SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ASSAY SYSTEMS
CANCER

Received
8 August 2014

Accepted
21 October 2014

Published
7 November 2014

Correspondence and requests for materials should be addressed to Z.C. (zcheng@seas.upenn.edu)

Monitoring Phospholipase A_2 Activity with Gd-encapsulated Phospholipid Liposomes

Zhiliang Cheng & Andrew Tsourkas

Department of Bioengineering, School of Engineering and Applied Sciences, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States.

To date, numerous analytical methods have been developed to monitor phospholipase A_2 (PLA_2) activity. However, many of these methods require the use of unnatural PLA_2 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 PLA_2 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 PLA_2 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 PLA_2, 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 PLA_2 activity in biological samples with minimal sample preparation.

Phospholipase A_2 (PLA_2) is a heterogeneous group of enzymes that specifically recognize and catalytically hydrolize the *sn*-2 acyl bond of glycerophospholipids, releasing free fatty acids and lysophospholipids. Changes in PLA_2 activity have been associated with numerous pathological conditions including atherosclerosis[1], pancreatitis[2], acute sepsis[3], and cancers[4]. Thus it has been suggested that PLA_2 activity can serve as a diagnostic and prognostic disease biomarker.

Currently, a wide range of analytical techniques have been developed to monitor PLA_2 activity. Some of the most widely used methods include electrochemistry[5], colorimetry[6], 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 PLA_2 reaction kinetics. Therefore, these assays might not monitor the "true activity" of PLA_2. 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], light[13], metal-ion[14], redox potential[15] 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 time[16]. 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 Gd^{3+}, 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 T1[17].

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

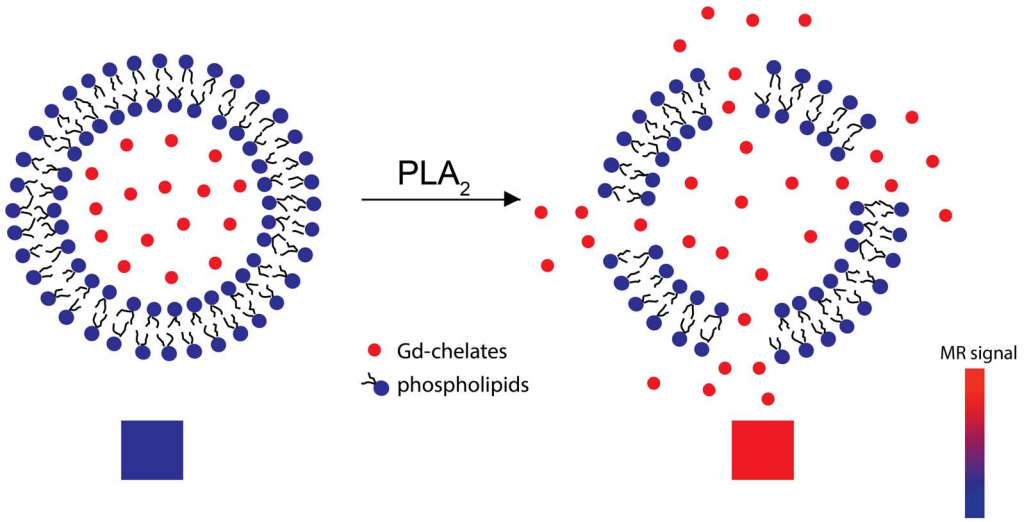

Figure 1 | Schematic diagram of PLA_2 responsive liposomes with encapsulated Gd agents. The addition of PLA_2 to the Gd-encapsulated liposomes leads to hydrolysis of the phospholipids that form the liposomal membrane. The resulting release of the encapsulated Gd leads to a measurable change in the T1 relaxation time of the sample.

permeability to develop a new activatable MR contrast agent for monitoring phospholipase A_2 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 PLA_2-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 PLA_2 on liposome stability and Gd relaxvitity 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 PLA_2 (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 (Ca^{2+}) since the activity of this PLA_2 enzyme is calcium-dependent[20,21]. If Gd-encapsulated liposomes were incubated with PLA_2 in the absence of Ca^{2+}, no cloudy appearance was observed (see Supplementary Fig. S1). This indicates the interaction between Gd-encapsulated liposomes and PLA_2 was calcium-dependent. Further studies were performed by incubating the Gd-encapsulated liposomes with both PLA_2 and enzyme inhibitor MAPF. Under this condition, the liposome suspension remained clear, confirming that PLA_2 activity was specifically responsible for the change in sample turbidity.

