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Quantum dot cluster (QDC)-loaded phospholipid micelles as a FRET probe for phospholipase A₂ detection

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Abstract

A simple assay for phospholipase A₂ (PLA₂) enzyme was developed based on a fluorescence resonance energy transfer (FRET) probe using the quantum dot cluster (QDC)-loaded phospholipid micelles. The probe was prepared by encapsulating many small hydrophobic quantum dots (QDs) within the hydrophobic core of micelles that were formed from the coassembly of hydrogenated soy phosphatidylcholine phospholipids (HSPC) and fluorescent lipids (NBD-PC). QDCs formed within the micelle core served as the substrate for NBD fluorescence quenching through FRET. The QDC-loaded micelles showed very low background fluorescence. As the PLA₂ enzyme selectively digested lipids, the NBD fluorescence was recovered from its quenched state, leading to the sensitive detection of PLA₂. This assay provided a limit of detection (at a signal-to-noise ratio of 3) of 3 U/L for PLA₂. In the presence of a PLA₂ inhibitor, the fluorescent response of the sensor for PLA₂ decreased, indicating that the assay could also be used for screening the PLA₂ inhibitors.

Graphical Abstract

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
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Introduction

Phospholipase A₂ (PLA₂) is an important enzyme of the phospholipase superfamily that catalyzes the hydrolysis of sn-2 position acyl chains of phospholipids.¹ PLA₂ dysregulation is a feature of several human diseases, including autoimmune diseases,² cardiovascular diseases,³ neurological disorders,⁴ and some cancer types.⁵ Given its diverse biological roles, many assays have been developed to determine the PLA₂ level by measuring its digestive activity or by measuring protein concentration through an immunoassay. Typical methods for measuring PLA₂ activity include discontinuous radiochemical,⁶ spectrophotometric,⁷ electrochemical,⁸ chromatographic,⁹ magnetic relaxation,¹⁰ and fluorescent¹¹ methods. Among these, the fluorescent method has attracted increasing attention because of its sensitivity, simplicity, and suitability for in-vivo analysis.¹² Fluorogenic substrates are alternative probes for the real-time analysis of enzyme kinetics and their inhibitors. Several PLA₂ probes based on a phosphatidylcholine skeleton have already been developed based on FRET technique.^{13,14,15} PLA₂ primarily acts on aggregated phospholipids organized into lipid bilayers, such as the cell membrane; thus, in addition to their substrate chemistry, PLA₂ is also sensitive to the nanoscale intermolecular spatial arrangement of the substrate.¹⁶ PLA₂ activity on free lipid monomers is relatively lower than that on bilayers, monolayers, and micelles.¹⁷ Furthermore, the use of phospholipid monolayer-coated hydrophobic beads as substrates can decrease lag time for enzymatic hydrolysis.¹⁸ Therefore, micro- or nanoparticles composed of artificial lipid analogs that undergo fluorometric¹⁹ or colorimetric²⁰ changes upon hydrolysis have been synthesized to assay PLA₂ activity. Quantum dots (QDs) are extensively used as optical labels for biosensing because of their high fluorescence quantum yields, stability against photobleaching, and size-controlled luminescence properties.²¹ For example, single QD-loaded phospholipid vesicles,²² liposomes²³ or micelles^{24,25} were successfully used in bio-imaging applications in aqueous and biological systems. QD-encapsulated liposome as FRET probe has also been developed for monitoring the enzymatic activity of PLA₂.²⁶

Herein, we developed a fluorescent assay using quantum dot clusters (QDC)-loaded phospholipid micelle as probe for PLA₂ detection. The phospholipid layer was labeled with the fluorophore and photostable QDC was acted as quencher. Fluorescent assays of PLA₂ activity and its inhibitor were investigated.

Results and discussion

Principle for sensing PLA₂ using QDC-loaded phospholipid micelles

It is well known that inorganic nanomaterials, such as gold nanoparticles,²⁷ iron oxide nanocrystals²⁸ and graphene,²⁹ can quench fluorescence emission from a range of organic fluorophores with extraordinarily high efficiency. Here, a fluorescent probe for PLA₂ activity was designed using QDC as a quencher. As shown in Scheme 1, the probe consisted of hydrophobic QDC and phospholipid layer. To act as PLA₂-responsive fluorescent probe, small percentage of NBD-PC was also incorporated into micelle shell. The fluorophore NBD was attached to the sn-2 position of the phospholipid, which could be released by the PLA₂ hydrolysis. In its “off-state”, the QDC quenched the fluorescence of the NBD. However, quenching effect disappeared when NBD was released from phospholipid by the

PLA₂ hydrolysis, representing the “on-state”. The fluorescence response based on these QDC-loaded phospholipid micelles could be used for the detection of PLA₂.

