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Cell Membrane Permeable Fluorescent Ca^{2+} Probe Based on Bis-BODIPY with Branched PEG

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A new class of Ca^{2+} probes is developed based on PEG-BODIPY-BAPTA conjugates. These newly formed probes with BAPTA acid exhibit high sensitivity and selectivity for Ca^{2+} over other metal ions, can pass through the cell membranes of living cells without special procedures, and can monitor changes in intracellular Ca^{2+} signal.

Introduction

Ca^{2+} has extremely important biological functions as intracellular second messengers that simulate the regulation of many physiological functions.[1-3] Ca^{2+} fluorescent probes have become the basic technology in investigating the signalling pathways involving Ca^{2+} physiological functions, which are indispensable means for studying the spatiotemporal fluctuations of intracellular free Ca^{2+} concentration.[4]

The derivatives of Ca^{2+} chelators ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) are among the most successful fluorescent sensors that exhibit a spectral response upon binding Ca^{2+}.[5] However, the acid or salt form these probes cannot cross the cellular membranes, which is an essential requirement in allowing such molecules to be used within the cellular environment.[6] The bulk cell loading procedures are applicable to large populations of cells and include the following components: acetoxymethyl (AM) ester,[7] ATP-induced permeabilisation,[8] electroporation,[9] coupling to cell-penetrating,[10] and beads-loading methods.[11] The noninvasive and technically straightforward AM ester technique is by far the most popular method for loading fluorescent ion indicators.[12] This certain method, however, has disadvantages, including compartmentalisation, incomplete AM ester hydrolysis, and cellular leakage over time.[13-14]

We recently demonstrated that dendritic PEG-substituted dyes can be efficiently internalised by cells and accumulated in the cytoplasm.[15] We aimed membrane permeable fluorescent Ca^{2+} probes (**MPFCP**) by PEG encapsulation. 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) was selected as the fluorophore, while the 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) group was elected as the Ca^{2+} recognition site. BAPTA was employed in the experiment because of its high calcium affinity in physiological medium to be the calcium ligand in the current fluorescent probes.[5] Meanwhile, BODIPY is a well-known fluorophore characterised by useful properties, such as excellent photochemical stability, high molar absorptivity, high fluorescence quantum yield, and pH-independent fluorescence.[16] The BODIPY fluorophores have been successfully used to generate fluorescent indicators based on the photoinduced electron transfer (PET) mechanism.[17] Moreover, the wide range of excitation/emission wavelength choices available within the BODIPY fluorophore series allows several different colours of Ca^{2+} probes to be adopted in live cells.[18] Nevertheless, the acids or salts form probes based on BODIPY-BAPTA conjugates are cell-impermeable. Accordingly, the cellular uptake was enhanced by conjugating the probes with the branched polyethylene glycol groups. Two BODIPY fluorophores were also linked with one BAPTA chelator to design a bis-BODIPY probe, partly encapsulated with more PEG chains (Chart 1).

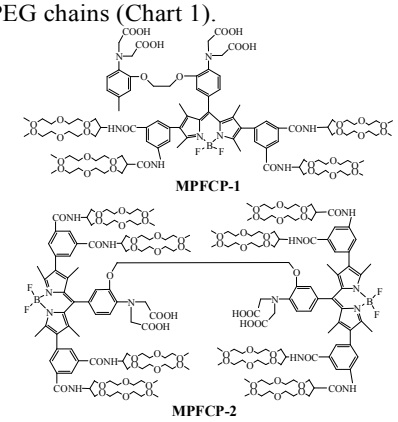
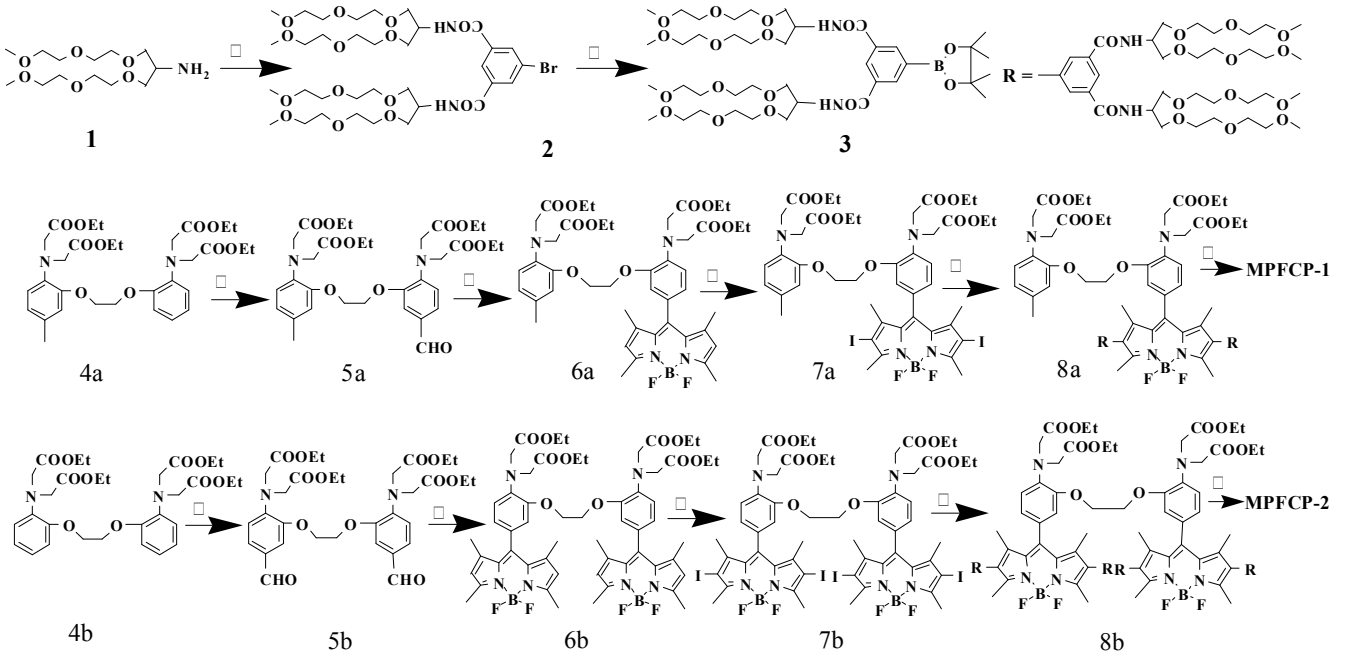


