Electronic Supplementary Material (ESI) for ChemComm.


# Target-Controlled Gating Liposome "off-on" Cascade Amplification for Sensitive and Accurate Detection of Phospholipase D in Breast Cancer Cell with Low-Background Signal

**Qingwang Xue,[a,b] Wei Jiang,[c*] Lei Wang,[a*]**

[a] *School of Pharmacy, Shandong University, Jinan 250012,P.R. China.*

[b] *Department of Chemistry, Liaocheng University, Liaocheng, 252059, Shandong, China.*

[c] *School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, P.R. China.*

Corresponding author:

Tel: +86-531-88363888; fax: +86-531-88564464.

Email: wangl-sdu@sdu.edu.cn; wjiang@sdu.edu.cn

## EXPERIMENTAL SECTION

### Chemicals and Materials

Phospholipase D (PLD), phospholipase C (PLC) and Phospholipase $A_2$ ($PLA_2$) were obtained from Sigma (Shanghai, China). L-α-phosphatidylcholine (PC), β-cholestanol (Chol) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Glucoamylase and amylose were purchased from Sigma-Aldrich (Shanghai, China). 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), chlorpromazine and O-Tricyclo[5.2.1.$0^{2,6}$]dec-9-yl dithiocarbonate potassium salt (D609) were obtained from Sigma-Aldrich (Shanghai, China). Glycine, cysteine, glutathione, lysozyme, immunoglobulin G (IgG), human serum albumin (HSA), immunoglobulin G (IgG), and bovine serum albumin (BSA) were purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). Other chemicals (analytical grade) were obtained from standard reagent suppliers. All aqueous solutions were prepared using ultrapure water with an electric resistance >18.2 MΩ, which was obtained through a Millipore Milli-Q water purification system.

### Instruments

Transmission electron micrograph (TEM) images was performed using a JEM-2100 transmission electron microscope (JEOL), operating at 200 kV with an ultrahigh-resolution pole piece and providing a point resolution of 2 Å. Atomic force microscopy (AFM) image was performed on a Bruker Multimode 8 atomic force microscope. Hydrodynamic dynamic radius ($R_H$) and zeta-potential (ξ) of the target-controlled gating liposome (TCGL) were obtained using a Malvern mastersizer 2000 (Malvern, UK).

### Preparation and Characterization of Target-Controlled Gating Liposome (TCGL)

According to the procedure from the literature with slight modifications,[1, 2] target-controlled gating liposome (TCGL) was prepared. Three lipids, L-α-

phosphatidylcholine (PC), β-cholestanol (Chol), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) with a molar ratio of 7:1:2 were added into 3 mL chloroform in the flask, and then mixed uniformly at 35 - 45 °C. The chloroform was evaporated in a rotary evaporator under vacuum for 30 min, and then the flask was flushed with argon stream for 2 h to remove any residual organic solvent traces. The dried lipid film was then rehydrated in 5 mL of 1 mg/mL glucoamylase solution and vortexed for 30 min in a bath sonicator (35 °C). To obtain narrowly distributed small unilamellar liposome vesicles, the resulting solution was extruded 11 times through a polycarbonate membrane with 100 nm pores (Nucleopore). Untrapped glucoamylases were removed by gel filtration through a Sephadex G-100 column. The collected fraction of TCGL was stored at 4 °C until use. For simplicity, we designated the concentration of the TCGL as 1×.

The morphology of TCGL was measured by TEM and AFM. The average hydrodynamic diameters and zeta potentials of the prepared TCGL were measured by dynamic light scattering measurements using a Malvern mastersizer.

**The proposed TCGL-PGM sensing assay for PLD activity**

20 μL 1× TCGL solutions was first added to 20 μL reaction buffer containing 5 μL of Tris-HCl buffer (100 mM Tris, 75 mM $CaCl_2$, pH 8.0) and 15 μL of substrate amylose (2.5 μg/μL). Then 10 μL PLD (with final concentrations ranging from 0 to 1000 U $L^{-1}$) was added, and incubated at 37 °C for 20 min for full reaction with occasional gentle shaking. Finally, the result was detected using a mini commercial PGM.

