

Superior Fluorescent Probe for Detection of Cardiolipin

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S Supporting Information

ABSTRACT: Cardiolipin (CL) is a unique phospholipid found in mitochondrial inner membrane. It is a key component for mitochondrial function in both respiration and apoptosis. The level of CL is an important parameter for investigating these intracellular events and is a critical indicator of a number of diseases associated with mitochondrial respiratory functions. 10-Nonyl acridine orange (NAO) is the only fluorescent dye currently available for CL detection. However, the performance of NAO is far from satisfactory in terms of selectivity and sensitivity. In this work, we report an aggregation-induced emission-active fluorogen, TTAPE-Me, for CL detection and quantification. With improved sensitivity and excellent selectivity to CL over other major mitochondrial membrane lipids, TTAPE-Me could serve as a valuable fluorescent sensor for CL quantification. The use of TTAPE-Me for the quantification of isolated mitochondria is also demonstrated.



Eukaryotic cells use ~5% of their genes to synthesize lipids.¹ Such heavy portion is invested because of the indispensable functions of lipids in cells. With their unique structures, lipids form bilayers to segregate the internal constituents from the extracellular environment as well as to compartmentalize discrete organelles.¹ In addition to their barrier function, lipids are also used for energy storage in lipid droplets and as messengers in signal transduction and molecular recognition processes.^{1,2}

Cardiolipin (CL) is a diphosphatidylglycerol lipid exclusively found in the mitochondrial inner membrane.³ This unique lipid consists of four unsaturated acyl chains and a polar head with two negative charges (Figure 1). CL regulates enzymatic activities involved in electron transport and oxidative phosphorylation.⁴ An interaction of CL with cytochrome *c* (cyt *c*) activates the peroxidase activity of the protein and triggers the mitochondria-mediated apoptosis.^{3,5–12} During apoptosis, the distribution of CL changes, which consequently affects ATP synthesis in mitochondria.¹¹ Meanwhile, the level of CL is decreasing during apoptosis, in a time-dependent manner correlated with the release of cyt *c* to the cytosol and the generation of reactive oxygen species.^{13–15} In addition to its important role in the apoptosis pathway, the level of CL is also

of clinical significance. The depletion of CL is a critical indicator of aging, Barth syndrome, and a number of diseases associated with mitochondrial respiratory function including heart ischemia, reperfusion, gliomas, and cardiac hypertrophy and failure.¹⁶ Tangier disease, on the other hand, is caused by the enhanced production of CL.¹⁷ It has also been reported that Parkinson's disease, HIV-1, and cancers are associated with the abnormalities of CL.¹⁴ Therefore, it is highly important to develop effective methods for the detection and quantification of CL.

However, specific detection of CL among numerous phospholipids is not trivial. Lipidomics profiling by high-resolution LC–MS has recently been developed for quantitative analysis of CL.¹⁸ This powerful method requires sophisticated instrumentation and experienced operators, which limit the scope of its application. Optical detection by fluorescence, on the other hand, is a relatively simple and accessible method while providing superior sensitivity.¹⁹ A

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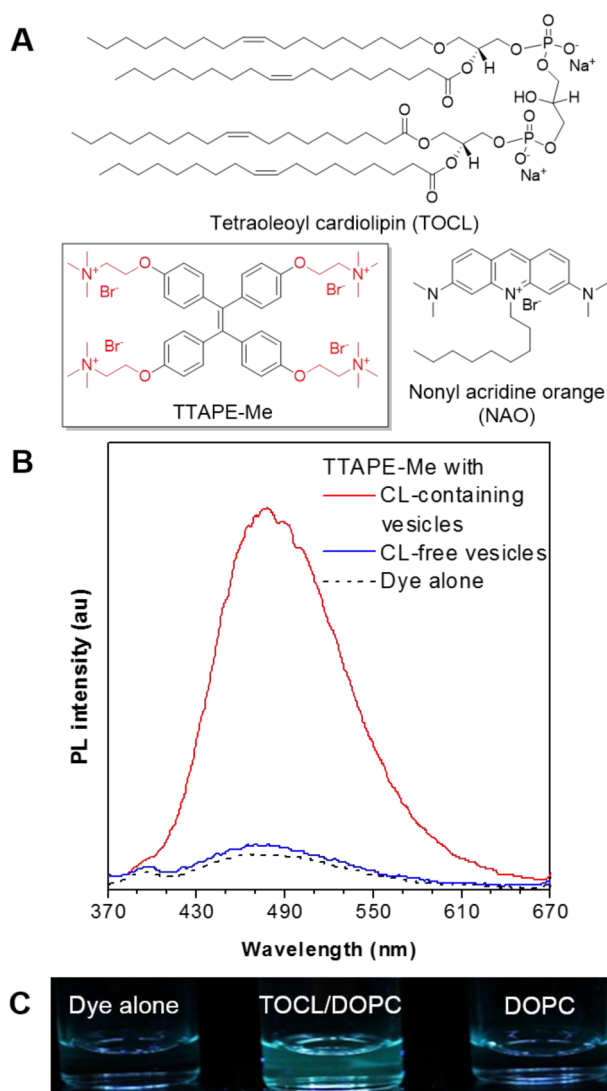