Liposomal samples with and without PLA_2 and MAPF were further characterized by measuring the liposome size by dynamic light scattering (DLS). DLS (Figure 2B) revealed that the Gd-encapsulated

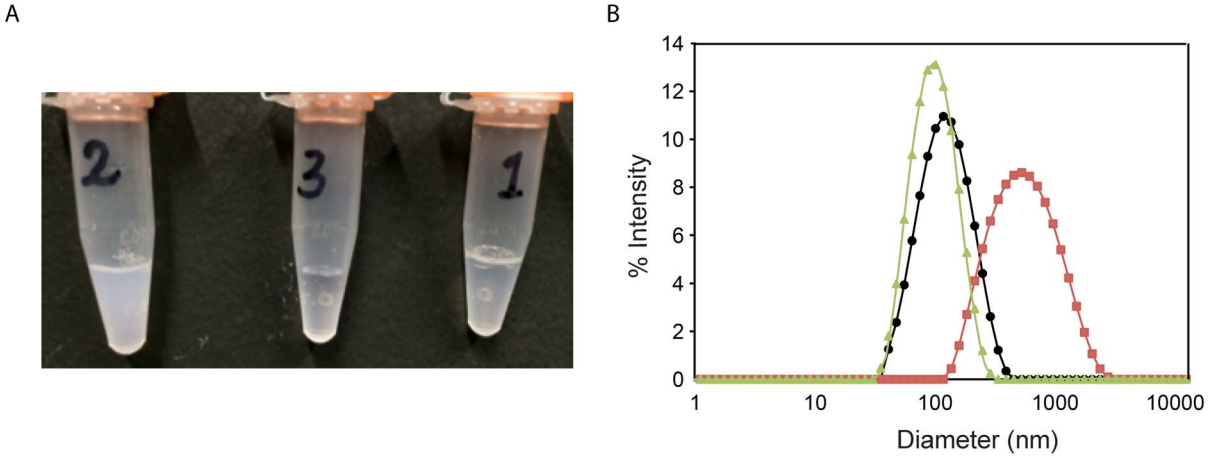

Figure 2 | (A) Images of liposome samples in the presence and absence of PLA_2.Gd-encapsulated liposomes were incubated with PLA_2 (2), PLA_2 in the presence of MAPF (1), or in buffer (3) at a temperature of 37°C. Images were acquired following a 24 h incubation. In all cases, the final phospholipid (DPPC) and Ca^{2+} concentration in the incubation media was 3.36 mg/mL and 1.67 mM, respectively. The final amount of PLA_2 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 PLA_2. The intensity-weighted hydrodynamic diameter of Gd-encapsulated liposomes incubated with PLA_2 (■), PLA_2 in the presence of MAPF (●), and buffer (▲) were acquired. For the DLS measurement, 0.1 mg/mL DPPC was used.