**Selectivity of the TCGL-PGM sensing assay and Influence of Inhibitors on PLD Activity**

To evaluate the specificity of the proposed TCGL-PGM sensing assay, some other biomolecules in place of PLD were added into the TCGL solution with the same experimental conditions and procedures.

To further study the inhibitors screening ability of the proposed TCGL-PGM

assay, various concentrations inhibitors FIPI were pre-incubated with PLD activity (400 U $L^{-1}$) in 10 μL of Tris-HCl solution (10 mM, pH 8.0) for 20 min at room temperature, and then the identical detection procedures were carried out.

**Preparation of the Cell Lysates**

Human breast cancer cell lines (MCF-7 and MDA-MB-231) and non-tumorigenic MCF-10A were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100μg/mL streptomycin and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. The cell density was determined by a hemacytometer. To prepare cell suspensions, $2 \times 10^7$ cells were washed twice with ice-cold PBS by centrifugation at 2000 rpm for 5 min. After discarding the supernatant carefully, the cells were resuspended in 2 mL of ice-cold 10 mM Tris-HCl buffer (pH = 8.0). The cell suspensions were ultrasounded four times in an ice bath for 50 s each with an ultrasonic cell disintegrator of 60 W ultrasonic powers. After that, the samples were centrifuged to collect the lysed protein in the supernatants at 12000 rpm for 20 min at 4 °C. Finally, the supernatants were transferred carefully to microcentrifuge tube and stored at -20 °C.

**Determination of PLD in Breast Cancer Cell**

5μL the cell lysates ($1 \times 10^7$ cells $mL^{-1}$, 1000-fold diluted by 10 mM Tris-HCl buffer, pH 8.0) were spiked with 10 μL of standard PLD solutions at concentrations of 0 - 1.0 $UL^{-1}$. D609 (2.5 μL, 0.4 mM) and chlorpromazine (2.5 μL, 2.0 mM) was then added to the spiked samples and pre-incubated for 20 min at room temperature to eliminate the interference from PLC and $PLA_2$ in the cell lysates. The mixture was then added to 30 μL of reaction system containing 20 μL of 1 × TCGL probe solutions, 5 μL of Tris-HCl buffer (pH 8.0, 100 mM Tris, 75 mM $CaCl_2$), and 5 μL of substrate amylose (7.5 μg/μL), and incubated at 37°C for 20 min. After that, the resulting solution was then subjected to glucose signal measurement by a mini commercial PGM.

## RESULTS AND DISCUSSION

### Calculation of the number of target-controlled gating liposomes (TCGL)

The lipid molecules (*Ntot*) number in a TCGL was calculated according to equation (1):

$$N_{tot} = \frac{[4\pi(\frac{d}{2})^2 + 4\pi\left[\frac{d}{2} - h\right]^2]}{a_L} \quad (1)$$

Where d is the hydrodynamic diameter, h is the bilayer thickness, and $\alpha_L$ is the average head group surface area per lipid. The lipid bilayer thickness was assumed to be 4 nm and $\alpha_L$ value for phosphatidylcholine, phosphoethanolamine and cholesterol were 0.65 ± 0.01 $nm^2$, 0.52 ± 0.01 $nm^2$, and 0.41 $nm^2$, respectively.[3] Based on those values and the molar fraction of each component, the αL value for TCGL were 0.6 $nm^2$/lipid. The *Ntot* was calculated to be 1.798 × $10^5$ lipid molecules per TCGL. The number of TCGL per milliliter (*Nlipo*) can be derived from the lipid concentration with Avogadro's number as shown in equation (2)

$$N_{lipo} = \frac{M_{lipid} \times N_A}{N_{tot} \times 1000} \quad (2)$$

Where $M_{lipid}$ is the molar concentration of lipid. The number of TCGL was calculated based on the number of lipid molecules in a liposome and the lipid concentration used to prepare the liposome solution. Given that the total concentration of lipid used is 84.6 μM, *Nlipo* was calculated to be 2.8 × $10^{11}$ liposomes per mL.

During the preparation process, the TCGL solution lost during extrusion and dialysis step was estimated to be around 12.5 ± 3% and 40.8 ± 2%, respectively. The data were obtained by preparing similar liposomes with fluorescent lipids and analyzed with UV-vis spectrometry. The number of liposome recovery after preparation is calculated to be 1.47 × $10^{11}$ liposomes per mL.