Figure 1. (A) Chemical structures of TOCL, TTAPE-Me, and NAO. (B) Emission spectra and (C) the corresponding fluorescence photographs of TTAPE-Me solutions in the absence and presence of vesicles, with and without CL. CL-containing and CL-free vesicles are composed of TOCL/DOPC (1:1 molar ratio, CL-containing) and pure DOPC (CL-free), respectively, in a 25 mM HEPES buffer at pH 7.4. [dye] = 10 μ M; [lipid]_{total} = 22 μ M; λ_{ex} = 350 nm.

fluorescent dye, 10-*N*-nonyl acridine orange (NAO, Figure 1), was introduced for CL detection and mitochondria staining in the early 1980s.²⁰ The green fluorescence of NAO is decreased in the presence of CL. The quantification of CL by NAO, however, is not realistic because both the excitation and emission maxima are dependent on the dye concentration; the linear relationship can be established only when the NAO/CL molar ratio is equal to 2.²¹ To quantify mitochondria with NAO, tortuous steps are involved, including mitochondria fixation, long time incubation, and centrifugation.²⁰ Furthermore, NAO suffers from small Stoke's shift and poor water solubility, making it less appealing to be used in biological systems. The working mechanism of NAO is still not clear, and the performance is difficult to improve even through different approaches.^{20–23} Although NAO has numerous drawbacks mentioned above, it has been used for so many years, even without a standard protocol, because no alternative has been developed so far.

In our search of alternatives, luminogens with aggregation-induced emission (AIE) characteristics have attracted our attention.^{24–26} Opposite to conventional dyes, the AIE luminogens are nonemissive when molecularly dissolved but become highly fluorescent in the aggregate state owing to the restriction of their intramolecular motions.^{27–31} Inspired by the specific interaction of cyt *c* with CL, in this work, we have designed and synthesized (Supporting Information Scheme S1) a positively charged AIE fluorogen, 1,1,2,2-tetrakis[4-(2-trimethylammonioethoxy)phenyl]ethene tetrabromide (TTAPE-Me, Figure 1) for CL detection.³² This dye turns on its fluorescence upon binding to CL-containing membranes, which may enable the detection and quantification of CL.

EXPERIMENTAL DETAILS

Materials and Methods. THF (Labscan) was purified by simple distillation from sodium benzophenone ketyl under nitrogen immediately before use. Trimethylamine was purchased from Aldrich and used as received. BSPOTPE were prepared according to our previously published procedures.³³ Synthetic lipids, DOPC (1,2-dioleoyl-*sn*-glycero-4-phosphocholine), TOCL (1,1',2,2'-tetraoleoyl cardiolipin), DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-*L*-serine (sodium salt)), DPPE (1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine), and soy PI (*L*- α -phosphatidylinositol (soy) (sodium salt)), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). SM (*N*-hexanoyl-D-sphingomyelin) was purchased from Sigma. Acridine orange 10-nonyl bromide (nonyl acridine orange) was purchased from Molecular Probes, Invitrogen. pUC 18 DNA, plasmids, was purchased from Takara Bio Inc. Water was purified by a Millipore filtration system. All the experiments were performed at room temperature unless otherwise specified. ¹H and ¹³C NMR spectra were measured on a Bruker ARX 300 spectrometer in D₂O. Mass spectra were recorded on a Finnigan TSQ 7000 triple quadrupole spectrometer operating in fast-atom bombardment (FAB) mode or a GCT Premier CAB 048 mass spectrometer operated in MALDI-TOF mode. Steady-state fluorescence spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer with a Xenon discharge lamp excitation. Particle size analysis was determined at room temperature by a ζ -potential analyzer (ZetaPALS, ζ -potential analyzer utilizing phase analysis light scattering; Brookhaven Instruments Corporation, U.S.A.).

