Gadolinium-DTPA Liposomes as a Potential MRI Contrast Agent

Work in Progress

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To evaluate the use of liposomes containing Gadolinium-DTPA (Gd-DTPA) as potential intravascular contrast agents, we synthesized and tested Gd-DTPA liposomes. A freeze-thaw extrusion process was used to synthesize neutral unilamellar vesicles. Using this technique, we prepared 0.4 micron vesicles with encapsulation efficiency as high as 39% for Gd-DTPA. In vitro dialysis showed that essentially 100% of the Gd-DTPA was retained with the liposomes after 72 hours of dialysis. MR imaging of in vitro samples showed concentration-dependent increase in signal intensity with Gd-DTPA liposomes. Imaging of rats after intravenous injection of Gd-DTPA liposomes showed sustained intravascular contrast enhancement of vascular structures and liver greater than free Gd-DTPA. There was no evidence of acute toxicity in rats during the imaging experiments or on follow-up of two months. Paramagnetic liposomes may be useful to enhance the vasculature, liver, and spleen.

Key words: magnetic resonance imaging; Gadolinium-DTPA; liposomes.

THE PARAMAGNETIC contrast agent Gadolinium-DTPA (Gd-DTPA) has been developed as an intravascular magnetic resonance (MR) contrast agent.[1,2] This contrast agent is distributed between the intravascular and extracellular fluid spaces and excreted renally.[1,2] Because of equilibration between intravascular and extravascular spaces, rapid renal excretion, and prolonged imaging times usually necessary for MR, Gd-DTPA has not improved detection of hepatic or splenic disease.[3] Intracellular delivery of the extracellular contrast agent Gd-DTPA might enhance target tissues and improve the ability to detect disease in organs such as the liver and spleen.[4] Confinement of Gd-DTPA to the intravascular space might improve enhancement of vascular structures and serve as a marker of organ or tissue perfusion.[5]

Liposomes and liposoluble materials have been used as vehicles to deliver iodinated contrast media to the liver and spleen for computed tomography (CT)[6–9] and to deliver radionuclides to the reticuloendothelial system for nuclear medicine. In CT, liposoluble contrast agents have improved the ability to detect hepatic neoplasia and metastases.[9,10] The relatively large concentration of iodine necessary to cause contrast enhancement on CT requires a large volume of liposomal lipid to deliver sufficient iodine to the liver and spleen to cause appreciable CT enhancement.

Gd-DTPA causes contrast enhancement on MRI T1-weighted images at concentrations approximately two orders of magnitude lower than the concentration of iodine required to cause contrast enhancement for CT.[1,2] Because of the stronger contrast enhancement effect of Gd-DTPA, which occurs at much lower concentrations than with CT contrast agents, it may be possible to use paramagnetic liposomes as a contrast agent for MR to cause contrast enhancement with a low dose of liposomes. To evaluate the possibility of developing a liposomal MR contrast agent, we conducted a preliminary study synthesizing Gd-DTPA liposomes using the freeze-thaw extrusion method[11] to prepare

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unilamellar lipid vesicles of known diameter. Stability was tested in vitro, and in vivo imaging was performed.

Materials and Methods

Unilamellar vesicles were prepared using the freeze-thaw extrusion process as follows: Egg phosphatidylcholine, 0.216 g, 0.077 g cholesterol, and wheat germ digalactosyldiglyceride, 0.023 g (molar ratio 55:40:5), were dissolved in 1 mL chloroform stock solution, 50 μl of ^{3}H-DPPC (0.25 mCi/2.5 mL toluene, NEN Canada) was added, the mixture evaporated initially under nitrogen, then held at reduced pressure for two hours to remove residual solvent.

The lipid mixture was dispersed in 4 mL 0.67 M Gd-DTPA pH 7.0, to which had been added 50 μL of ^{14}C-inulin (0.25 mCi/2.5 mL water, NEN Canada, by vortexing at room temperature to yield multilamellar vesicles [MLVs]. The MLVs were transferred to a 4.5 mL cryovial, then subjected to five cycles of freeze-thawing using liquid nitrogen. The frozen and thawed MLVs were sized by ten passes under nitrogen pressure through two stacked 0.4 micron polycarbonate filters (Nucleopore, Pleasanton, CA) using the extruder device (Lipex Biomembranes, Vancouver, BC). Two-thirds of this sized preparation was then passed ten times through two stacked 0.2 micron filters. Half of this latter preparation was then passed ten times through two stacked 0.1 micron filters.