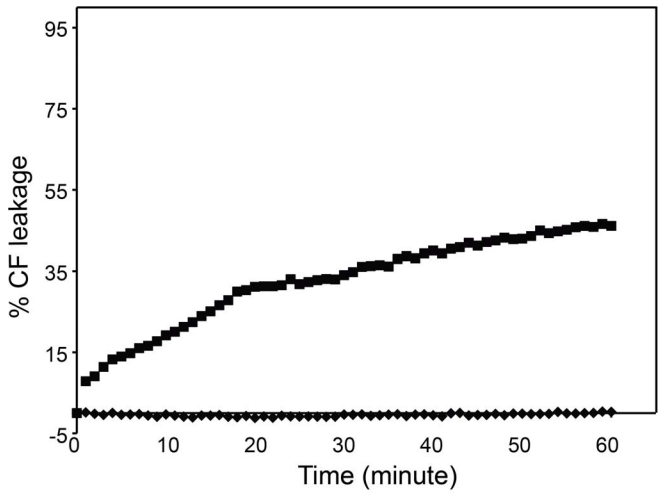

Figure 3 | Kinetics of CF release from liposomes in the presence and absence of PLA_2. Liposomes were incubated with PLA_2 (■) or 0.1 M Tris buffer (◆) at a temperature of 37°C and fluorescence was measured as a function of time. In both cases, the final phospholipid (DPPC) and Ca^{2+} concentration in the incubation media was 72 μg/mL and 1.5 mM, respectively. The final amount of PLA_2 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 PLA_2 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 PLA_2 hydrophlysis[5]. Aggregation of this insoluble product is presumably responsible for the cloudy appearance. In contrast, Gd-encapsulated liposomes incubated with both PLA_2 and MAPF did not exhibit any significant change in hydrodynamic diameter over a 24 h incubation period. Taken together, these studies indicated that PLA_2 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 PLA_2, 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 PLA_2 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 PLA_2.

The ability of Gd-encapsulated liposomes to monitor PLA_2 activity was evaluated by measuring T1 relaxation time as a function of time (Figure 4A). T_1 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 PLA_2 was added to Gd-encapsulated liposomes, and there was a ~45% change in T1 relaxation time after a 1 h incubation with PLA_2 (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 PLA_2. The released gadoteridol had fast exchange rates between the Gd-bound

