Monitoring Phospholipase A2 Activity with Gd-encapsulated Phospholipid Liposomes

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To date, numerous analytical methods have been developed to monitor phospholipase A2 (PLA2) activity. However, many of these methods require the use of unnatural PLA2 substrates that may alter enzyme kinetics, and probes that cannot be extended to applications in more complex environments. It would be desirable to develop a versatile assay that monitors PLA2 activity based on interactions with natural phospholipids in complex biological samples. Here, we developed an activatable T1 magnetic resonance (MR) imaging contrast agent to monitor PLA2 activity. Specifically, the clinically approved gadolinium (Gd)-based MR contrast agent, gadoteridol, was encapsulated within nanometer-sized phospholipid liposomes. The encapsulated Gd exhibited a low T1-weighted signal, due to low membrane permeability. However, when the phospholipids within the liposomal membrane were hydrolyzed by PLA2, encapsulated Gd was released into bulk solution, resulting in a measureable change in the T1-relaxation time. These activatable MR contrast agents can potentially be used as nanosensors for monitoring of PLA2 activity in biological samples with minimal sample preparation.

Phospholipase A2 (PLA2) is a heterogeneous group of enzymes that specifically recognize and catalytically hydrolyze the sn-2 acyl bond of glycerophospholipids, releasing free fatty acids and lysophospholipids. Changes in PLA2 activity have been associated with numerous pathological conditions including atherosclerosis1, pancreatitis2, acute sepsis3, and cancers4. Thus it has been suggested that PLA2 activity can serve as a diagnostic and prognostic disease biomarker.

Currently, a wide range of analytical techniques have been developed to monitor PLA2 activity. Some of the most widely used methods include electrochemistry5, colorimetry6, fluorimetry7,8, and radiometry techniques9,10. Some assays are rapid, simple and versatile, however, they often suffer from the several shortcomings. For example, fluorescent-labeled phospholipid substrates are not identical to natural lipid substrates and thus might alter the PLA2 reaction kinetics. Therefore, these assays might not monitor the “true activity” of PLA2. It would be desirable to develop an assay based on interactions with natural phospholipids. Application of many existing methods in complex biological environments is also a challenge.

A number of activatable magnetic resonance imaging (MRI) contrast agents have recently been developed that undergo a change in relaxivity in response to various biological process associated with disease. Many of these activatable MR contrast agents are based on chelated gadolinium (Gd), whereby external stimuli including pH11,12, light13, metal-ion14, redox potential15 and enzyme activity induce a transition in chelate conformation/structure leading to a change in the interactions between water and Gd and a corresponding change in T1 relaxation time16. For example, Meade and coworkers have developed a gadolinium complex (4,7,10-tri[acetic acid]-1-[2-β-galactopyranosylethoxy]-1,4,7,10-tetraazacyclododecane) bearing a galactopyranosyl moiety (Egad) that blocks the ninth coordination site of Gd3+, inhibiting water access to the paramagnetic ion. The contrast agent is irreversibly turned ‘on’ when the blocking moiety was removed by exposure to β-galactosidase, causing a 20% decrease in T117.

Recently, we developed a highly efficient MR contrast agent based on Gd-encapsulated vesicles, whereby hundreds of thousand Gd-chelates were encapsulated within a single ~100 nm vesicle18,19. To overcome the detrimental effects of the slow water exchange rate through the vesicle bilayer on longitudinal relaxivity, Gd-labeled macromolecules were encapsulated into porous vesicles. Due to fast water exchange across the porous membrane, these nanovesicles had an increased relaxivity compared to when Gd was encapsulated within nonporous vesicles. In this study, we take advantage of this relationship between Gd relaxivity and liposome...
permeability to develop a new activatable MR contrast agent for monitoring phospholipase A2 activity. Specifically, the clinically approved Gd-based MR contrast agent, gadoteridol (molecular weight 558.7), was encapsulated within nanometer-sized phospholipid liposomes composed of 90mol% DPPC and 10mol% DSPE-PEG2000. It was hypothesized that PLA2-mediated hydrolysis of the liposomal membrane would result in the release of the entrapped Gd-chelates into bulk solution and lead to a corresponding reduction in the T1-relaxation time, i.e. increase in MR signal (Figure 1). The effect of PLA2 on liposome stability and Gd relaxivity was tested under a variety of different conditions.