Preparation of Liposomes (Large Unilamellar Vesicles, LUVs). Chloroform stocks of different lipids (10 mg/mL) were mixed in a desired molar ratio and dried under a stream of nitrogen gas. The lipid film was hydrated in a 25 mM HEPES buffer, pH 7.4, to a final lipid concentration of 2.2 mM. The lipids mixtures were incubated at 37 °C for 30 min, followed by bath sonication for 1 h. The LUVs were obtained by extruding 11 times through 100 nm pore size polycarbonate filters (SPI-Pore) at 50 °C on a prewarmed lipid extruder.

Isolation of Yeast Mitochondria. Wild-type *Saccharomyces cerevisiae* (YPH 500) cells were cultured in yeast extract peptone dextrose medium (YPD; 2% bacto-peptone, 1% yeast extract, and 2% glucose) to an OD of 2.0, and mitochondria were isolated according to the mitochondria preparation protocol by Meisinger et al.³⁴ YPH 500 cells were harvested by centrifugation (5 min at 3000g) and resuspended in prewarmed DTT buffer (100 mM H₂SO₄, 10 mM DTT, pH 9.4) at 30 °C for 20 min. The cells pellets were resuspended and incubated with 2.5 mg of Zymolyase-20T per gram of wet cell paste in zymolyase buffer (1.2 M sorbitol, 20 mM

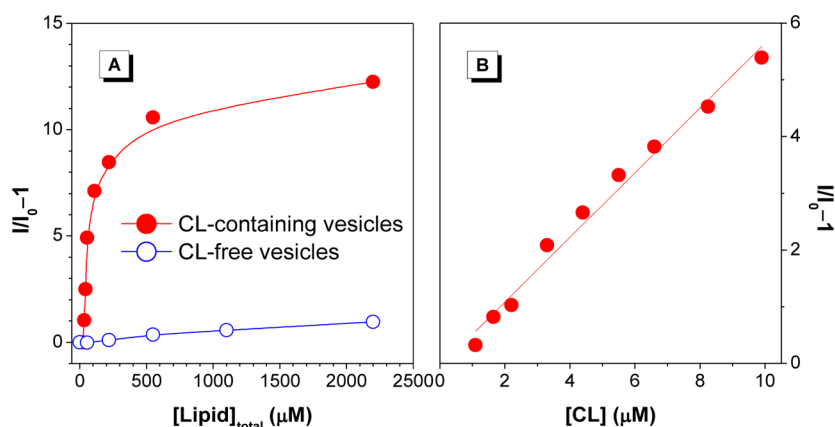


Figure 2. (A) Plot of the fluorescence enhancement ($I/I_0 - 1$) of TTAPE-Me at 480 nm with CL-containing and CL-free vesicles. (B) Linear region of the $I/I_0 - 1$ value vs CL concentration. $[\text{dye}] = 10 \mu\text{M}$; $\lambda_{\text{ex}} = 350 \text{ nm}$.