For each of the sized vesicle preparations, external Gd-DTPA was removed by chromatography on Sephadex G50F (Pharmacia, Uppsala, Sweden) which had been pre-swollen by stirring for 2 hours at room temperature in saline buffer: 10 mM HEPES, 6 mM KCL, 139 mM NaCl pH 7.4. G50F was packed into 23 × 1.5 cm Biorad columns and equilibrated with at least 50 mL of buffer prior to addition of sample. The sample load was 1 mL. Vesicles were eluted with saline buffer in the column void volume and a sample taken for scintillation counting to determine trap volumes as previously described.[11] For one batch of 0.4 micron vesicles, nonencapsulated Gd-DTPA was removed by dialysis of the extruded vesicles against 4 L of saline buffer at 4° C with constant stirring. The external dialysis medium was changed eight times during 48 hours.

The size of the 0.4 micron vesicles was assessed by electron microscopy using negative staining techniques.

In vitro stability of the 0.4 micron liposomes was tested by dialysis performed over 72 hours against normal saline (6 × 1 L) at 4° C. The stability was calculated by counting the ^{153}Gd-DTPA activity remaining in the liposomes at the end of 72 hours.

Imaging

All in vitro and in vivo imaging was performed on a Siemens 1.5 Tesla magnet (Iselin, NJ). Spin echo technique was used for both T1- and T2-weighted imaging. T1-weighted sequences used parameters TR/TE: 400 milliseconds/16 milliseconds, four acquisitions, 256 × 256 matrix 16 cm field view for 2-mm thick slices using a 7.5-cm surface coil. For T1 calculations, images were acquired using a fixed TE of 28 milliseconds with TR of 300, 500, 1500 and 3500 milliseconds. For T2 calculations, a fixed TR of 3500 milliseconds was used with TE values of 28, 45, 70, 90 and 150 milliseconds. T1 and T2 calculations were performed using the software on the Magnetom system (Iselin, NJ) by selecting a region of interest on the CRT monitor. For measuring signal intensity of liver and muscle before and after-contrast medium, the cursor was placed on the same regions of paraspinal muscle and liver to avoid position-related changes in signal intensity. The observers were unaware which rat and dose were being evaluated at the time of measurement.

Phantoms containing various concentrations of either 0.4 micron Gd-DTPA liposomes of free Gd-DTPA were prepared in phosphate buffered normal saline (pH 7.4), heparinized whole human blood and 10% low temperature gelling agar gel and drawn into 20-cc syringes. Samples were arranged in circular array plexiglass containers. All phantoms were scanned using the body coil.

Male buffalo rats, weighing approximately 450 grams, were scanned using a 7.5-cm circular surface coil. The animals were scanned in the supine position, and signal intensities of liver, paraspinal muscle, kidney, renal pelvis, bladder, and vascular structures were measured before and after contrast. Rats were imaged before and after contrast injection of either 0.4 micron Gd-DTPA liposomes (eight rats) or free Gd-DTPA (two rats).

Injections were performed by tail vein injection using a 26-gauge butterfly needle. Gd-DTPA liposomes were injected over a period of about 2 minutes; liposomes were diluted with an equal volume of normal saline immediately before injection. After imaging, animals were observed for eight weeks for any signs of delayed toxicity.

Results

The lipid and Gd-DTPA concentrations in the different vesicle preparations are shown in Table 1. The 0.4 micron vesicles contained the highest concentration of Gd-DTPA. The 0.4 micron vesicle preparations had 39% efficiency for encapsulation of Gd-DTPA and removal of free Gd-DTPA by dialysis resulted in the highest concentration of Gd-DTPA *reflecting the high trapped volume of the 0.4 micron vesicles*. The 0.4 micron vesicles were *essentially* 100% stable to prolonged dialysis. *The predialysis sample (1 mL volume) contained 22,230 counts of ^{153}Gd activity per 100 seconds, and after 72 hours of dialysis the sample contained 22,201 counts/100 seconds.* Because of high Gd-DTPA concentration and encapsulation efficiency, the 0.4 micron liposomes were used for subsequent imaging experiments. Electron microscopy of the 0.4 micron vesicles showed a mean diameter of 289 ± 39 nanometers.

