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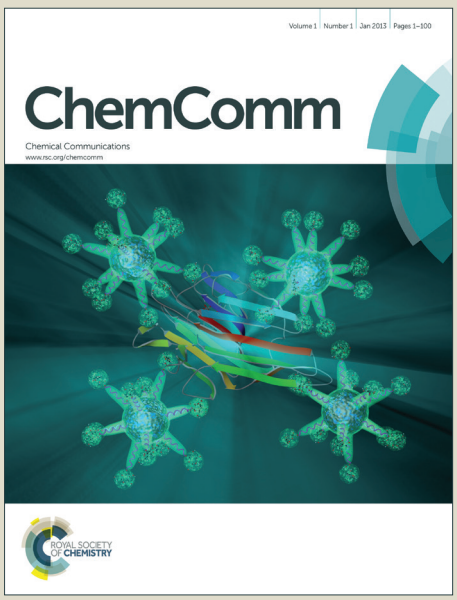

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PLA2-responsive and SPIO-loaded phospholipid micelles

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A PLA_2-responsive and superparamagnetic iron oxide (SPIO) nanoparticle-loaded phospholipid micelle was developed. The release of phospholipid-conjugated dye from these micelles was triggered due to phospholipid degradation by phospholipase A_2. High relaxivity of the encapsulated SPIO could enable non-invasive magnetic resonance imaging.

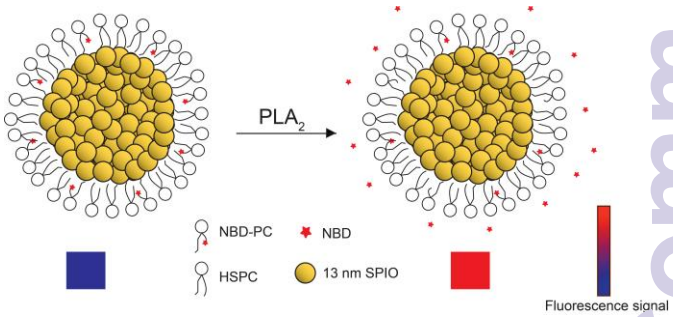
Phospholipase A_2 (PLA_2) specifically recognizes and catalytically hydrolyzes phospholipids at the sn-2 position to form fatty acid and lysophospholipid products. Elevated levels of PLA_2 have been associated with a number of diseases and pathologies including atherosclerosis,[1] pancreatitis,[2] acute sepsis,[3] and cancer.[4] For example, in prostate cancer PLA_2 levels have been found to be up to 22-fold higher than physiological levels.[5-7] Thus it has been suggested that PLA_2 enzymes can serve as a novel diagnostic and therapeutic target for certain diseases.

To date, a substantial amount of effort has been made in developing novel drug delivery system that seeks to concentrate the therapeutic agents in the desired sites while reducing the relative concentration in healthy tissues. One promising approach involves the use of stimulus-responsive nanoparticles that require endogenous or external stimuli to trigger drug release. The ability to limit drug release to a particular location can lead to lower off-target toxicity and improve the therapeutic index.[8] While many types of stimuli including pH, temperature, light, ultrasound and proteolysis have been explored,[9-11] few studies have utilized PLA_2 to trigger drug release.[12]

Successful nanoparticle-based drug delivery is a very complicated process that involves the distribution, metabolism and excretion of the drug. The ability to non-invasively track nanoparticles following their administration is therefore expected to be highly valuable. A promising strategy for achieving this goal is to develop multifunctional nanoparticles that combine therapeutic and diagnostic functions within a single nanoformulation, i.e. "theranostic" agents.[13] It is expected that theranostic agents could provide important insight into localization of the drug and allow pathological processes to be monitored longitudinally.

Currently, a wide range of nanoparticle platforms including dendrimers,[14] liposomes,[15] polymersomes,[16] micelles,[17] emulsions,[18] and silica nanoparticles,[19] have been tested as platforms for drug nanocarriers. Among the many nanoparticular systems, phospholipid-based micelles are particularly attractive due to the recognized and tested biocompatibility of many phospholipids as well as their controlled size, ability to solubilize hydrophobic drugs/imaging agents, and favorable pharmacokinetics.[20, 21]

Herein, we report the design and testing of a PLA_2-responsive and superparamagnetic iron oxide (SPIO) nanoparticle-loaded phospholipid micelle (Scheme 1). It was expected that PLA_2-responsive phospholipid micelles loaded with SPIO nanoparticles could provide drug release specifically in response to PLA_2 activity and also enable non-invasive magnetic resonance (MR) imaging.



Scheme 1 Schematic diagram of PLA_2-responsive and SPIO-loaded phospholipid micelle.