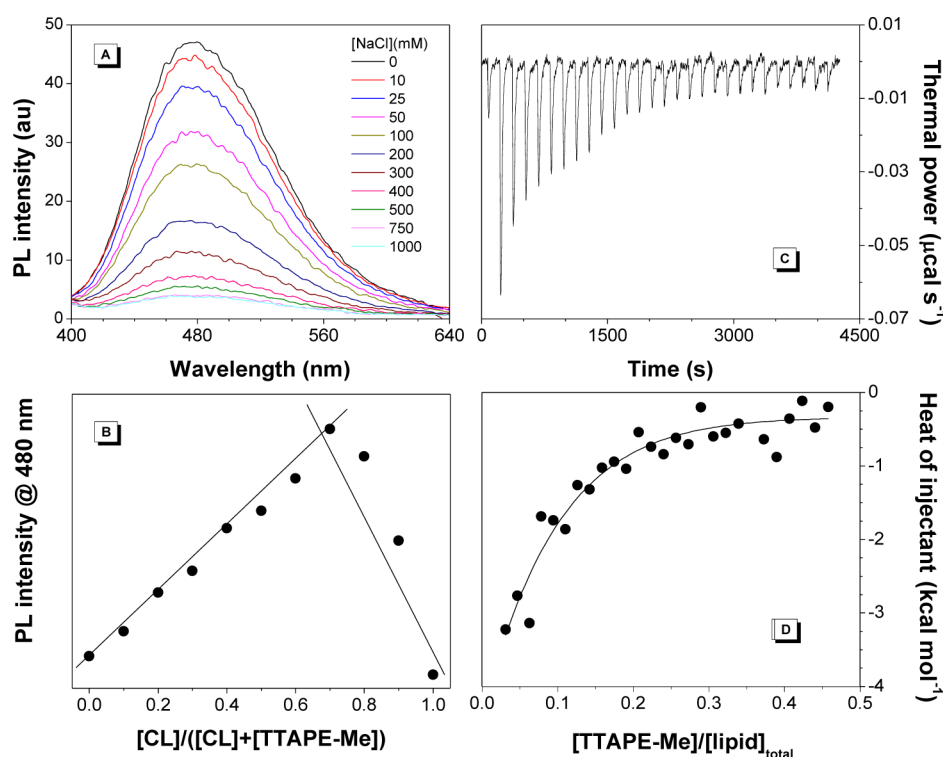


Figure 3. (A) Emission spectra of TTAPE-Me with CL-containing vesicles in the presence of varying concentrations of NaCl. $[\text{dye}] = 10 \mu\text{M}$; $[\text{lipid}]_{\text{total}} = 22 \mu\text{M}$; $\lambda_{\text{ex}} = 350 \text{ nm}$. (B) Job plot for determination of the binding stoichiometry of TTAPE-Me to CL-containing vesicles. The total concentration of TTAPE-Me and CL is kept at $20 \mu\text{M}$. (C) Calorimetric curves at 25°C for titration of CL-containing vesicles with serial injections of TTAPE-Me. (D) Binding isotherm as a function of $[\text{TTAPE-Me}]/[\text{lipid}]_{\text{total}}$ molar ratio. CL-containing vesicles are composed of TOCL and DOPC (1:1 molar ratio).

potassium phosphate, pH 7.4) for 1 h under shaking at room temperature. Homogenization of spheroblasts was performed by 15 strokes on ice with 40 mL tight glass douncer (Kontes) in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.2% BSA). Mitochondria were finally resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) at final concentration of $\sim 5 \text{ mg/mL}$. The protein content was determined by a biuret procedure using bovine serum albumin as a standard.³⁵

Isothermal Titration Calorimetry Measurements. Calorimetric titration experiments were performed at $25.00 \pm 0.01^\circ\text{C}$ on a MicroCal VP-ITC apparatus. Lipid vesicle solutions for

the ITC experiments were prepared in 25 mM HEPES buffer solutions at pH 7.4. For a typical titration, a series of $5 \mu\text{L}$ aliquots of TTAPE-Me solution were injected into the lipid vesicle solution at a 150 s intervals. The heat for each injection was determined by the integration of the peak area in the thermogram with respect to time. Blank titration was conducted by injecting TTAPE-Me into the sample cell containing only buffer solution under the same conditions. The interaction heat was corrected by subtracting the blank heat from that for the TTAPE/lipid vesicle titration. The binding constants were derived by fitting the isotherm curves with Origin 7.0 software.