### Characterization of the Target-Controlled Gating Liposome

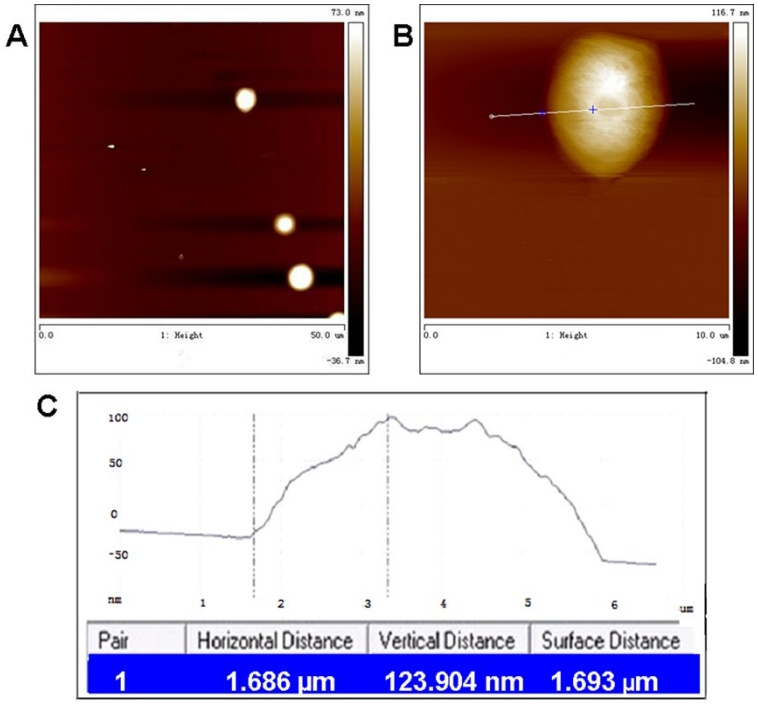



**Fig. S1.** AFM images of (A) the TCGL and (B) single TCGL, and height profiles (C) of single TCGL.

The diameter and surface charge zeta-potential (ξ) of TCGL were performed by using light scattering (DLS) measurements. It gave an average hydrodynamic diameter of approximately 135 ± 20.5 nm in size and a reduced surface charge zeta-potential (ξ) (−24.8 ± 1.5 mV) **(Table S1)**. The result indicated that the TCGL were well-dispersed in water solution. Considering the thickness of a lipid bilayer,[4] the average volume of a single TCGL is approximately $1.07 \times 10^{-12}$ µL, which was calculated according to previous literature reports.[5] These data gave immediate evidence for the loading capacity of glucoamylase.

**Table S1.** Characterizations of glucoamylase-free liposomes, liposome-glucoamylase complexes (TCGL).

| Sample | mean diameter (nm)[a] | polydispersity index [a] | Zeta potential (mV) [a] |
|---|---|---|---|
| glucoamylase-free Lip | 116 ± 12.6 | 0.206 ± 0.017 | −26.7 ± 1.2 mV |
| Lip-glucoamylase complex | 135 ± 20.5 | 0.237 ± 0.026 | −24.8 ± 1.5 mV |

[a] Mean ± standard deviation, n=3; Mean diameter is the hydrodynamic diameter;

Polydispersity index indicates the quantification of dispersity; Zeta potential indicates the average surface charge.