Results
The Gd-based MR contrast agent, gadoteridol, was encapsulated within phospholipid vesicles composed of 90mol% DPPC and 10mol% DSPE-PEG2000 via lipid film hydration. Nanometer-sized unilamellar gadoteridol-encapsulated vesicles (LUV) were then formed by subjecting the sample to multiple freeze–thaw cycles and extrusion through a 100 nm polycarbonate filter. The resultant sample was a clear suspension. However, the sample immediately turned cloudy upon the addition of PLA2 (Figure 2A), indicating the formation of aggregated liposomes or the formation of insoluble products. It should be noted that the incubation media also contained calcium ions (Ca2+) since the activity of this PLA2 enzyme is calcium-dependent20,21. If Gd-encapsulated liposomes were incubated with PLA2 in the absence of Ca2+, no cloudy appearance was observed (see Supplementary Fig. S1). This indicates the interaction between Gd-encapsulated liposomes and PLA2 was calcium-dependent. Further studies were performed by incubating the Gd-encapsulated liposomes with both PLA2 and enzyme inhibitor MAPF. Under this condition, the liposome suspension remained clear, confirming that PLA2 activity was specifically responsible for the change in sample turbidity.

Liposomal samples with and without PLA2 and MAPF were further characterized by measuring the liposome size by dynamic light scattering (DLS). DLS (Figure 2B) revealed that the Gd-encapsulated

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**Figure 1** | Schematic diagram of PLA2 responsive liposomes with encapsulated Gd agents. The addition of PLA2 to the Gd-encapsulated liposomes leads to hydrolysis of the phospholipids that form the liposomal membrane. The resulting release of the encapsulated Gd leads to a measurable change in the T1 relaxation time of the sample.

**Figure 2** | (A) Images of liposome samples in the presence and absence of PLA2. Gd-encapsulated liposomes were incubated with PLA2 (2), PLA2 in the presence of MAPF (1), or in buffer (3) at a temperature of 37°C. Images were acquired following a 24 h incubation. In all cases, the final phospholipid (DPPC) and Ca2+ concentration in the incubation media was 3.36 mg/mL and 1.67 mM, respectively. The final amount of PLA2 in the incubation media was 5 units. For the inhibition study, a final concentration of 0.3 mg/mL MAPF was used. (B) Dynamic light scattering (DLS) measurements of liposomes in the presence and absence of PLA2. The intensity-weighted hydrodynamic diameter of Gd-encapsulated liposomes incubated with PLA2 (■), PLA2 in the presence of MAPF (●), and buffer (▲) were acquired. For the DLS measurement, 0.1 mg/mL DPPC was used.
Liposomes alone had a mean diameter of 105 nm. The sample of Gd-encapsulated liposomes incubated with PLA2 had a very large peak (around 500 nm in diameter) with a broader size distribution, consistent with the cloudy appearance observed in Figure 2A. It has been previously reported that the low solubility of phospholipid hydrolysis product, such as free fatty acids, could be formed upon PLA2 hydrolysis. Aggregation of this insoluble product is presumably responsible for the cloudy appearance. In contrast, Gd-encapsulated liposomes incubated with both PLA2 and MAPF did not exhibit any significant change in hydrodynamic diameter over a 24 h incubation period. Taken together, these studies indicated that PLA2 was able to interact specifically with Gd-encapsulated liposomes.

