 X 256 matrix, 30-cm field of view, and 10-mm sections. We were careful to include PBS standards in several positions within the sample holder, because field inhomogeneities would be manifested as variations in the signal intensity of the standards. T1 values were calculated with a least-square fit to the relation $A|1 - \exp[-M(t)/B]|$, where $M(t)$ is the intensity for successive times t = TR.

Vesicle Stability

In vitro stability of the vesicle preparations in saline was determined at various times after preparation by means of dialysis (Spectrapor [Spectrum Medical Industries, Los Angeles] 10-mm tubing; molecular weight cutoff, 12,000-14,000) over 120 hours against five 1-L changes of normal saline at 4°C. The stability was calculated on the basis of either the percentage of Gd-153 DTPA activity remaining inside appropriately labeled vesicles or by determining the H-3 DPPC/C-14 inulin ratio by scintillation counting.

RESULTS

Table 1 shows various parameters associated with the vesicles used in this study. The trap volumes of the vesicles are an intrinsic property of the vesicles prepared by this method,

whereas the effective concentration of Cd-DTPA, and hence the trapping efficiency, depend on the lipid concentration. The lipid dose required to achieve a given value for $[Gd]_{eff}$ is inversely related to the trapping efficiency. Increase the trapping efficiency twofold, and the lipid dose will decrease by a similar factor. Table 1 also shows that the vesicles exhibit excellent retention of their internal contents after prolonged storage in saline at 4°C.

The relaxivity of free Cd-DTPA and Cd-DTPA entrapped within lipid vesicles is indicated in Figure 1. There are three points to note. First, in all instances, the relaxivity of entrapped Cd-DTPA was less than that of free Gd-DTPA. Second, the smaller the vesicle, the greater the relaxivity. Last, 100-nm egg PC vesicles exhibited a relaxivity approximately 40% greater than that of 100-nm egg PC/cholesterol (6:4) vesicles. There is an approximately linear relationship between the rclaxivity of the egg PC/cholesterol (6:4) vesicles and their surface-area-to-volume ratio, as shown in Figure 2. Our relaxivity value of 2.79 $sec^{-1} \cdot mmol/L^{-1}$ for free Gd-DTPA is lower than the value of approximately 5 $sec^{-1} \cdot mmol/L^{-1}$ reported for higher field strengths by Villinger et al (6). The fact that Tl values were measured using an imaging device rather than a spectrometer may in part explain this disparity. However, for the purpose of comparing the relaxivities of vesicles of differing size, it is the relative changes in relaxivity that are important rather than the absolute values.

DISCUSSION

Gd-DTPA causes a concentration-dependent decrease in tissue Tl and T2, with a relatively greater decrease for tissues with longer values of Tl and T2 (eg, tumor) than for tissues with shorter relaxation times (eg, hepatic parenchyma). Since Tl is longer than T2 for biological tissues, the absolute changes in Tl are greater than those in T2. Hence, contrast enhancement is better observed with Tl-weighted sequences, for example, spin-echo sequences with short TE and medium TR values.

In practice, the use of Gd-DTPA for the enhancement of hepatic metastases is complicated by several factors. There is no mechanism for the active uptake of Cd-DTPA by the liver; hence, the contrast enhancement obtained is dependent on the plasma concentration of Gd-DTPA, and since all contrast agents are to various extents toxic, there is clearly a limit to how much Gd-DTPA can be administered (3,7).

For imaging with Tl-weighted sequences, a decrease in T1 will lead to an increase in the relative signal intensity whereas a decrease in T2 will decrease the relative signal intensity. At low concentrations of Gd-DTPA there will be an increase in signal intensity via T1, but at concentrations above approximately l-2 mmol/L (see Appendix), T2 effects dominate and there is a decrease in signal intensity. Thus, the change in signal intensity produced by Gd-DTPA exhibits a maximum as a function of concentration (8). This presents difficulties with regard to early postbolus imaging, since Gd-DTPA can readily cross biological membranes, thereby equilibrating with the extracellular space, and is also rapidly excreted by the kidneys (2,3,7); these conditions produce concomitant changes in tissue concentration and hence, signal intensity. Another consequence of this rapid equilibration is that the Gd-DTPA, if uniformly distributed across tumor and parenchyma, would obscure rather than enhance contrast by decreasing the longer T1 of tumor more than the shorter T1 of parenchyma. This is graphically illustrated in another study described in this issue (9), in which free Gd-DTPA was observed to decrease the contrast between in situ liver metastases and the surrounding tissue. For enhancement of contrast what is required is a means to direct contrast agent to parenchyma, rather than tumor, so as to maximize the T1 differences between the two tissues.