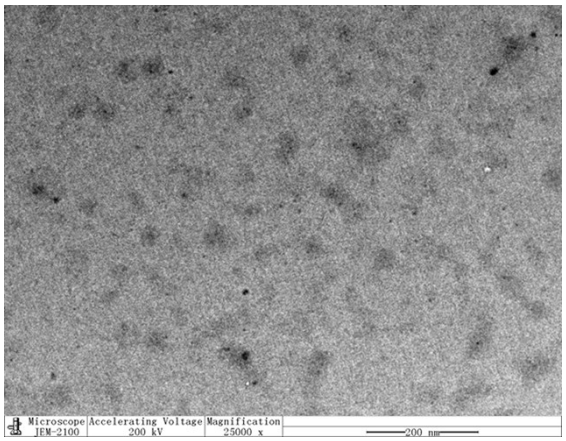



**Fig. S2.** TEM image of the target-controlled gating liposome (TCGL) after incubation with PLD.

The TCGL encapsulation efficiency (EE) and the glucoamylase loading capacity per TCGL are important parameters for the sensing assay. The former was estimated by using the ratio of the glucoamylase amount encapsulated in the TCGL to the glucoamylase amount added in the preparation of the TCGL. The latter was calculated according to the experimental data and the theoretical calculations by reference to previous literature reports.[6] Due to the hydrolysis of amylose by glucoamylase to produce glucose for quantitative readout by the PGM, the PGM signal was thus used to evaluate the glucoamylase. The amount of encapsulated glucoamylase in the TCGL was determined by using the glucoamylase calibration curve, which was obtained by measuring the PGM signal of the standard glucoamylase solutions. The TCGL was decomposed with PLD (**Fig. S2)**. The encapsulation efficiency was calculated to be 46.7% for TCGL prepared. According to the theoretical calculations, we also calculated the amount of encapsulated glucoamylase in the TCGL. Here the volume of glucoamylase is crucial to calculate the loading capacity of glucoamylase molecules per TCGL. In order to estimate the volume of glucoamylase, we assumed that the glucoamylase are spherical in shape. The structure of glucoamylase was obtained from the crystal structural data 1LF6 of the Protein Data Bank (PDB).[7] Based on this structure, the diameter of glucoamylase is measured to be

approximately ~8.0 nm. Meanwhile, considering the average volume of a single liposome is $1.07 \times 10^{-12}$ μL, the loading capacity was calculated to be ~$4.0 \times 10^3$ glucoamylase molecules/liposome for TCGL prepared. The results are consistent with the data obtained on the basis of the calculation using the data obtained experimentally (**Fig. S3B)**, which indicate that the TCGL prepared in this work have a high loading efficiency.

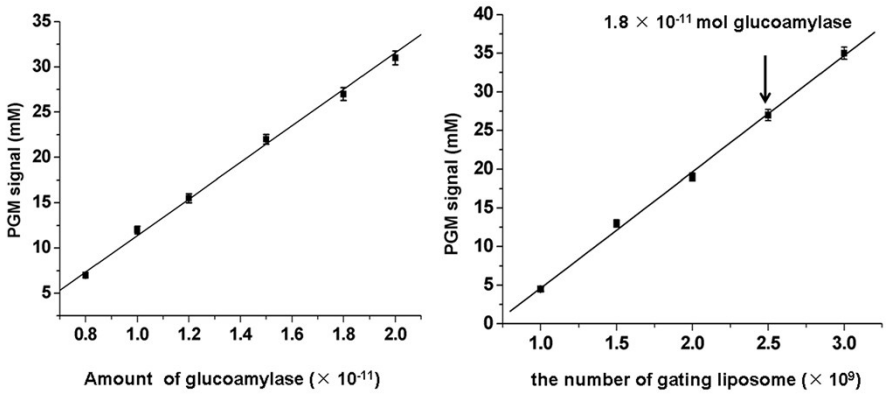



**Fig. S3.** PGM signal intensity *vs* glucoamylase amount (A) and the number of gating liposome (B). The experiments were carried out in 15 μL of substrate amylose (2.5 μg/μL).

The TCGL stability during storage was studied by monitoring the PGM signal of glucose increase at a definite time point. The PGM signal stems from what the release of glucoamylase catalyzes the hydrolysis of amylose to produce. The percent release of glucoamylase was determined by using the following equation.

$$\textbf{Percent release} = \frac{I - I_0}{I_{max} - I_0} \times 100\ \%$$

Where $I_0$ is the initial PGM signal, $I$ and $I_{max}$ is the PGM signal at a definite time point and after disrupting the TCGL in the presence of PLD, respectively. The leakage percentages were determined to be 0.1% after storage for 15 days at 4 °C **(Fig. S4)**. The result indicated acceptable stability of the synthesized TCGL. Furthermore, we detected the concentration of target PLD 1000 U/L using the TCGL stored at different time periods. Compared with the fresh TCGL made, the relative error of the relative PGM signal intensity $(I - I_0)$ was 1.4% after 20 days **(Fig. S5)**. This indicated that the TCGL were stable for 20 days. When the stored time of TCGL were for a

longer time, the leakage of the encapsulated glucoamylase molecule in TCGL was severe. Thus, to guarantee the zero-background, the TCGL could be used in 15 days.

