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Development of a Cholesterol-Conjugated Fluorescent Sensor for Site-Specific Detection of Zinc Ion at the Plasma Membrane

Shohei Iyoshi,[†] Masayasu Taki,*[,†,‡] and Yukio Yamamoto[†]

Graduate School of Human and Environmental Studies and Graduate School of Global Environmental Studies, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan

taki.masayasu.4c@kyoto-u.ac.jp

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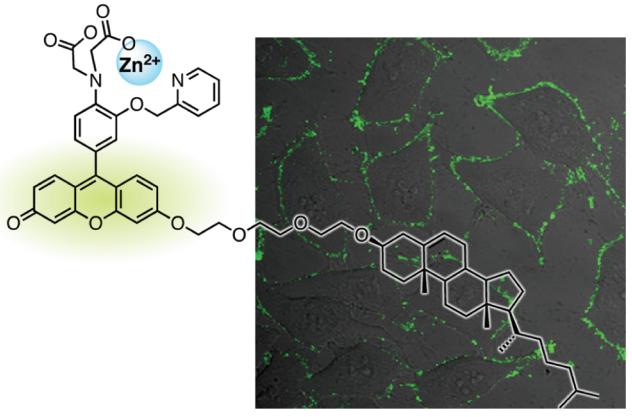
ABSTRACT



A cholesterol-conjugated fluorescence Zn^{2+} sensor based on the fluorescein platform was designed and synthesized. The cholesterol moiety is essential for localizing the Zn^{2+} sensor to the cell membrane, allowing the sensor to probe changes in the Zn^{2+} concentration in a localized area of the cell.

Zinc is the second most abundant heavy metal in living organisms and plays a significant role as a component of many metalloenzymes and transcription factors.[1] In recent years, the importance of zinc ions in brain function,[2] the immune system,[3] and apoptosis regulation[4] has also been revealed, and these findings have attracted the attention of many researchers. However, details of the mechanisms of zinc homeostasis have yet to be clarified. In order to understand this equilibrium between zinc uptake and efflux, chemical tools that can detect a change in zinc

[†] Graduate School of Human and Environmental Studies.
[‡] Graduate School of Global Environmental Studies.

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concentration in particular subdomains within a cell are desired.

Fluorescence imaging is the most suitable method for monitoring the local dynamics of zinc in living cells because of its high spatial resolution.[5] A straightforward approach to achieve this is to attach a fluorescent zinc sensor, which includes small organic molecules and protein-based indicators, to a target protein known to be expressed on specific compartments within the cell. For instance, Lippard et al. have reported SNAP-tag-based site-specific zinc sensors, which have a benzylguanine unit as a substrate of O^6-alkylguanine transferase (AGT).[6] They successfully carried out chemical labeling of the AGT-fused protein expressed on mitochondria or Golgi apparatuses and visualization of a change in zinc concentrations at a local intracellular region. Palmer et al. have developed genetically targeted ratiometric zinc sensors based on the fluorescence resonance energy transfer (FRET) mechanism and have monitored the releasable pool of Zn^{2+} in mitochondria.[7] Although these genetically encoded techniques are advantageous in that the sensor distributions can be controlled precisely, the localization strategies require a complex gene modification procedure, including plasmid preparation and gene transfection, to arrange the observation objects. Another strategy to localize fluorescent molecules to cellular subdomains is to use fluorescent zinc sensors with a functional group that can interact with specific organelles.[8] A labile Zn^{2+} pool within mitochondria has been monitored by using the mitochondria-specific fluorescent zinc sensor Rhodzin-3, which is based on a positively charged rhodamine fluorophore.[8a] Although this approach may lack the certainty of sensor localization within the target organelle, this method requires no gene modification and, more importantly, enables multicolor imaging of a single cell by changing the fluorescence sensor moiety and organelle-targeting group.[9] However, only a few zinc sensors based on this approach have been reported, and none have yet been reported for the plasma membrane.