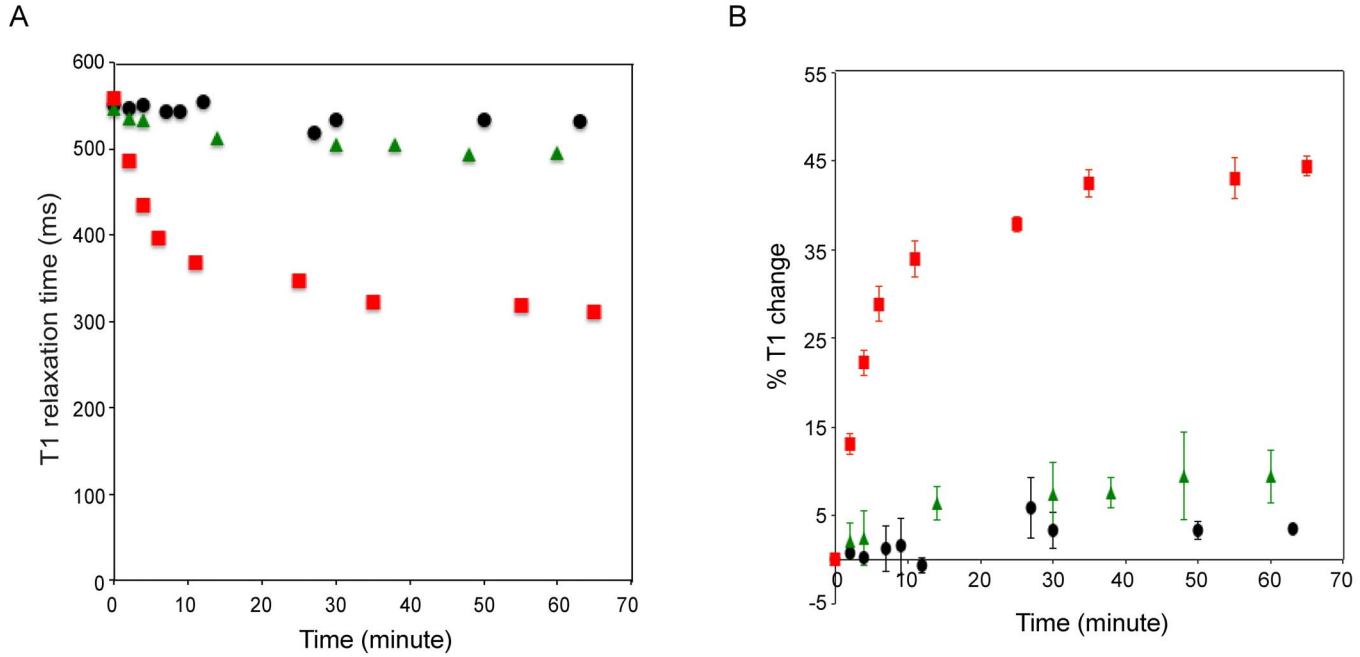

Figure 4 | Temporal change in T1 relaxation time (A) and percent change in T1 relaxation time (B) of Gd-encapsulated liposomes in the presence and absence of PLA_2. Liposomes were incubated with PLA_2 (■), PLA_2 in the presence of MAPF (▲), and buffer (●). For all samples, the final phospholipid (DPPC), Gd and Ca^{2+} concentration in the incubation media was 3.36 mg/mL, 2.4 mM and 1.67 mM, respectively. The final amount of PLA_2 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 PLA_2-mediated mechanisms, experiments were also performed with MAPF. As expected, MAPF inhibited PLA_2 activity and reduced the percent change in T1 relaxation time. These results confirm that the observed changes in T1 were PLA_2-specific. As shown in Figure 5, the percent change in T1 relaxation time increased as a function of PLA_2 concentration in the range of 0 to 2.23 μg/mL, which demonstrated that varying amounts of PLA_2 could be detected by measuring the percent change in T1 relaxation time. Previous studies have shown that PLA_2 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 PLA_2 activity. In contrast to other existing assays that use radioactive or fluorescence-labeled probes, phospholipids were no modified, which could lead to a more accurate measurement of PLA_2 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 A_2 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 chemical were used as received. All of the aqueous solutions were prepared with DI water.

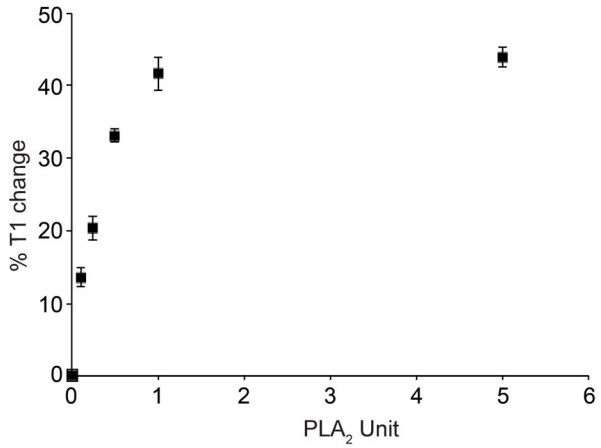

Figure 5 | T1 relaxation time of Gd-encapsulated liposomes as a function of PLA_2 concentration. Liposomes were incubated with various concentrations of PLA_2 for 1 h before T1 relaxivity was measured. For all samples, the final phospholipid (DPPC), Gd and Ca^{2+} concentration in the incubation media was 3.36 mg/mL, 2.4 mM and 1.67 mM, respectively.

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 H_2O (50°C), followed by extrusion 21 times through two stacked 100 nm Nuclepore 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 PLA_2 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 $([Ix - I_0]/[I_t - I_0]) \times 100$ where I_0 is the fluorescence intensity of the vesicle suspension containing CF at the initial time, Ix 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).