To study the release of small encapsulated agents from liposomes in the presence and absence of PLA2, the small fluorescent dye, carboxyfluorescein (CF, molecular weight 376), was entrapped within the aqueous lumen of the liposomes at self-quenching concentrations (i.e., 100 mM CF). The release of CF was then monitored fluorometrically. It was found that intact liposomes exhibited less than a 1% release of the encapsulated CF over 1 hour (Figure 3), confirming that small analytes are inefficient at traversing the liposomal membrane. These findings imply that the low-molecular weight MR contrast agent gadoteridol would also be retained within these liposomes since the molecular weight of gadoteridol is even slightly higher than that of CF. In contrast, nearly 50 ± 2% (mean ± standard deviation, n = 3) of CF was leaked within 1 h following the addition of PLA2 to the liposome sample. These results confirm that the permeability/porosity of the liposome can be increased by upon the hydrolysis of the phospholipid membrane by PLA2.

The ability of Gd-encapsulated liposomes to monitor PLA2 activity was evaluated by measuring T1 relaxation time as a function of time (Figure 4A). T1 relaxation times were acquired using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). When Gd-encapsulated liposomes were incubated in 0.1 M Tris buffer at a temperature of 37 °C little to no change in T1 was observed over a 24 h time period. This indicated the encapsulated Gd was stably retained within the liposome aqueous interior. Further, the encapsulated Gd had a low T1-weighted signal due to the slow water exchange rate through the liposome bilayer. In contrast, T1 relaxation time decreased when PLA2 was added to Gd-encapsulated liposomes, and there was a ~45% change in T1 relaxation time after a 1 h incubation with PLA2 (Figure 4B). This change was presumable due to the release of encapsulated Gd into surrounding bulk solution upon the hydrolysis of the phospholipid membrane by PLA2. The released gadoteridol had fast exchange rates between the Gd-bound

![Figure 3](https://www.nature.com/scientificreports/srep06958/figure/3)

**Figure 3 | Kinetics of CF release from liposomes in the presence and absence of PLA2.** Liposomes were incubated with PLA2 (■) or 0.1 M Tris buffer (○) at a temperature of 37 °C and fluorescence was measured as a function of time. In both cases, the final phospholipid (DPPC) and Ca²⁺ concentration in the incubation media was 72 μg/mL and 1.5 mM, respectively. The final amount of PLA2 in the incubation media was 1 unit. At the end of experiment, maximum CF fluorescence was determined by the addition of Triton X-100.

![Figure 4](https://www.nature.com/scientificreports/srep06958/figure/4)

**Figure 4 | Temporal change in T1 relaxation time (A) and percent change in T1 relaxation time (B) of Gd-encapsulated liposomes in the presence and absence of PLA2.** Liposomes were incubated with PLA2 (■), PLA2 in the presence of MAPF (▲), and buffer (○). For all samples, the final phospholipid (DPPC), Gd and Ca²⁺ concentration in the incubation media was 3.36 mg/mL, 2.4 mM and 1.67 mM, respectively. The final amount of PLA2 in the incubation media was 1 unit (2.23 μg/mL). The percent change of T1 (% T1 change) was calculated as ([T0 – Tt]/T0) × 100, where T0 is the T1 relaxation time of the Gd-encapsulated liposomes at the initial time, Tt is the T1 relaxation time at any given time.
water and the surrounding bulk water, resulting in a higher T1-weighted signal (low values of T1) compared with T1-weighted signal from the Gd-encapsulated liposomes. Notably, it is also possible that a decrease in T1 relaxation time stemmed from an increase in the porosity of the outer membrane of the liposomes, as opposed to Gd release. An increase in membrane permeability can also lead to a significant improvement in the water-exchange rate between encapsulated gadoteridol and the surrounding bulk water, leading to a reduction in the T1-relaxation time. However, considering the similarity in size between gadoteridol and carboxyfluorescein (558.7 vs. 376 Da), it is likely that gadolinium release was the predominant mechanism. To further confirm that the measured changes in T1 relaxation time were specifically caused by PLA2-mediated mechanisms, experiments were also performed with MAPF. As expected, MAPF inhibited PLA2 activity and reduced the percent change in T1 relaxation time. These results confirm that the observed changes in T1 were PLA2-specific. As shown in Figure 5, the percent change in T1 relaxation time increased as a function of PLA2 concentration in the range of 0 to 2.23 μg/mL, which demonstrated that varying amounts of PLA2 could be detected by measuring the percent change in T1 relaxation time. Previous studies have shown that PLA2 concentrations in this range had a strong association with coronary artery disease (CAD)21.