In this context, we designed and synthesized a new small-molecule-based fluorescent zinc sensor, LF-Chol (Figure 1). Because cholesterol has been known to preferentially interact with a subset of membrane lipids,[10] we expected that the conjugated fluorescent zinc sensor moiety of LF-Chol would localize specifically to the cell membrane. We chose an APTRA (*o*-aminophenol-*N*,*N*,

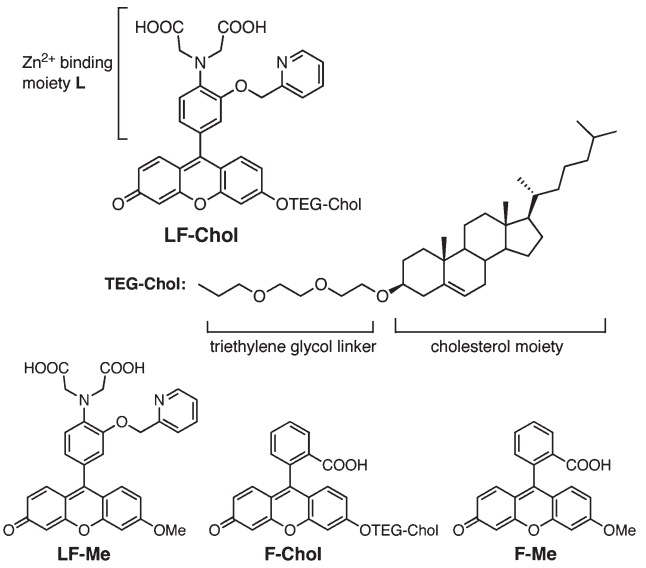

Figure 1. Molecular design of **LF-Chol** and its reference compounds, **LF-Me**, **F-chol**, and **F-Me**.

O-triacetic acid)-based zinc-chelating structure in which one acetic acid ligand is substituted with a 2-pyridylmethyl group (**L** in Figure 1), because this pyridine-containing chelator has sufficient selectivity and high affinity (dissociation constant K_d sub-nM) for zinc ions, as we reported previously.[11] In addition, owing to the highly hydrophilic character of the two remaining carboxyl groups of the zinc chelator, the zinc sensor moiety is expected to be located at the extracellular region in the vicinity of the plasma membrane, which may allow for detection of zinc efflux from the cell.

In order to evaluate the photophysical properties of LF-Chol as a fluorescent zinc sensor, a reference molecule LF-Me (shown in Figure 1), in which the TEG-cholesterol moiety was substituted with a simple methyl group, was initially prepared (Scheme 1). Compound **2** was prepared according to the improved procedure we reported previously.[11] It was necessary to transform the *tert*-butyl esters in compound **2** into the corresponding ethyl esters **3** because the formation of the xanthene platform as a fluorophore by Friedel–Crafts reaction would be carried out under acidic conditions. Formylation of ethyl ester **3** under Vilsmeier conditions followed by the Friedel–Crafts reaction afforded fluorescein derivative **5**, which is also used for the synthesis of LF-Chol. Methylation of the phenolic oxygen atom and subsequent hydrolysis afforded LF-Me, which was purified by semipreparative reversed-phase HPLC (H_2O/CH_3CN containing 0.1% TFA) for spectroscopic measurements.[12]

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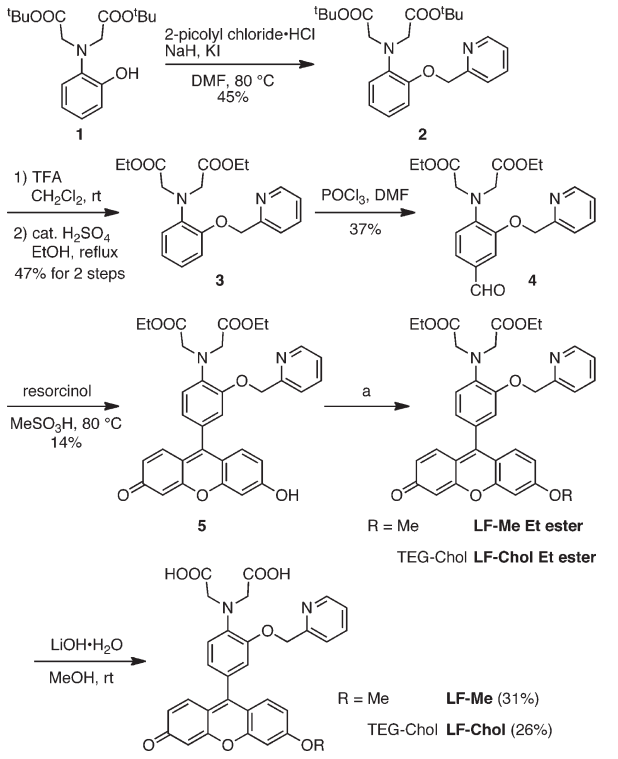
(12) Since available amounts of LF-Me and LF-Chol were too small for ^{13}C and $^{1}H/^{13}C$ NMR analyses, respectively, the overall purity of the product was confirmed by HPLC and the product was identified by mass analysis (see Supporting Information).