Overview of QDC-loaded phospholipid micelles

Hydrophobic CdSe/ZnS QDs were characterized with mean diameters of approximately 16 nm, as measured by TEM (Fig. 1a). Quantum dots-loaded phospholipid micelles were prepared by emulsification of QD and phospholipid in aqueous solution.^{28,30} The size and spatial arrangement of particles in the micelles was shown in Fig. 1b. The QD-loaded micelles were formed consisting of a core with densely packed QD nanoparticles. The core with densely packed QD nanoparticles was called QDC. DLS measurements revealed that the hydrodynamic size of the final micelles was of 100 nm (Fig. 1c), indicating that the QDC-loaded phospholipid micelles was formed. The low polydispersity (0.17) is a measure of the relative uniformity between the QDCs formed, which is suggestive of growth occurring via monomer-cluster growth mechanism.³¹ Notably, the QDC-loaded micelles were highly stable in storage (4 °C) for at least 30 days, with no apparent change in the average hydrodynamic diameter or size distribution. Therefore, the hydrophobic nature of the QDs could prevent the QDC-loaded phospholipid micelles from breaking up in the aqueous environment, while the presence of the lipid at the surface of the QDC-loaded phospholipid micelles maintained stability against coalescence.³² For comparison, single QD-loaded phospholipid micelles were also prepared, as shown in Fig. S1 (Supporting information). Compared with QD, no significant change in size was observed for single QD-loaded phospholipid micelles.

Sensing of PLA₂ activity

CdSe/ZnS QD had an emission maximum at 625 nm with a broad absorption range below 650 nm (Fig. S2 and S3, supporting information). NBD fluorophore had an emission maximum at 545 nm with 460 nm excitation (Fig. S4, supporting information). Therefore, probe with 460 nm excitation resulted in FRET between the QD and NBD molecules. As shown in Fig. 2 (curve a), total quenching of NBD fluorescence and a significant emission with a maximum at 625 nm (typical for QD emission) was observed. Ratio of QD and NBD fluorescence reached 11-fold, suggests that the energy transfer was complete. The addition of PLA₂ (100 U/L) resulted in an increase in NBD fluorescence that was accompanied by a decrease in QDC fluorescence (Fig. 2, curve b). A 10-fold increase in the NBD fluorescence was obtained after 1 h. This indicates that NBD was released to solution and the fluorescence of NBD was restored. Fig. 2 (curve c) shows the fluorescence spectra of probe after addition of Triton X100. It is apparent the emission intensity of the NBD significant increases whereas the luminescence intensity of the QD further decreases, and a 17-fold increase in the NBD fluorescence was obtained. This indicates that the complete release of the NBD on treatment of the probes with a detergent. The remaining QD fluorescence resulted from direct excitation of QDCs. Control fluorescence measurements on single QD-NBD micelles were conducted to explore the impact of single QD on NBD fluorescence. Compared with the probe, an obvious NBD fluorescence was observed and ratio of QD and NBD fluorescence is 2-fold (Fig. 2, curve d), indicates that single QD could not effectively quench NBD fluorescence. Further, when probes were incubated in HEPES buffer, no change in fluorescence was observed over a 24 h time period. Therefore, it was expected

that the PLA₂-triggered release of NBD could be monitored fluorometrically because of the efficient quenching of NBD fluorescence in the probes.

The ability for the PLA₂ assay of probes was evaluated by measuring the fluorescence intensity as a function of time. The PLA₂ activity assay relies on the intensity changes in the NBD fluorescence, and measurements were conducted at room temperature for enzyme concentrations of 50 U/mL. As shown in Fig. 3A, the initial fluorescence intensity of the probe was rather weak, but the fluorescence increase was observed after addition of PLA₂ within 60 min. PLA₂ is a family of water-soluble enzymes that acts with calcium ion (Ca²⁺) as a cofactor, therefore, repeated measurement in the absence of Ca²⁺ is a useful control study. As expected, no change in fluorescence was observed in the presence of the calcium chelator EDTA (Ethylenediaminetetraacetic acid), indicating that the fluorescence of the system is dependent on the PLA₂-mediated hydrolysis of the lipids, rather than simply the presence of the enzyme. To further test the specificity of this probe toward PLA₂, change of the probe fluorescence in the presence of PLC or PLD (each 100 U/L) was investigated. Almost no response was observed in these cases, indicating that phospholipases PLC and PLD did not interfere in the determination of PLA₂ although they can catalyze the hydrolysis of phospholipids.

The sensitivity of the assay was investigated using varying PLA₂ concentrations. The results were shown in Fig. 3B. The fluorescence responses increased with increasing PLA₂ concentration from 5 to 400 U/L. Compared with about 7.1-fold increase in fluorescence intensity using iron oxide nanocrystals as quencher,²⁸ a 23-fold increase was obtained using the probe when 300 U/L PLA₂ was determined due to low background signal of the probe. The peak fluorescence readouts at 545 nm were dynamically increased with increasing concentrations of PLA₂ within the range of 5–200 U/L, and a correlation coefficient of 0.994 was obtained (Figure S5). The detection limit of 3 U/L was achieved based on the signal-to-noise ratio (S/N = 3). This simple and sensitive assay for PLA₂ activity allowed us to extend its application, for example, developing an enzyme inhibitor assay. LY311727, an inhibitor of phosphatidylcholine-specific PLA₂, was tested to examine the inhibition of PLA₂ activity. Fig. 3C shows the effect of LY311727 concentration on the activity of PLA₂. As can be seen, the activity of PLA₂ decreased with an increase in LY311727 concentration. These results suggest that the probe has a potential for screening inhibitors of PLA₂.