Results from scans of phantoms containing Gd-DTPA liposomes are shown in Fig. 1. Gd-DTPA liposomes had a similar effect on signal intensity of samples prepared in either saline, whole blood, or agarose gel. Concentration-dependent increases in signal intensity were observed with increasing concentration of Gd-DTPA liposomes. Maximal signal intensity was observed at a concentration of about 5 mM Gd-DTPA. T1 and T2 calculations showed linear variations with both free and encapsulated Gd-DTPA. Comparing the effect on signal intensity of T1-weighted images with similar concentrations of liposomally encapsulated and free Gd-DTPA in vitro samples, it was apparent that an approximately fivefold higher concentration of liposome Gd-DTPA was required for similar increase in signal intensity as for free Gd-DTPA. For free Gd-DTPA, maximum signal intensity occurred at a concentration of 1 mM, whereas for the liposomally encapsu-

TABLE 1. Liposome Characteristics

Filter Pore Size (nm)	Lipid Concentration (μm/mL)	Gd-DTPA concentration (μm/mL)
100	35	38
200	39	42
400	44	47
400*	116	124

*Unbound Gd-DTPA removed by chromatography, dialysis used in all other cases.
nm = nanometers; μm/mL = micromoles per milliliter.

lated Gd-DTPA, a maximum was observed at an effective concentration of 5 mM Gd-DTPA (Fig. 1).

Scans of rats before and after contrast injection with Gd-DTPA liposomes showed enhancement of liver, renal cortex, and vascular structures (Fig. 2). A dose of 0.1 millimoles/kg or greater of Gd-DTPA in liposomes resulted in a dramatic increase in signal intensity (Table 2). Maximal enhancement was observed immediately after injection of contrast agent, but enhancement of liver and vascular structures was sustained for 2 hours after contrast in two animals imaged 2 hours after injection of liposomal Gd-DTPA. Scans of two control animals after injection of 0.1 and 0.2 millimoles/kg of free Gd-DTPA showed enhancement of renal cortex, renal pelvis, and urinary bladder but no appreciable enhancement of liver or vascular structures.

During intravenous injection of Gd-DTPA liposomes, there was no obvious sign of respiratory distress. The rats were observed for two months after injection of liposomes and then killed. They showed no gross evidence of toxicity from Gd-DTPA liposomes. Other toxicity was not monitored.

Discussion

In this preliminary study, Gd-DTPA liposomes caused sustained intravascular contrast enhancement, which was observed in venous structures and small vessels although not appreciably in large arteries. The vascular enhancement observed with Gd-DTPA liposomes is most consistent with increasing the relaxivity and signal from blood. Vascular enhancement was not observed in the two control animals given similar doses of intravenous free Gd-DTPA. The greater vascular contrast enhancement observed with liposomal Gd-DTPA likely reflects its relative confinement to the vascular space as opposed to free Gd-DTPA, which is rapidly equilibrated between intravascular and extravascular fluid and is excreted rapidly by the kidneys. Vascular enhancement from Gd-DTPA liposomes could be clinically useful in the evaluation of organ or tissue perfusion. For example, in the case of stroke or altered brain perfusion, it might be possible to use Gd-DTPA liposomes to detect areas of underperfused cerebral tissue. Similarly, other organs such as liver or kidneys might be evaluated. It might