Scheme 1. Synthesis of LF-Me and LF-Chol



[a] For LF-Me Et ester: MeI, K_2CO_3, DMF, 60 °C, overnight, 51%. For LF-Chol Et ester: **7**, K_2CO_3, DMF, 60 °C, overnight, 41%.

Under physiological conditions (50 mM HEPES, pH 7.20, 0.1 M KNO_3), the fluorescence of LF-Me is nearly quenched ($\Phi < 0.005$) because of the photoinduced electron transfer (PET) process and is enhanced (ca. 45-fold) upon coordination with Zn^{2+} ($\Phi = 0.18$), as shown in Figure 2. A metal selectivity experiment revealed that the fluorescent response of LF-Me was highly Zn^{2+}-selective and that the existence of other biologically relevant metal ions did not disturb the Zn^{2+} sensing process (Figure S1). A 1:1 metal-to-ligand complex was confirmed by Job's plot (Figure S2), and on the basis of this finding, the dissociation constant K_d for Zn^{2+} was determined to be 126 nM by a curve-fitting analysis of the fluorescence intensity spots at 517 nm against the free Zn^{2+} concentration, $[Zn^{2+}]_{free}$, in a Zn^{2+}/NTA (NTA = nitrilotriacetic acid) buffer solution system (Figure 2 inset).[11] This K_d value reveals that the sensor is suitable for detecting $[Zn^{2+}]_{free}$ between 32 and 520 nM,[13] which is appropriate to the amount of Zn^{2+} secreted by exocytosis to the extracellular milieu.[14] These profiles of LF-Me support the view that the Zn^{2+}-sensing moiety of LF-Chol possesses sufficient ability to detect Zn^{2+} under physiological conditions.

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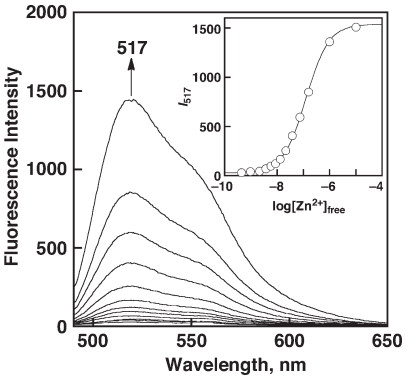

Figure 2. Emission spectra of LF-Me (5 μM) excited at 470 nm in Zn^{2+}/NTA buffered system (50 mM HEPES, pH 7.20, 0.1 M KNO_3; 10 mM NTA, 0−9.5 mM $ZnSO_4$) and in 50 mM HEPES buffer (pH 7.20) containing 4 μM $ZnSO_4$. Inset: plots of fluorescence intensities at 517 nm with best-fit curves for the dissociation constant of 1.26×10^{-7} M.

To confirm the localization ability of LF-Chol to the plasma membrane, we next conducted *in vivo* imaging experiments with a fluorescent reference compound, F-Chol (Figure 1), in which the Zn^{2+}-binding moiety **L** was replaced with a 2-carboxyphenyl group. HeLa cells stained with F-Chol exhibit strong emission from the plasma membrane region as determined by confocal scanning laser microscopy (Figure 3). This result indicates that the cholesterol moiety strongly interacts with a subset of membrane lipids, whereas the fluorescein moiety is expected to be located at the extracellular region because of the high polarity of the carboxyl group. In contrast, no fluorescence signal was detected in any area (Figure S3) when the cells were incubated in the presence of F-Me (Figure 1), which lacks the cholesterol moiety from F-Chol. This finding also supports the crucial role of cholesterol in dye localization. On the basis of the above observations, we concluded that LF-Chol is sufficiently capable of detecting extracellular Zn^{2+} in the vicinity of the plasma membrane.