Conclusion

In conclusion, measuring the T1 relaxation time of Gd-encapsulated liposomes provides a simple method to monitoring PLA2 activity. In addition, considering the method simply relies on changes in the relaxation time of water, the assay can be carried out in more complex environments such as turbid or biological media with little to no interference. In addition, this method could potentially be utilized for in vivo applications due to deep tissue imaging capabilities of MR.

Methods

Chemicals. 1,2-dipalmitoyl-sn-glyero-3-phosphocholine (DPPC) and 1,2-distearyrl-sn-glyero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol))-2000 (DSPE-PEG2000) were obtained from Avanti Polar Lipids (Alabaster, AL). Phospholipase A2 from Naja mossambica mossambica and methyl arachidonyl fluorophosphate (MAPF) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Prohance (or Gadoteridol) was obtained from Bracco Diagnostics Inc. All other chemical were used as received. All of the aqueous solutions were prepared with DI water.

Preparation of nanometer-sized liposomes. Liposomes were prepared using the film hydration method. Briefly, a lipid stock solution containing 90% DPPC/10% DSPE-PEG2000 (molar ratio) was dissolved in chloroform and subsequently dried under a stream of nitrogen at vacuum desiccation for a minimum of 4 h. The resultant dried lipid films were rehydrated with 0.5 M concentration of Gadoteridol or 100 mM carboxyfluorescein (CF) for 30 min. Samples were subjected to 10 freeze–thaw–vortex cycles in liquid nitrogen and H2O (50 °C), followed by extrusion 21 times through two stacked 100 nm Nucleapore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids). Nonentrapped compound was removed via centrifugal filter devices (Amicon Ultra-4, 50 000 MWCO) and size exclusion chromatography using Sepharose CL-4B (Sigma-Aldrich).

Leakage Assay. Measurements of the PLA2 induced release of CF trapped within the liposomes were carried out as follows: CF-loaded liposomal suspensions were incubated in 0.1 Tris (pH 7.4) buffer. The fluorescence intensity at 525 nm was measured using an excitation at 490 nm. The amount of CF released (% CF released) was calculated as ((I0 - I)/I0) × 100 where I0 is the fluorescence intensity of the vesicle suspension containing CF at the initial time, I is the fluorescence intensity at any given time, and I0 is the fluorescence intensity after addition of an aqueous solution of Triton X-100 to the suspension.

Instrumentation. Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano from Malvern Instruments. The scattering angle was held constant at 90°. Fluorescence spectra measurements were done on a SPEX Fluoromax-3 spectrophotometer (Horiba Jobin Yvon). T1 relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). The gadolinium concentration in samples was determined by ICP-OES analysis using a Genesis ICP-OES (Spectro Analytical Instruments GMBH; Kleve, Germany).

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Figure 5 | T1 relaxation time of Gd-encapsulated liposomes as a function of PLA2 concentration. Liposomes were incubated with various concentrations of PLA2 for 1 h before T1 relaxation was measured. For all samples, the final phospholipid (DPPC), Gd and Ca2+ concentration in the incubation media was 3.36 mg/mL, 2.4 mM and 1.67 mM, respectively.
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**Author contributions**

Z.C. performed all experiments and analyzed data. Z.C. and A.T. wrote the paper.

**Additional information**

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