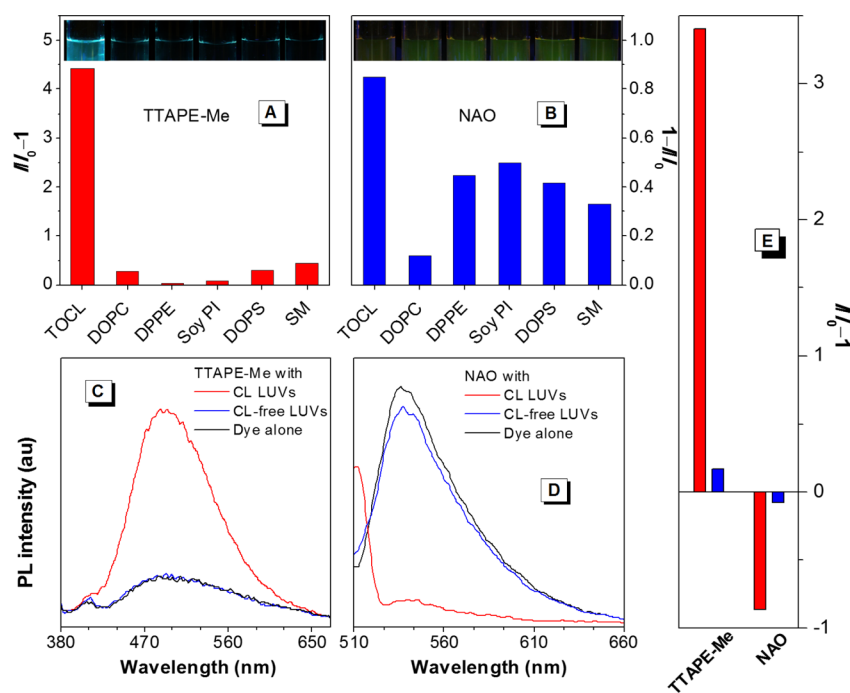


Figure 4. (A and B) Change in fluorescence intensity of (A) TTAPE-Me and (B) NAO with vesicles of different lipid contents (left to right: 20% TOCL, 100% DOPC, 40% DPPE, 2% soy PI, 1% DOPS, and 2% SM; the rest of each type of vesicle is filled by DOPC). Insets: Corresponding fluorescence photographs of TTAPE-Me and NAO solutions in the presence of these LUVs. (C and D) Emission spectra of (C) TTAPE-Me and (D) NAO with CL-containing and CL-free all-component vesicles. (E) Bar chart of the change in fluorescence intensity shown in panels C and D (red, CL-containing vesicles; blue, CL-free vesicles). [dye] = 10 μ M; [lipid]_{total} = 22 μ M; for TTAPE-Me, λ_{ex} = 350 nm, λ_{em} = 480 nm; for NAO, λ_{ex} = 499 nm, λ_{em} = 530 nm.

RESULTS AND DISCUSSION

With the aid of the quaternary ammonium substituents, TTAPE-Me is completely soluble and thus nonfluorescent in aqueous solution, in accord with the general property of AIE luminogens.²⁴ To determine whether TTAPE-Me can specifically detect CL, we prepare two types of large unilamellar vesicles (LUVs, 100–200 nm in diameter, Supporting Information Figure S1). The CL-free vesicles were prepared by pure 1,2-dioleoyl-*sn*-glycero-4-phosphocholine (DOPC, Supporting Information Chart S1), which is the most abundant phospholipid in eukaryotic membranes. CL-containing vesicles were fabricated by the mixture of 1,1',2,2'-tetraoleoyl cardiolipin (TOCL, Figure 1) and DOPC, in which the zwitterionic DOPC is used to stabilize the vesicles. As shown in Figure 1, the emission of TTAPE-Me is turned on in the presence of CL-containing vesicles.

The fluorescence of TTAPE-Me increases dramatically upon the increase of the total lipid concentration of the CL-containing vesicles, while with the CL-free vesicles, the emission of TTAPE-Me remains rather weak (Figure 2A). The fluorescence enhancement ($I/I_0 - 1$) of TTAPE-Me at 480 nm is in a linear fashion in the CL concentration of 0–10 μ M (Figure 2B), which lies in the physiological range of CL in mitochondrial membrane.²¹ Linear detection of CL can also be obtained with the varying content of TOCL in the vesicles (Supporting Information Figure S2, 2–50% TOCL). The results imply that flexible TTAPE-Me to CL ratio is allowed in the detection and quantification of CL, in contrast to the conventional NAO which requires strict 2:1 ratio of NAO/CL for quantitative measurement. Moreover, the detection of CL can be done immediately upon mixing the vesicles with the probe without any extra treatment.