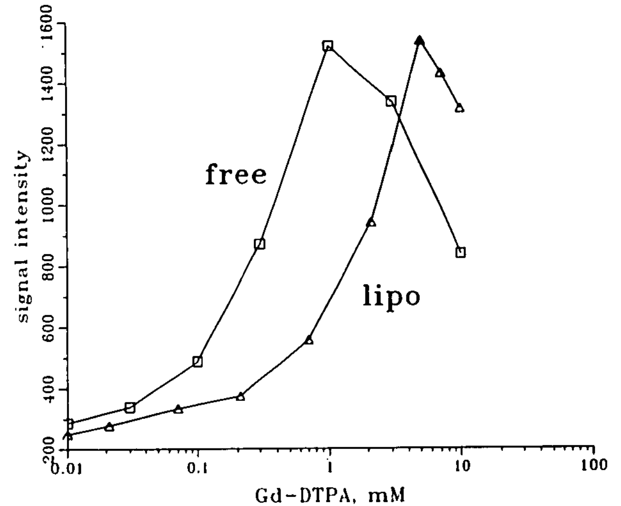

Fig. 1. Signal intensity vs. Gd-DTPA concentration (TR 400 milliseconds/TE 16 milliseconds) for free Gd-DTPA and liposomal (lipo) Gd-DTPA in normal saline phantom. Peak signal intensity for free Gd-DTPA occurs at 1 millimolar concentration and liposomal Gd-DTPA at 5 millimolar indicating shielding of Gd-DTPA by liposome membrane.

also be useful to evaluate tumors with an intravascular contrast agent as a measure of perfusion.

Liposomes are cleared principally by the reticuloendothelial system and should be accumulated preferentially by the liver and spleen.[12] In this study, the liver showed appreciable contrast enhancement. Because of the small size of the spleen in these animals we were not able to assess for contrast enhancement in the spleen. Intracellular delivery of contrast agent to the liver and spleen may be helpful in improving the detection of intrahepatic and splenic neoplasia by MR.

The relatively lower in vitro contrast enhancement effect of Gd-DTPA liposomes compared with free Gd-DTPA may reflet an increased correlation time for water exchange with the entrapped paramagnetic species because of the presence of liposomal membrane. In our subsequent work we plan to evaluate smaller vesicles to see if this will increase the contrast enhancement effect by allowing a larger proportional surface area for water exchange to facilitate proton/paramagnetic center interaction. It also may be possible to alter the lipid membrane constituents within the liposomes to facilitate water transport. Decreasing the proportion of cholesterol in the liposomal membrane should increase water permeability,[13] but this also will likely decrease the stability of the liposomes.[14]

The freeze-thaw extrusion process[11,15,16] used in this preliminary study resulted in the formation of liposomes with encapsulation efficiency as high as 39% and good in vitro stability as shown by 100% retention of Gd-DTPA within the vesicles after 72 hours of dialysis. The encapsulation that