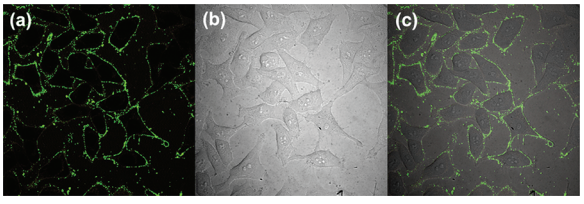

Figure 3. (a) Confocal fluorescence image of HeLa cells loaded 2.5 μM F-Chol. (b) Bright-field transmission image of the cells shown in (a). (c) Overlay of images (a) and (b).

LF-Chol was prepared by using the common compound **5** for LF-Me (Scheme 1). A coupling reaction with a cholesterol derivative **7** followed by hydrolysis of the

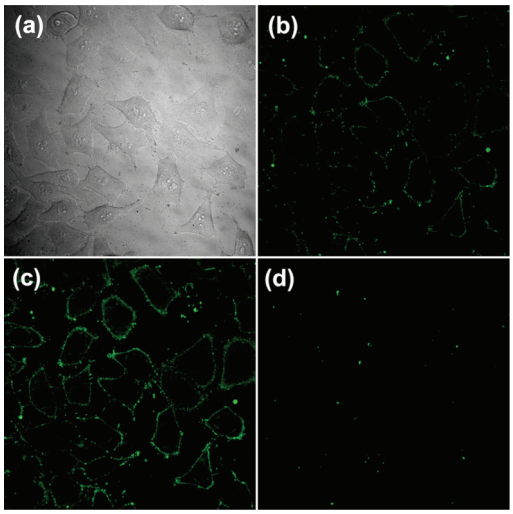

Figure 4. Live-cell fluorescence imaging of extracellular Zn^{2+} at the plasma membrane by confocal microscopy. (a) Bright-field transmission image of HeLa cells. (b) Cells stained with 2.5 μM LF-Chol. (c) Images obtained immediately after the addition of 20 μM Zn^{2+} to the same media shown in (b). (d) After addition of 100 μM EDTA to the same cells shown in (c).

ethyl esters with LiOH gave the final fluorescence sensor LF-Chol as a brown solid. The crude product was purified by ODS column chromatography. For application to *in vivo* imaging, a sample of the compound was further purified by HPLC.[12] Cells loaded with 2.5 μM of LF-Chol emitted slight but detectable fluorescence in the plasma membrane, as shown in Figure 4b. The staining pattern is similar to that observed with F-Chol, demonstrating membrane localization of this zinc sensor. The addition of Zn^{2+} (final concentration: 20 μM) to the same media yielded a fluorescence increase in the local region of the cells, indicating that the zinc-sensing moiety of LF-Chol complexed with extracellular Zn^{2+} while retaining sensor localization in the plasma membrane (Figure 4c). In fact, a decrease in emission was observed upon addition of 100 μM EDTA, which is a membrane-impermeable chelator for Zn^{2+}. This strongly supports the view that the Zn^{2+}-binding moiety of LF-Chol existed on the extracellular side and detected extracellular Zn^{2+} in a reversible manner (Figure 4d).[15]

We expect that LF-Chol can contribute to the visualization of zinc ion influx or efflux in the extracellular plasma membrane region, including zinc release from neurons and zinc secretion from pancreatic β-cells. Currently, we are working on the preparation of LF-Chol derivatives for detecting Zn^{2+} in other intracellular organelles such as the nucleus and mitochondria, which may be achieved with Hoechst derivatives and cationic functional groups, respectively. In addition, because this sensor-localization technique enables Zn^{2+} imaging without any genetic encoding techniques, it should be possible to perform multicolor imaging of local Zn^{2+} at more than one cellular compartment in a single cell by using zinc sensors with differently colored fluorophores. Such an imaging technique may help in understanding the Zn^{2+}-trafficking pathway between cell compartments.

(15) It should be noted that some bright fluorescent spots still remained in Figure 4d. Because LF-Chol becomes neutral and increases the hydrophilicity upon complexation with Zn^{2+}, a part of the metallated sensor may permeate the cell membrane.

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Supporting Information Available. Detailed description of synthetic procedure and NMR spectra of the new compounds, the results of a metal selectivity experiment and Job's plot, and fluorescence images of cells with F-Me. This material is available free of charge via the Internet at http://pubs.acs.org.