TTAPE-Me is amphiphilic with the hydrophobic core of tetraphenylethene (TPE) and four quaternized ammonium moieties to promote its water affinity. Initially, we speculate that both hydrophobic and electrostatic interactions might contribute to the interaction of TTAPE-Me and CL. As Figure 3A shows ionic strength indeed affects the fluorescence intensity of TTAPE-Me. With the increase of NaCl concentration, the fluorescence of the dye diminishes, which confirms that TTAPE-Me binds to CL via electrostatic attraction. The Na⁺ ions compete with the bound dye molecules. Once the dye is released into solution, the intramolecular motions are no longer restricted and the fluorescence is turned off. Because both CL and DOPC contain long alkyl chains, whereas TTAPE-Me binds to CL only, the role of hydrophobic interactions is less significant. Furthermore, there is no any significant changes in fluorescence intensity of TTAPE-Me even after one and a half hours of incubation with CL-containing LUVs (Supporting Information Figure S3). As a control, another water-soluble AIE luminogen, BSPOTPE, with two negative charges, is used, which exhibits no remarkable fluorescence enhancement with CL-containing vesicles (Supporting Information Figure S4).

According to the AIE principle,²⁵ only the bound TTAPE-Me is fluorescent, and thus the fluorescence can report the binding of TTAPE-Me to CL-containing vesicles. The emission intensity at varying ratios of TTAPE-Me to CL-containing LUVs is then recorded and correlated as shown in Figure 3B. The Job plot has a peak at ~ 0.67 , corresponding to a 2:1 binding ratio for TOCL to TTAPE-Me. The binding ratio perfectly matches their charge ratio, further supporting that the binding of TTAPE-Me toward CL is primarily driven by electrostatic interactions. To determine how strong the affinity

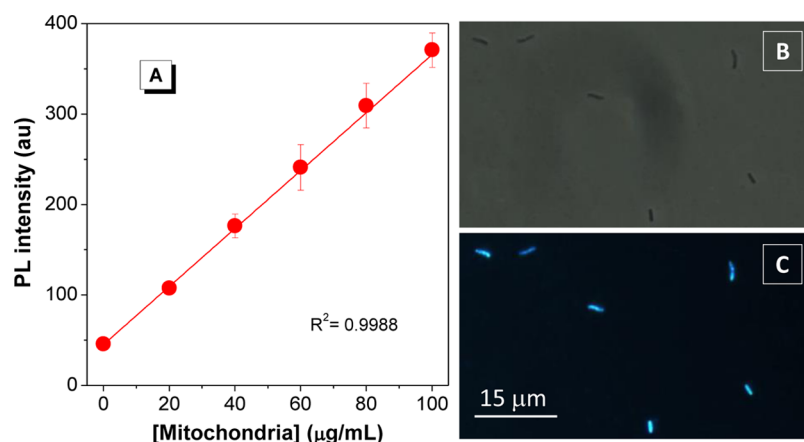


Figure 5. (A) Emission intensity of TTAPE-Me at 480 nm with different amounts of yeast mitochondria in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). Images of TTAPE-Me stained yeast mitochondria were taken (B) under daylight and (C) with UV illumination. [dye] = 10 μ M; λ_{ex} = 350 nm.

is, we have employed isothermal titration calorimetry (ITC). The result in Figure 3C indicates that the interaction of TTAPE-Me to CL is an exothermic process. Integration of the area of each injection peak followed by the subtraction of the dilution heat of dye molecules generated the binding curve (Figure 3D). Fitting of the curve gave a dissociation constant of 2.08×10^{-6} M.

