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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, fluorimetry^{7,8}, and radiometry techniques^{9,10}. 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 pH^{11,12}, 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 vesicle^{18,19}. 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

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.

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-dependent^{20,21}. 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

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.

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.

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 hydrolysis⁵. 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 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 $[(T_0 - T_t)/T_0] \times 100$, where T_0 is the T₁ relaxation time of the Gd-encapsulated liposomes at the initial time, T_t 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)²².

Conclusion

In conclusion, measuring the T1 relaxation time of Gd-encapsulated liposomes provides a simple method to monitoring PLA₂ activity. In contrast to other existing assays that use radioactive or fluorescence-labeled probes, phospholipids were not modified, which could lead to a more accurate measurement of PLA₂ activity. Moreover, since this 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-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-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 fluorophosphonate (MAPF) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Prohance (or Gadoteridol) was obtained from Bracco Diagnostics Inc. All other

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 relaxivity 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.

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 using a direct stream of nitrogen prior to 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 Nuclepore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids). Nontrapped 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 $([I_x - I_0]/[I_t - I_0]) \times 100$ where I_0 is the fluorescence intensity of the vesicle suspension containing CF at the initial time, I_x is the fluorescence intensity at any given time, and I_t 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 spectrofluorometer (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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Author contributions

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

Additional information

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