Chart 1 Chemical structures of MPFCP probes



Scheme1 (□) 5-Bromoisophthalic acid, DMF, 0 °C, EDCI, 0.5 h; compound 1, HOBt, rt, 24 h; (□) [Pd(dppf)$_2$Cl$_2$], KOAc, bis(pinacolato)diboron, DMF, 90 °C, overnight; (□) POCl$_3$, DMF, rt, 0.5 h, 45 °C, 20 h; (□) 2,4-dimethylpyrrole, TFA, anhydrous CH_2Cl_2, N_2, rt, 12 h; DDQ, N_2, 40 min; triethylamine, $BF_3{\cdot}Et_2O$, 40 min;(□) iodine monochloride, MeOH/CH_2Cl_2, rt, 5 min; (□) compound 3, 2M K_2CO_3, Pd(PPh$_3$)$_4$, toluene, ethanol, N_2, 80 °C, 15 h; (□) 0.1M KOH aq, ethanol, rt, 12 h.

Results and discussion

PEG-BODIPY-BAPTA conjugates were synthesised according to Scheme 1. PEG 1,[19] BAPTA ethyl ester 4a and 5-methyl-BAPTA ethyl ester 4b,[20] and 5-bromoisophthalic acid [21] were combined according to published procedures. PEG bromobenzene 2 was obtained via the N-(3-dimethylaminopropyl)-N'-ethylcarbonate-mediated coupling of 5-bromoisophthalic acid and PEO chains. Compound 2 was converted into diboronate ester 3 under the Miyaura reaction conditions in the presence of bis(pinacolato)diborane, Pd(dppf)$_2$Cl$_2$, and KOAc with dry DMF as the solvent. The formyl derivatives of BAPTA are the key intermediates for synthesising BODIPY-BAPTA Ca^{2+} probes. Compound 4 was formylated by following the Vilsmeier–Haack procedure, yielding 5,5'-diformyl-BAPTA ethyl ester 5. BODIPY-BAPTA conjugates were then obtained via a three-step one-pot reaction. BAPTA aldehyde was initially reacted with 2,4-dimethyl pyrrole to afford a dipyrromethane intermediate that was oxidised with p-chloranil to a dipyrromethene intermediate, which was then reacted with BF_3 to produce BODIPY-BAPTA 6. BODIPY dyes were further iodised, affording 80% of diiodo-BODIPY 7a and 70% of tetraiodo-BODIPY 7b. PEG-BODIPY-BAPTA esters 7 were synthesised with Suzuki coupling between PEG phenylboronic acid ester 3, diiodo-BODIPY 7a, and tetraiodo-BODIPY 7b. Meanwhile, the synthesised PEG-BODIPY-BAPTA esters 8 were transformed into the corresponding K salts via saponification under mild conditions with KOH in a mixture of methanol and H_2O. The subsequent ion exchange and lyophilisation processes induced **MPFCP**.