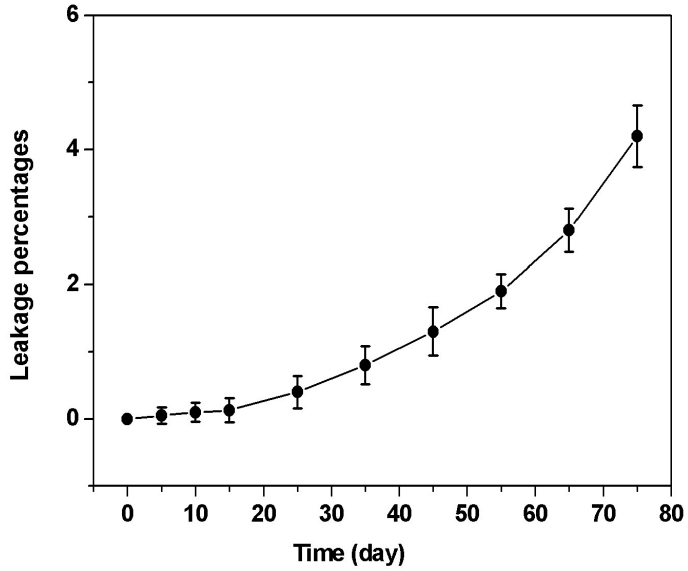



**Fig. S4**. Leakage percent of the target-controlled gating liposome (TCGL) at different storage day (0 - 80 days).

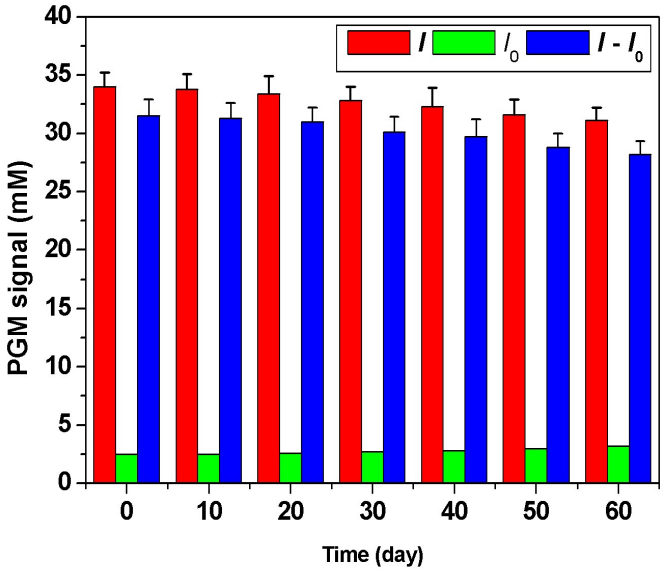



**Fig. S5**. PGM signal intensity for detection of target PLD 1000 U/L using the target-controlled gating liposome (TCGL) stored for different time periods by PGM. Experimental conditions: 20 μL of 1× TCGL solutions, 15 μL of substrate amylose (2.5 μg/μL).

In addition, the glucoamylase activity encapsulated was also investigated

because the glucoamylase activity encapsulated in the TCGL is directly related to the sensitivity of the proposed TCGL-PGM sensing assay. Here we compared completely the released glucoamylase in the TCGL with that in free solution with the same experimental condition **(Fig. S6)**. According to the previous literature,[8] a $KI/I_2$ solution was used to monitor the amylose breakdown. It was confirmed that the glucoamylase encapsulated in the TCGL has similar reactivity to that in free solution.