1. Kugiyama, K. *et al.* Circulating levels of secretory type II phospholipase A(2) predict coronary events in patients with coronary artery disease. *Circulation* **100**, 1280–1284 (1999).
2. Agarwal, N. & Pitchumoni, C. S. Assessment of severity in acute pancreatitis. *Am J Gastroenterol* **86**, 1385–1391 (1991).
3. Green, J. A. *et al.* Circulating phospholipase A2 activity associated with sepsis and septic shock is indistinguishable from that associated with rheumatoid arthritis. *Inflammation* **15**, 355–367 (1991).
4. Abe, T. *et al.* Group II phospholipase A2 is increased in peritoneal and pleural effusions in patients with various types of cancer. *Int J Cancer* **74**, 245–250 (1997).
5. Mirsky, V. M., Mass, M., Krause, C. & Wolfbeis, O. S. Capacitive Approach To Determine Phospholipase A(2) Activity toward Artificial and Natural Substrates. *Anal Chem* **70**, 3674–3678 (1998).
6. Aili, D., Mager, M., Roche, D. & Stevens, M. M. Hybrid nanoparticle-liposome detection of phospholipase activity. *Nano Lett* **11**, 1401–1405 (2011).
7. Rose, T. M. & Prestwich, G. D. Fluorogenic phospholipids as head group-selective reporters of phospholipase A activity. *ACS Chem Biol* **1**, 83–91 (2006).
8. Chemburu, S. *et al.* Conjugated polyelectrolyte supported bead based assays for phospholipase A2 activity. *J Phys Chem B* **112**, 14492–14499 (2008).
9. Cosentino, M. J. & Ellis, L. C. A rapid simple radiometric assay for phospholipase A2 activity. *Prostaglandins* **22**, 309–322 (1981).
10. Aufenanger, J., Zimmer, W. & Kattermann, R. Characteristics and clinical application of a radiometric Escherichia coli-based phospholipase A2 assay modified for serum analysis. *Clin Chem* **39**, 605–613 (1993).
11. Nwe, K., Huang, C. H. & Tsourkas, A. Gd-labeled glycol chitosan as a pH-responsive magnetic resonance imaging agent for detecting acidic tumor microenvironments. *J Med Chem* **56**, 7862–7869 (2013).
12. Zhang, S., Wu, K. & Sherry, A. D. A Novel pH-Sensitive MRI Contrast Agent. *Angew Chem Int Ed Engl* **38**, 3192–3194 (1999).
13. Tu, C. Q., Osborne, E. A. & Louie, A. Y. Synthesis and characterization of a redox- and light-sensitive MRI contrast agent. *Tetrahedron* **65**, 1241–1246 (2009).
14. Dhingra, K. *et al.* Towards extracellular Ca2+ sensing by MRI: synthesis and calcium-dependent 1H and 17O relaxation studies of two novel bismacrocyclic Gd3+ complexes. *J Biol Inorg Chem* **13**, 35–46 (2008).
15. Berkovic, G., Krongauz, V. & Weiss, V. Spiropyrans and spirooxazines for memories and switches. *Chem Rev* **100**, 1741–1753 (2000).
16. Louie, A. Y. *et al.* In vivo visualization of gene expression using magnetic resonance imaging. *Nat Biotechnol* **18**, 321–325 (2000).
17. Moats, R. A., Fraser, S. E. & Meade, T. J. A "smart" magnetic resonance imaging agent that reports on specific enzymatic activity. *Angew Chem Int Edit* **36**, 726–728 (1997).
18. Cheng, Z. & Tsourkas, A. Paramagnetic porous polymersomes. *Langmuir* **24**, 8169–8173 (2008).
19. Cheng, Z., Thorek, D. L. & Tsourkas, A. Porous Polymersomes with Encapsulated Gd-labeled Dendrimers as Highly Efficient MRI Contrast Agents. *Adv Funct Mater* **19**, 3753–3759 (2009).
20. Raghupathi, R. & Franson, R. C. Inhibition of phospholipase A2 by cis-unsaturated fatty acids: evidence for the binding of fatty acid to enzyme. *Biochimica et biophysica acta* **1126**, 206–214 (1992).

21. Farber, S. A., Olson, E. S., Clark, J. D. & Halpern, M. E. Characterization of Ca2+-dependent phospholipase A2 activity during zebrafish embryogenesis. *J Biol Chem* **274**, 19338–19346 (1999).
22. Charniot, J. C. *et al*. Interpretation of lipoprotein-associated phospholipase A2 levels is influenced by cardiac disease, comorbidities, extension of atherosclerosis and treatments. *Int J Cardiol* **168**, 132–138 (2013).

Acknowledgments

This work was supported in part by the National Institutes of Health NIBIB R01EB012065 (AT), NCI R01CA157766 (AT), NCI R01CA175480 (ZC) and the American Cancer Society (#IRG-78-002-33, ZC).

Author contributions

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

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Cheng, Z. & Tsourkas, A. Monitoring Phospholipase A_2 Activity with Gd-encapsulated Phospholipid Liposomes. *Sci. Rep.* **4**, 6958; DOI:10.1038/srep06958 (2014).