The effect of LY311727 on the activity of PLA₂ was examined and the fluorescence intensities decreased gradually with the increase of concentration of LY311727, indicating the inhibition of PLA₂ activity by LY311727 (Figure S6). The IC₅₀ value was calculated to be 52 μM.

Experimental

Chemicals and reagents

Hydrogenated soy phosphatidylcholine (HSPC), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD PC) were purchased from Avanti Polar Lipids, Inc. Inhibitor 3-[3-(2-amino-2-oxoethyl)-1-benzyl-2-ethylindol-5-yl]oxypropylphosphonic acid (LY311727) was obtained from Santa Cruz

Biotechnology, Inc. Phospholipase A₂ (PLA₂) from *Naja mossambica*, Phospholipase C (PLC) from *Clostridium perfringens*, Phospholipase D (PLD) from cabbage and Triton X-100 were purchased from Sigma-Aldrich Co. Hydrophobic CdSe/ZnS QD (Q1625, 16 nm) was purchased from Wuhan Jiayuan quantum dots Corporation, Ltd. Stock solution of HSPC was prepared by dissolving HSPC in chloroform to a concentration of 50 mg/mL. Other chemicals and reagents were commercially available and were of analytical grade. Water was obtained by Millipore Milli-Q purification system.

Apparatus

Fluorescence spectra measurements were done on a F7000 spectrofluorometer (Hitachi, Japan). Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano from Malvern Instruments. Transmission electron microscopy (TEM, JEM-1010) was used to determine the size and morphology of QD and QDC-loaded phospholipid micelles.

Synthesis of QDC-loaded phospholipid micelles

QDC-loaded phospholipid micelles were prepared using an oil-in-water emulsion-based self-assembly method. A mixture containing HSPC (0.88 mg), NBD PC (0.1 mg) and QD (1 mg) in 150 μ L chloroform was injected into a glass vial containing 3 mL of water, and the sample was sonicated until a homogenous mixture was obtained. The chloroform was then allowed to evaporate overnight. Following that, QDC-loaded phospholipid micelles samples were centrifuged at 1000 rpm for 30 minutes to remove large aggregates. To obtain the QDC-loaded micelle, the resulting supernatant was centrifuged at 3000 rpm for half hour, and the pellet was resuspended in water (1 mL). To obtain the single QD-loaded micelle, the resulting supernatant was then centrifuged at 10000 rpm for half hour, and the pellet was resuspended in water (1 mL). The collected samples of QDC- and QD-loaded phospholipid micelles were stored in the dark at 4 °C.

Sensing of PLA₂ and inhibitory assay

Aliquots (900 μ L) of 10.0 mM HEPES (N-2-hydroxyethylpiperazine-N-ethane-sulphonic acid) buffer (pH 7.4) containing 2.0 mM CaCl₂ and probes (50 μ L) were firstly prepared. PLA₂ (50 μ L) were then added to the solution, and fluorescence was then recorded.

For the inhibitor assay, LY311727 was preincubated with PLA₂ (50 unit/L) in 50 μ L of HEPES buffer for 20 min before adding to the probe sample.

Conclusions

We developed a fluorescent probe based on the phospholipid micelle encapsulated QDC for the PLA₂ activity assay. Fluorescence of NBD-labeled phospholipid was quenched by QDC, as the phospholipid layer is digested by the enzyme and fluorescence of NBD is restored. Compared with single QD, use of QDC enables improve quenching efficiency. The probe provided high sensitivity (LOD: 3 U/L). In addition to determining phospholipase levels, the probe can be used to identify and study PLA₂ inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of QDC-loaded phospholipid micelles. a) TEM image of quantum dot. b) TEM image of a QDC-loaded phospholipid micelle. c) Dynamic light scattering profile of QDC-loaded phospholipid micelles in HEPES buffer.

Fig. 2.

Fluorescence spectra of (a) probes, (b) probes after addition of PLA_2 (100 U/L), (c) probes after addition of Triton X100, and (d) the single QD-loaded phospholipid micelles.

Fig. 3. Fluorescence response of the probes to PLA₂: A) in the absence (a) and presence (b) of Ca²⁺; B) with different enzyme concentrations; C) in the presence of different concentrations inhibitor. PLA₂ concentration, 50 U/L. Ex, 460 nm, Em, 545 nm.

Scheme 1.

Schematic of QDC-loaded phospholipid micelles and analytical principle of the probe for activity assay of PLA_2 .