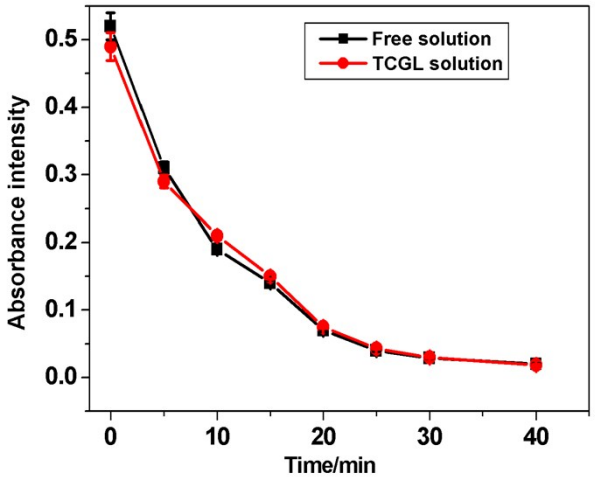



**Fig. S6**. Comparison of glucoamylase activity in free solution and TCGL solution after complete release by monitoring the absorbance at 547 nm of the amylose-$KI/I_2$ solution.

**Optimization of the designed TCGL-PGM sensing assay for PLD activity**

In the proposed sensing system, the release of glucoamylase encapsulated in TCGL plays important roles. To efficiently and rapidly release glucoamylase encapsulated in TCGL by PLD, the concentration of $Ca^{2+}$ for the implementation of TCGL-PGM sensing system was optimized due to the catalytic activity of the PLD is highly dependent on the presence of $Ca^{2+}$. Activated by $Ca^{2+}$, PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA). **Fig. S7A** shows the response of $Ca^{2+}$ concentration to the PGM signal intensity in the TCGL-PGM sensing system for PLD. It is clear that the PGM signal response increases substantially when the $Ca^{2+}$ concentration changes from 0 to 15 mM. No significant increase in the sensor response occurred from 7.5 to 15 mM. As a result, the optimal concentration of $Ca^{2+}$ for the assay was selected as 7.5 mM in subsequent studies.

To further verify the signal mechanism of the proposed sensing system, a time-course experiment was conducted. The TCGL solutions were incubated into a mixture containing 1000 U $L^{-1}$ PLD and 2.5 μg/μL substrate amylase in Tris-HCl buffer (pH 8.0, 100 mM Tris, 75 mM $CaCl_2$) at 37 °C for a certain period of time ranging from 5 to 40 min. After that, the PGM signal was detected using a mini commercial PGM and plotted against the time of incubation. As shown in **Fig. S7B**, the PGM signal increased with a longer time of incubation before reaching saturation after 20 min. At first, a weak PGM signal was observed because the amount of released glucoamylase molecules was low. Subsequently, an immediate rise in PGM signal intensity was observed, signifying the implemention of the hydrolysis of amylose to glucose after quick cleavage of the TCGL and release of glucoamylase molecules. After 20 min, the PGM signal intensity increased to a platform due to the depletion of the reaction components, such as glucoamylase activity or amylose. These results further confirm that the designed sensing method occurred as expected and the detection of PLD can be accomplished within 20 min.