To further evaluate the specificity of TTAPE-Me toward CL, we have examined the response of TTAPE-Me to other major lipids found on mitochondrial membranes: 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), *L*- α -phosphatidylinositol (soy PI), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), and *N*-hexanoyl-D-sphingomyelin (SM) (Supporting Information Chart S1).³² Six different types of LUVs composed of each of the above lipids as well as TOCL and DOPC at the exact percentage as mitochondrial membrane were fabricated (the rest are filled by DOPC). As can be seen in Figure 4A, the fluorescence of TTAPE-Me is selectively turned on with the TOCL vesicles, while other vesicles with the lipid components at their physiological concentrations do not cause any pronounced change of the fluorescence. Whereas DOPS and PI are also negatively charged, they carry only one charge per molecule and share only 1% and 2% of the total mitochondrial membrane lipids, respectively (CL for ~20%).³⁶ Hence, the presence of such small percentage of DOPS and soy PI does not affect the selectivity and sensitivity of TTAPE-Me for CL detection. Photographs taken under UV illumination clearly show that only TOCL-containing vesicles can turn on the fluorescence of TTAPE-Me (Figure 4A inset). On the other hand, one might think that the negatively charged mitochondrial DNA (mtDNA) may interfere with the detection of CL by TTAPE-Me. However, in our control experiment, we do not observe any fluorescence enhancement of TTAPE-Me in the presence of plasmids, a model for the circular double-stranded mtDNA, at a wide range of concentrations (Supporting Information Figure S5), implying the presence of mtDNA would not complicate the CL quantification. The specificity of TTAPE-Me toward CL has also been further investigated by using other charged biomolecules. As shown in Supporting Information Figure S6, the selectivity is not interfered by other charged biomolecules such as CL-free LUVs, charged amino acids, and proteins, at the same concentrations.

Meanwhile, to mimic the mitochondrial membranes, we have prepared CL-containing and CL-free all-component LUVs,

which are composed by the mixture of all the above-mentioned lipids at their physiological ratios (Supporting Information Figure S7). With the CL-free all-component LUVs, the emission spectrum of TTAPE-Me remains identical to that of the free dye in buffer, while with CL-containing LUVs, the greenish-blue fluorescence is enhanced by over 3-fold (Figure 4, parts C and E). Parallel experiments are conducted with NAO as the probe. NAO is a turn-off sensor whose fluorescence is decreased in the presence of CL (Figure 4D). However, not only CL, other lipids such as DPPE, PI, or DOPS, can also induce the decrease of NAO signals to a large extent (Figure 4B). The decrease of the fluorescence intensity and the change in emission color is difficult to discern by naked eyes (Figure 4B inset). Thus, in addition to its poor selectivity, the sensitivity of NAO is not comparable to that of TTAPE-Me (Figure 4E).²¹

In addition to CL detection, we demonstrate the utility of TTAPE-Me for mitochondria quantification. Individual mitochondria from *S. cerevisiae* strain YPH 500 have been isolated and calibrated by biuret test.²² As shown in Figure 5A, the emission of TTAPE-Me increases linearly with the increase of mitochondria concentration. Isolated mitochondria can be clearly visualized under fluorescence microscope with TTAPE-Me as the stain (Figure 5, parts B and C). These results show that by using TTAPE-Me, quantification of mitochondria can be accomplished in a simple and easy manner with high sensitivity and low background noise. In contrast, time-consuming and intricate procedures are involved when using NAO for the quantification of isolated mitochondria.²² The isolated mitochondria are fixed by formaldehyde, followed by multiple rinsing, centrifugation, and resuspension steps. The signal-to-noise ratio of NAO-based detection is much smaller probably due to the strong background of NAO in solution.²²

CONCLUSIONS

In conclusion, a water-soluble AIE fluorogen, TTAPE-Me, has been developed for the detection and quantification of CL, a unique phospholipid in mitochondrial inner membrane. The fluorescence of TTAPE-Me is selectively turned on by CL-containing vesicles, and the intensity is proportional to the concentration or fraction of CL. As a fluorescence turn-on sensor, TTAPE-Me can be used for quantitative analysis and visualization of isolated mitochondria. Compared with NAO,

the only dye commercially available for CL sensing, TTAPE-Me provides much higher sensitivity and selectivity as well as well-defined working mechanism without any difficult or ambiguous protocols. With these advantages, TTAPE-Me could be a great alternative to NAO for specific detection and quantification of CL, which will find an array of applications in clinical diagnosis and mitochondria-related research.

■ ASSOCIATED CONTENT

■ Supporting Information

Synthetic route of TTAPE-Me, chemical structures of phospholipids, particle size analysis of LUVs, emission spectra of TTAPE-Me and BSPOTPE dyed LUVs with different CL contents, and emission spectra of TTAPE-Me with different amounts of DNA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interests.

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