Oleic acid-stabilized hydrophobic SPIONs were synthesized with mean diameters of approximately 13 nm. Transmission electron microscopy (TEM) images revealed a narrow distribution of spherical SPIOs (Supp. Figure S1A). Using an oil-in-water emulsion method,[22] the small hydrophobic SPIONs were encapsulated within the hydrophobic core of micelles formed with the phospholi

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hydrogenated soy phosphatidylcholine (HSPC) and the fluorescent lipid 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-PC). The fluorophore NBD was attached to the sn-2 position of the phospholipid with a spacer (C6) between NBD and the lipid backbone, which was used to mimic a PLA_2-responsvie prodrug. It was hypothesized that PLA_2 could specifically recognize and catalytically hydrolyze the sn-2 acyl bond of NBD-PC, resulting in the release of NBD. SPIO-loaded phospholipid micelles possessed a mean hydrodynamic diameter of 100 nm and low polydispersity (PDI <0.25) as determined by dynamic light scattering (DLS; Figure 1A). Due to the amphipathic properties of phospholipid molecules, these SPIO-micelles were highly soluble in aqueous solutions. TEM further confirmed the encapsulation of the SPIO within the micelles (Supp. Figure S1B). The SPIO-micelles observed by TEM were approximately spherical in shape with tightly packed clusters of SPIO encapsulated within the hydrophobic core. To assess the paramagnetic properties of the SPIO-loaded phospholipid micelles, the amount of iron (Fe) within the sample was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The transverse r2 relaxivity was then calculated as the slope of the curves 1/T2 vs. Fe concentration, as shown in Figure 1B. T2 (transverse relaxation times) were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz) and at 40 °C. It was found that SPIO-loaded micelles had an r2 of 90 $mM^{-1}s^{-1}$. It should be noted that since tens of hydrophobic SPIO nanoparticles can be encapsulated within each 100 nm micelle, the SPIO-loaded micelles can generate a relaxivity that is amplified by a factor of 10 or more on a per particle basis, compared to a single SPIO nanoparticle. Therefore, it is expected that these agents are sufficiently sensitive for noninvasive MR imaging and tracking.

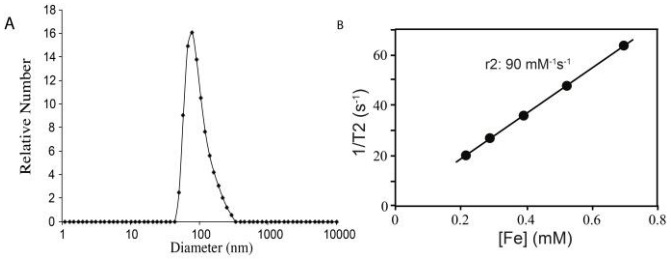

Figure 1. Characterization of SPIO-loaded phospholipid micelles. (A) Number-weighted size distribution of SPIO-loaded phospholipid micelles. (B) Relaxivity determination for SPIO-loaded phospholipid micelles.

As seen in Figure 2A, dissolution of the SPIO-micelles with surfactant Triton X-100 led to a significant increase in fluorescence. These findings imply that the NBD is highly quenched within the SPIO-micelle. There are two factors that likely contribute to the highly quenched state of NBD, self-quenching due to the high loading density (i.e. 10mol%) of NBD-PC within the micelles and quenching through interactions with the encapsulated SPIO nanoparticles. To explore the impact of self-quenching alone on NBP fluorescence, phospholipid micelles doped with 10mol% NBP-PC were prepared in the absence of SPIO nanoparticles and fluorescence was recorded before and after the addition of Triton X-100. It was found that in the absence of SPIO nanoparticles, fluorophore-fluorophore interactions alone led to more than 90% quenching of NBP fluorescence. Therefore, it is likely that this was the primary mechanism responsible for a low NBP signal in the micelles (Supp. Figure S2). The presence of SPIO nanoparticles further reduced fluorescence of the micelles. As a result, dissolution of the SPIO-micelles led to a ~30-fold increase in NBP fluorescence compared with ~15-fold for the empty micelles (Figure 2B). Because of the efficient quenching of NBP fluorescence in the SPIO-micelles, it was expected that the PLA_2-triggered release of NBD could be monitored fluorometrically.

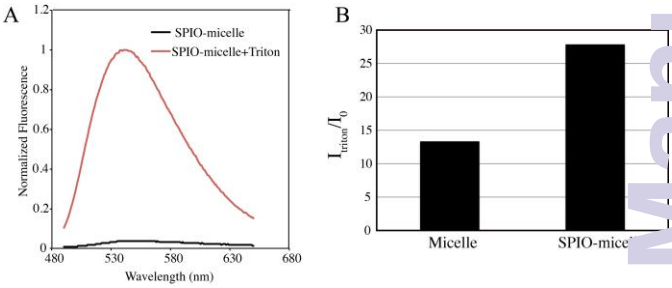

Figure 2. (A) Fluorescence quenching of NBD-PC in SPIO-loaded phospholipid micelles. The normalized fluorescence quenching was determined by subsequently lysing the micelles with Triton-X and measuring the total unquenched fluorescence intensity of the NBD-PC. (B) Quantitative analysis of fluorescence enhancement of NBD-PC in micelles or SPIO-loaded micelles, following lysis with Triton-X.