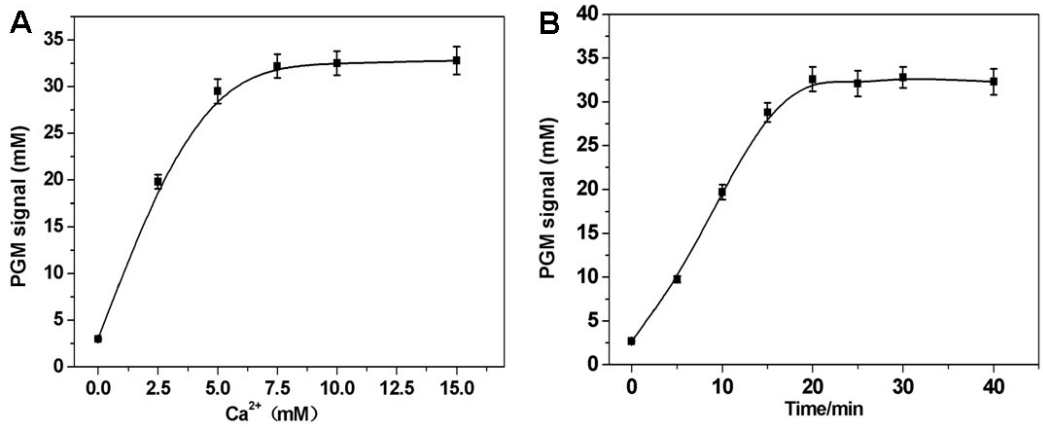
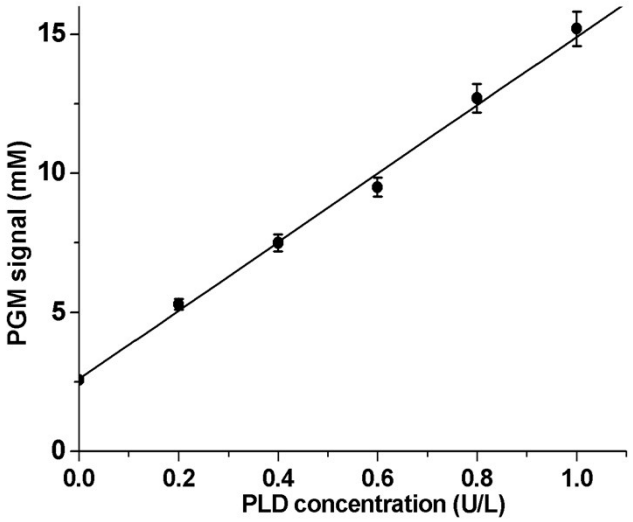
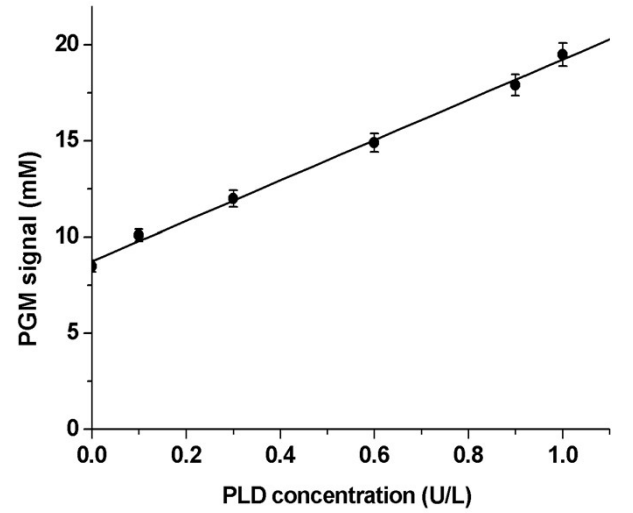



**Fig. S7**. (A) $Ca^{2+}$ concentration for the implementation of the proposed TCGL-PGM sensing assay for PLD. (B) Variance of PGM signals intensity with the reaction time of the proposed sensing assay for PLD. Conditions: the concentration of PLD used is 1000U/L. Error bars show the standard deviation of three experiments.

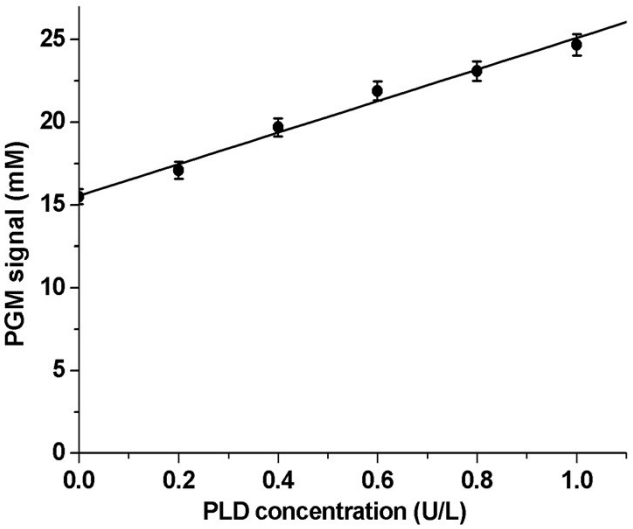
**Analysis of Real Samples**




**(A)**




**(B)**




**(C)**

**Fig. S8.** PGM signal intensity of the proposed TCGL-PGM sensing system toward

PLD (0 - 1.0 U/L) spiked in (A) MCF-10A, (B) MCF-7, and (C) MDA-MB-231 cell lysates. All experiments were performed in Tris-HCl buffer solution (pH 8.0, 100 mM Tris, 75 mM $CaCl_2$). Error bars represent the standard deviation from three repetitive experiments.