The rationale for using lipid vesicles to deliver contrast agent to the liver and spleen is twofold. First, it is well documented that the Kupffer cells and hepatocytes are able to endocytose particulates, including lipid vesicles (10). Vesicles therefore provide a carrier vehicle 'for the delivery of contrast agent, in this instance Gd-DTPA, to the target organs, liver and spleen. Second, because the contrast agent is preferentially delivered to the reticuloendothelial system, the total dose of contrast agent that need be given should be less than that for the free contrast agent, assuming both to be equally effective at equivalent concentrations within the target organ. The use of lipid vesicles should therefore ameliorate any toxicity due to bolus infusion of the free contrast agent, although it is possible that the lipid itself may cause toxicity either directly or by altering the clearance of the Gd-DTPA. In addition, intercellular dechelation of the Gd-DTPA complex may occur, thus liberating the highly toxic gadolinium ion. This possibility may be addressed by a combination of nuclear MR (NMR) dispersion experiments and dual-label (Gd-153 DTPA and Gd-[carbon-14]-DTPA) biodistribution and clearance studies; these studies are currently in progress.

The expectation, then, is that if the vesicles survive in the circulation sufficiently long to deliver the Gd-DTPA to the reticuloendothelial system, the contrast agent will be delivered to the Kupffer ceils and, depending on the size of the vesicle, also to the hepatocytes. Necrotic tissue within a tumor will not endocytose the vesicles, hence the basis for the differential contrast, although the degree of vascularization of a tumor and blood pool enhancement will also be important in determining the degree of differential contrast between tumor and surrounding tissue. In the absence of experimental results in a suitable animal model it is difficult to ascribe the relative significance of uptake versus blood pool effects; however, it should be noted that since lipid vesicles have limited exit routes from the circulation (ie, via fenestrated epithelia) (10) the washout problems observed with free Gd-DTPA should be ameliorated, with liposomal Gd-DTPA serving as a better marker of vascularity. Once endocytosed, the Gd-DTPA will decrease the T1 and T2 of the water within the cell, causing the parenchyma to appear hyperintense relative to tumor on Tl-weighted images.

Once endocytosed, the vesicles *need* not be physically degraded for the entrapped Gd-DTPA to act as an effective contrast agent. To a first approximation we may assume that the ability of the entrapped Gd-DTPA to interact with the cell water can be represented by the three-dimensional solution for the Fick diffusion equation from a point source, that is, $d = \sqrt{6DT}$, where d is the average distance a water molecule with diffusion coefficient D can diffuse in time T. With reasonable assumptions for the diffusion coefficient of water (3×10^{-5} $cm^2 \cdot sec^{-1}$), on the NMR timescale of approximately 10^{-5} sec, a water molecule will diffuse 4×10^{-5} cm. For Gd-DTPA entrapped within a 100-nm-diameter vesicle, the relative volumes potentially accessible (d^3) and internal (r^3) is of the order $[(4 \times 10^{-5})/(5 \times 10^{-8})]^3$ *or* -500.

Alternatively stated, when approximately only 0.2% of the total volume of the solution is occupied by the internal volume of the vesicles, all of the water in solution will be affected by the Gd-DTPA entrapped within the vesicles. We may calculate from the lipid concentrations and trap volumes that at the lowest $[Gd]_{eff}$ used for the **T1 measurements (0.25** mmol/L), the percentage of the total volume of the solution occupied by the internal volume of the lipid vesicles was approximately 0.04%. For all of the vesicles, the relaxation data fitted, with a high correlation coefficient (>.95), to a single exponential at the lowest $[Gd]_{eff}$ used, indicating the validity of these order of magnitude calculations, despite the simplicity of the model.