When SPIO-loaded micelles were incubated in 10 mM HEPES buffer, little to no change in fluorescence was observed over a 24 hr time period. However, a significant increase in fluorescence intensity was observed immediately upon the addition of PLA_2 (Figure 3A). It should be noted that secreted PLA2 was used in this study. The PLA_2-induced change in fluorescence was presumably due to the release of NBD into the surrounding bulk solution upon the hydrolysis of the phospholipid membrane by PLA_2. The released NBD led to fluorescence dequenching, resulting in a higher fluorescence intensity compared with fluorescence intensity from the SPIO-loaded-micelles. Furthermore, this change was time and concentration dependent. The intensity of fluorescence increased with increasing PLA_2 concentration in the range of 0 to 300 unit/L. In addition, the rate of increase in fluorescence increased with PLA_2 concentration.

To further confirm that the measured changes in fluorescence signal were specifically caused by PLA_2-mediated mechanisms, experiments were also performed under two different conditions. First, SPIO-loaded micelles were incubated with PLA_2 in the media containing the calcium chelator EGTA (ethylene glycol tetraacetic acid). As expected, no change in fluorescence was observed since the activity of this PLA_2 enzyme is calcium-dependent (Figure 3B),[23, 24] Second, studies were performed by incubating the SPIO-micelles with both PLA_2 and increasing concentrations of the enzyme inhibitor LY311727. Under these conditions, LY311727 inhibited PLA_2 activity and led to a reduction in the extent of fluorescence activation in a concentration dependent manner (Figure 3C). These results confirm that the observed changes in fluorescence were PLA_2-specific.

In this work we developed PLA_2-responsive SPIO-loaded phospholipid micelles. The release of phospholipid-conjugated dye from these micelles was triggered due to phospholipid hydrolysis by PLA_2. Furthermore, many small hydrophobic SPIO nanoparticles were encapsulated within the micelle core, which could serve as highly efficient MR contrast agent for in vivo imaging. As a consequence, future work will be aimed at developing PLA_2-responsive multifunctional micelles containing therapeutic (i.e. phospholipid-conjugated prodrug), imaging (i.e. SPIO), and targeting agents.

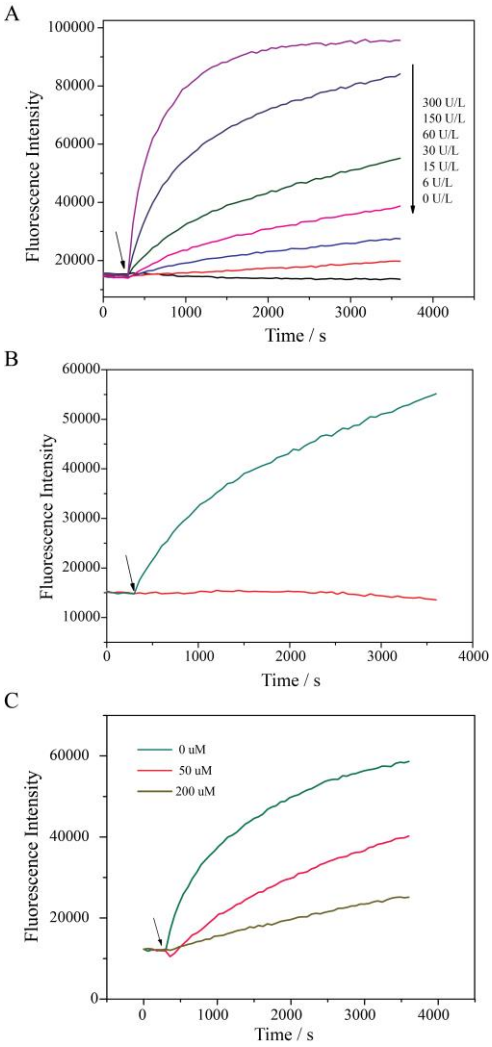

Figure 3. PLA_2 responsive SPIO-loaded phospholipid micelles. (A) SPIO-loaded phospholipid micelles were incubated with PLA_2 from 0 to 300 U/L. (B) PLA_2 was added to SPIO-loaded phospholipid micelles in the absence (blue curve) and presence of excess EGTA (red curve). (C) PLA_2 was added to SPIO-loaded phospholipid micelles in the presence of PLA_2 inhibitor LY 311727 from 0 to 200 μM. For all studies, the final concentration (Fe) of SPIO-loaded micelles and Ca^{2+} in the HEPES buffer (10 mM, pH 7.4) was 16 μg/mL and 2 mM, respectively. For B&C, the final concentration of PLA_2 in the buffer was 60 U/L. The black arrow indicated the time for the adding PLA_2. All experiments were performed at room temperature.

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