**Table S2. Determination of the Concentrations of PLD in Cell Extracts by the Commercial Amplex Red PLD Assay Kit and the proposed TCGL-PGM method.**

| Cell line[a] | spiked [PLD] (U/L) | TCGL-PGM method [mean ± SD ( U/L , n= 5)] | Commercial Amplex Red PLD assay kit [ mean ± SD (U/L, n= 5)] | Student's t-test values between the two approaches[b] |
|---|---|---|---|---|
| MCF-10A | 0 – 1.0 | 399.4 ± 29.7 | 422.6 ± 31.8 | 1.21 |
| MCF-7 | 0 – 1.0 | 1524 ± 107.6 | 1620 ± 115.6 | 1.36 |
| MDA-MB-231 | 0 – 1.0 | 2987.2 ± 212.5 | 3159.4 ± 232.5 | 1.56 |

[a] Concentration of cell lines is $1 \times 10^7$ cells/mL.

[b] The Student's t-test value is 2.306 at the 95% confidence level

## Inhibitory Assays of PLD

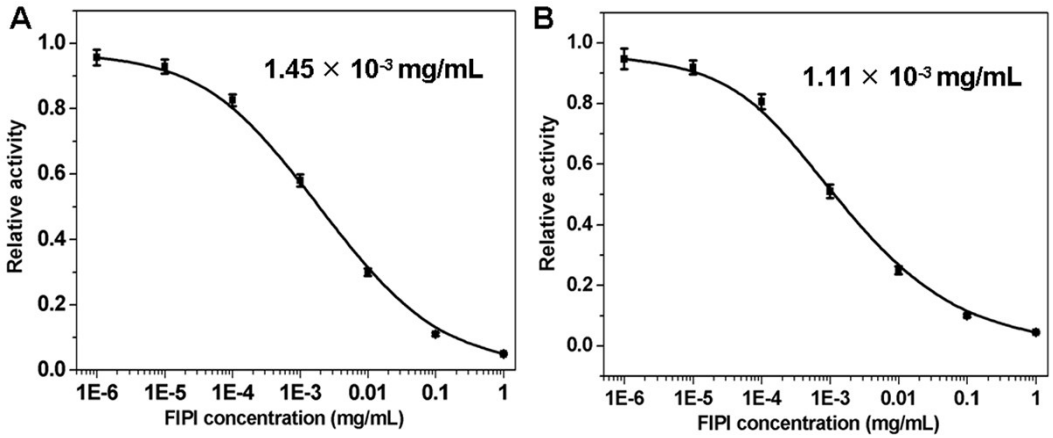



**Fig. S9** Dose-dependent inhibition curve of PLD: **(A)** the proposed TCGL-PGM sensing method, **(B)** the commercial Amplex Red PLD assay. Conditions: 400 U/L PLD and different FIPI concentrations ($10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$, 0.1, and 1 mg/mL). Error bars represent the standard deviation from three repetitive experiments.

## References

(1) Leekumjorn, S.; Sum, A. K. *Biophys J* 2006, *90*, 3951-3965.

1. M. P. Bui, S. Ahmed and A. Abbas, *Nano letters*, 2015, 15, 6239-6246.
2. E. Kim, J. Yang, J. Park, S. Kim, N. H. Kim, J. I. Yook, J. S. Suh, S. Haam and Y. M. Huh, *ACS nano*, 2012, 6, 8525-8535.
3. S. Leekumjorn and A. K. Sum, *Biophysical journal*, 2006, 90, 3951-3965.
4. K. A. Edwards and A. J. Baeumner, *Analytical chemistry*, 2007, 79, 1806-1815.
5. Y. Zhao, D. Du and Y. Lin, *Biosensors & bioelectronics*, 2015, 72, 348-354.
6. H. Qi, X. Qiu, D. Xie, C. Ling, Q. Gao and C. Zhang, *Analytical chemistry*, 2013, 85, 3886-3894.
7. A. E. Aleshin, P. H. Feng, R. B. Honzatko and P. J. Reilly, *Journal of molecular biology*, 2003, 327, 61-73.
8. L. Yan, Z. Zhu, Y. Zou, Y. Huang, D. Liu, S. Jia, D. Xu, M. Wu, Y. Zhou, S. Zhou and C. J. Yang, *Journal of the American Chemical Society*, 2013, 135, 3748-3751.