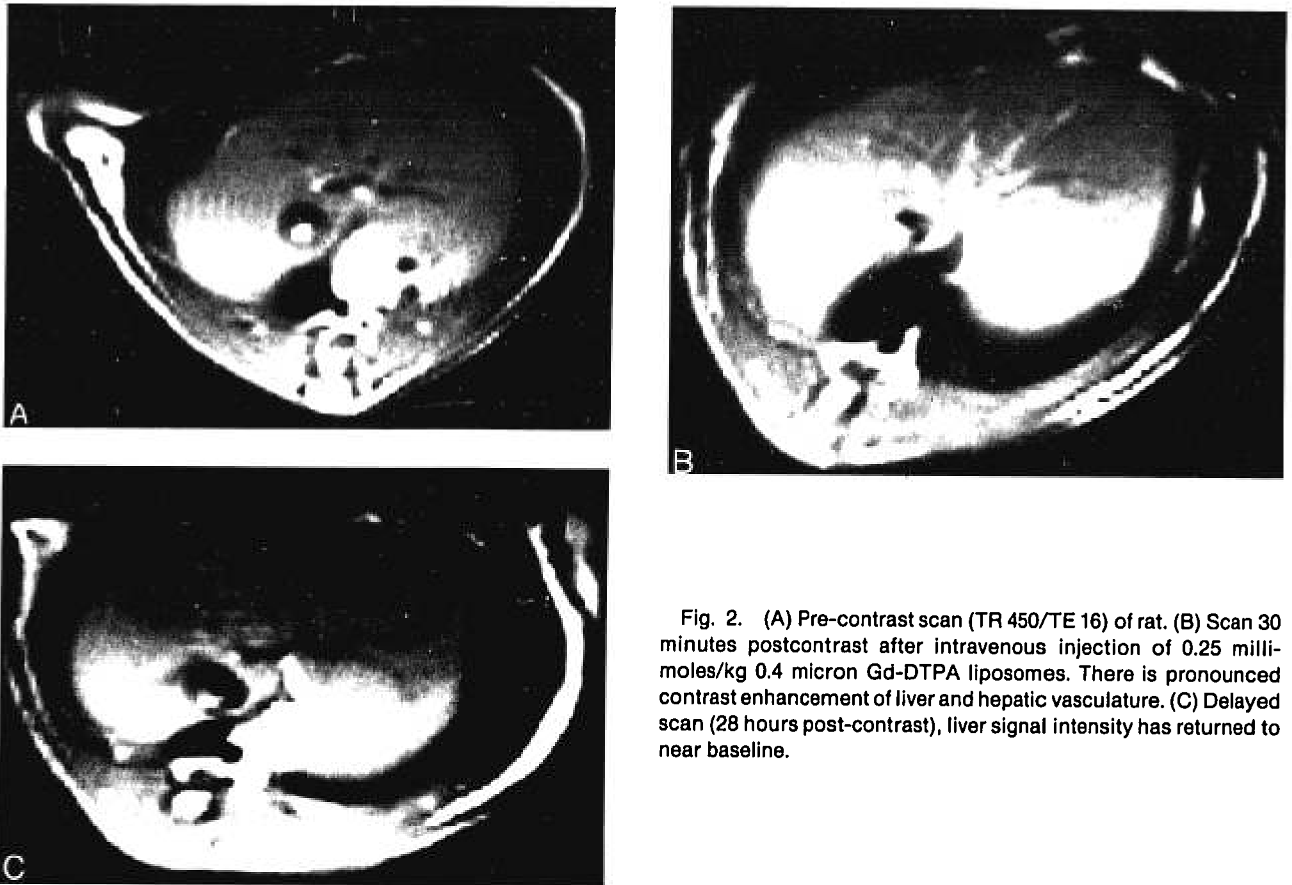

Fig. 2. (A) Pre-contrast scan (TR 450/TE 16) of rat. (B) Scan 30 minutes postcontrast after intravenous injection of 0.25 millimoles/kg 0.4 micron Gd-DTPA liposomes. There is pronounced contrast enhancement of liver and hepatic vasculature. (C) Delayed scan (28 hours post-contrast), liver signal intensity has returned to near baseline.

TABLE 2. Dose Gd-DTPA Liposomes and Contrast Enhancement

Dose (mM/Kg)	Liver	Muscle	Liver/Muscle
0.054 lipo (n = 1)	5.2%	3.9%	1.3%
0.080 lipo (n = 1)	71.1%	31.0%	30.6%
0.100 lipo* (n = 5)	10.0 to 71.1% $\bar{x}$ = 25.0%	$\bar{x}$ = 11.6%	$\bar{x}$ = 39.0%
0.130 lipo (n = 1)	29.0%	−46.2%	239.8%
0.254 lipo (n = 1)	232.0%	151.4%	153.2%
0.109 Free Gd-DTPA	0.0%	22.4%	−19.3%
0.218 Free Gd-DTPA	11.6%	3.1%	8.2%

Liver enhancement: (liver SI post - liver SI pre) ÷ (liver SI pre). Muscle enhancement (paraspinal muscle): (muscle SI post - muscle SI pre) ÷ muscle pre. Liver/muscle refers to ratio of liver enhancement to muscle enhancement. Post-contrast SI measured from scans 30 minutes after intravenous injection of contrast agents.

SI = signal intensity; lipo = liposomal Gd-DTPA; x = mean.

*Five rats were injected with dose of 0.1 millimoles Gd-DTPA/Kg encapsulated within liposomes.

Mean values for enhancement are depicted.

we report in this study is much higher than our previous experience using sonication techniques to prepare liposomes.[17] It appears that the freeze-thaw extrusion procedure results in vesicles with good in vivo tolerance. Although an LD_{50} was not measured in this preliminary work, there was no sign of acute toxicity in the rats during the imaging experiments. Clearly extensive toxicity and biodistribution work must be done to evaluate paramagnetic liposomes.

Gd-DTPA liposomes caused sustained intravascular contrast enhancement and enhancement of the liver. This contrast agent could potentially be useful for assessing tissue, tumor, or organ perfusion and to improve detection of hepatic and splenic tumors or metastases. The freeze-thaw extrusion process results in liposomes with high stability, high encapsulation efficiency, and good in vivo tolerance. In our future work, we hope to define parameters that will improve the contrast enhancement effect of the liposomal Gd-DTPA so that even small doses of liposomes might be used for contrast enhancement.

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