The relaxivity of the entrapped Gd-DTPA was always less than that for free Gd-DTPA. This fact is presumably a simple consequence of a decrease in the exchange rate between bulk water and the entrapped gadolinium nucleus **due to** the permeability barrier imposed by the intervening vesicle membrane. The linear variation of relaxivity with the surface-area-to-volume ratio follows directly from a consideration of the water flux across the vesicle membrane. For a lipid vesicle of area A and volume *V* containing n molecules at concentration C, the flux J across the membrane per unit time t is defined as follows: $J \propto [dn(t)/dt] \cdot (1/A) \equiv [dC(t)/dt] \cdot (V/A)$. That is, the flux of water is linearly dependent on the surface-area-to-volume ratio of the vesicles. At a given lipid concentration, the smaller the vesicle, the larger the surface-area-to-volume ratio and the greater the flux across the membrane per unit time. If it is assumed that it is the water transport across the membrane that is rate limiting, the relaxivity would be expected to exhibit a similar linear dependency. Last, 100-nm egg PC vesicles exhibit a relaxivity approximately 40% greater than do 100-nm egg PC/cholesterol (6:4) vesicles. Clearly, in this comparison, surface-area-to-volume ratio is not the significant variable; rather, the differences in relaxivity most probably reflect the presence of cholesterol, which has been shown to decrease the permeability coefficient for water in model membrane systems (11). In addition, differences in the observed relaxivity could reflect differences in the transmission of bulk susceptibility effects across the bilnyer (6). In the absence of T2 measurements we cannot exclude this possibility, but if T2* effects were dominant, 1 /T1 (or 1 /T2) should increase as the square of the radius of the vesicles (12). In fact, we observed (Fig 2) that 1 /T1 scales as the inverse of the vesicle radius **(surface area/volume ∝ l/R); thus, the observed functional dependence of the relaxivity is inconsistent with a T2*-dominated relaxation process.**

CONCLUSION

We have shown that Gd-DTPA entrapped within lipid vesicles can function as an effective relaxation agent. Both vesicle size and composition affect the relaxivity. At a given concentration of lipid and entrapped Gd-DTPA, smaller vesicles exhibit greater relaxivity. The relaxivity is approximately linearly dependent on the surface-area-to-volume ratio of vesicles 70-400 nm in diameter.

It is well established that vesicle size, lipid dosage, and vesicle composition all influence the pharmacodynamics of liposomes injected intravenously (10) and hence their effectiveness as contrast agents. While one liposomal formulation may be designed to maximize hepatosplenic uptake and contrast enhancement of these organs, another might be designed to minimize such uptake, to be stable in the circulation, and hence to serve as a blood pool contrast agent. Subsequent stages of this work will investigate these various possibilities and address the issues of biodistribution and toxicity in appropriate animal models. In addition, we are currently investigating alternate methods for the encapsulation of Gd-DTPA to improve the trapping efficiency of the vesicles.

APPENDIX

We calculated as follows the concentration of **Cd-DTPA for which the signal intensity is a maximum with a spin-echo** pulse sequence. **We seek** an extrenum to

$$M = (1 - e^{(TE-TR)/T1})e^{-TE/T2},$$

where TE, TR, T1, and T2 have the usual meanings. Let $TE = k_1$, $TR = k_2$, $1/T1 = x$, and $1/T2 = k_3x$:

$$M = (1 - e^{(k_1-k_2)x})e^{-k_1k_3x}.$$

Thus,

$$\frac{dM}{dx} = (1 - e^{(k_1-k_2)x}) \times -k_1k_3e^{-k_1k_3x} + e^{-k_1k_3x} \times -(k_1 - k_2)e^{(k_1-k_2)x}$$

$$= -k_1k_3e^{-k_1k_3x} + (k_1k_3 - k_1 + k_2)e^{-k_2k_3x}.$$

This exhibits an extrenum **when**

$$x = 1/T1 = \frac{\ln(k_1k_3) - \ln(k_1k_3 - k_1 + k_2)}{(k_1k_3 - k_2k_3)}.$$

The concentration of Gd-DTPA for maximum signal is given by the equation $[Gd\text{-}DTPA] = (1/T1 - 1/T1_w)/R$, where $1/T1_w$ is the reciprocal of the T1 relaxation time for water in the absence of contrast agent and R is the relaxivity of the contrast agent. Using a value of $1/T1_w$ of 0.22 and R of 2.79 $sec^{-1} \cdot mmol/L^{-1}$, one may **calculate** a maximum signal intensity at approximately 2 **mmol/L Gd-DTPA for TE** and **TR** values used in this study. ■

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