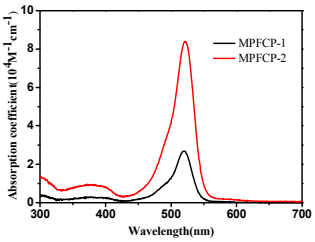


Figure 1 UV-vis absorption spectroscopy of **MPFCP** in buffer aqueous solution

These **MPFCP** probes had relatively high water solubility. The optical properties of these probes in buffer aqueous solution were investigated by applying ultraviolet–visible (UV-vis) absorption spectroscopy and photoluminescence (PL) spectroscopy. The photophysical data are summarised in Table S1. The absorption maximum of **MPFCP-1** emerged at 520 nm with a molar extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} (Figure 1). **MPFCP-2**, with two BODIPY fluorophores, exhibited a high molar absorption coefficient of 8.4×10^4 M^{-1} cm^{-1}, which was approximately three times the absorptivity of **MPFCP-1**. Meanwhile, the absorption maximum of **MPFCP-2** was slightly red-shifted to 525 nm. Ca^{2+}-dependent UV-vis absorption and PL spectroscopy were also analysed. The UV-vis absorption of **MPFCP** scarcely changed when the concentration of free Ca^{2+} was increased (Figure S2).

The fluorescence intensity of **MPFCP** without free Ca^{2+} solution was exceedingly small to be distinguished from the baseline. Contrarily, these **MPFCP** probes showed relatively high fluorescence increase upon Ca^{2+} binding (Figure 2). In particular, **MPFCP-1** showed a fluorescence increase of about 80-fold upon

Ca^{2+} binding with the maximum emission wavelength at 544 nm. **MPFCP-2,** two fluorophore units in **BAPTA**, had a slightly red-shifted emission maximum at 548 nm and a 100-fold fluorescence enhancement in the presence of Ca^{2+}, which was over 2-fold larger than the fluorescence increase previously reported for BODIPY-based Ca^{2+} indicators.[22] The lone electron pair of amino group of the BAPTA moiety is involved in Ca^{2+} binding. Therefore, calcium coordination partially blocks the PET process and yields an increased fluorescence.[17] Moreover, the bulky branched PEG chains at the C-2 and C-6 positions of BODIPY prevent fluorescence quench via the nonradiative pathways and fluorophore stacking.[15, 23]

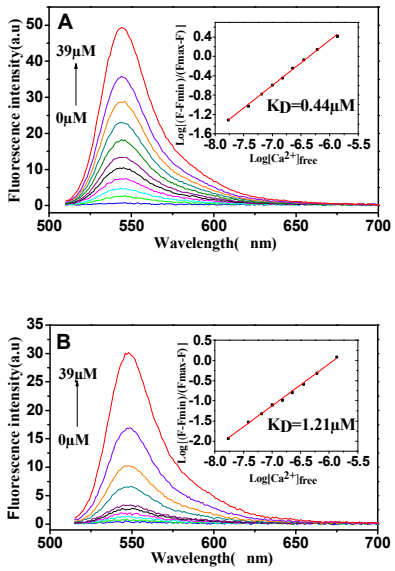


Figure 2 Ca^{2+}-dependent PL spectroscopy and dissociation constants K_d of **MPFCP-1** (A) and **MPFCP**-2 (B) in the presence of free Ca^{2+} at various concentrations (0, 0.017, 0.038, 0.065, 0.100, 0.150, 0.225, 0.351, 0.602, 1.35, 39μM) in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (30mM) containing KCl (100 mM) and ethyleneglycol tetraacetic acid (EGTA; 10mM) with the concentrations of probes 1μM at pH 7.2 and 22 °C.

The parent compound **BAPTA** had a low dissociation constant (Kd of **0.11** μM) for Ca^{2+}.[5a] The K_d of **MPFCP-1** was 0.44 μM, which was significantly close to that of a typical fluorescent Ca2+ probes with **BAPTA** chelators (e.g., Fluo-4 (K_d of 0.35 μM)).[12] By contrary, **MPFCP-2**, two **BODIPY** fluorophores with one **BAPTA** chelator, had a K_d of 1.21 μM for Ca^{2+}, which was 3-fold larger than that of **MPFCP-1**. The increase of K_d is usually ascribed to the slightly electron-withdrawing properties of fluorophore.[5a] Higher K_d value corresponds to the lower affinity for Ca^{2+}, reducing the buffering of intracellular $Ca^{2+}$and incurring a faster response to Ca^{2+}.[24]

Ca^{2+} probes were exposed to ionic environment that potentially interfered with Ca^{2+} chelation. The effects of other metal ions on calcium ion chelating were explored. Figure 3 displays the normalised relative response of **MPFCP** to physiological metal ions. The result of the investigation demonstrated that Ni^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, K^+ and Na^+ slightly influenced the fluorescence intensity of **MPFCP**. By contrary, the metal ions such as Cd^{2+}, Pb^{2+}, and Cu^{2+} significantly affected such an intensity, suggesting that Ca^{2+} did not displace the already complexed Cd^{2+}, Pb^{2+}, and Cu^{2+}. Fortunately, the concentrations of free Cd^{2+}, Pb^{2+}, and Cu^{2+} ions were negligible in the cell. However, the intracellular free Mg^{2+} concentration, typically ranging from about 0.1 mM to 6 mM, was at least 1000-fold of Ca^{2+} concentration.[12] The interference caused by Mg^{2+} was thoroughly examined. The fluorescence activation ratio of **MPFCP-1** for Ca^{2+}/Mg^{2+} was 6, whereas **MPFCP**-2 had 48 times activation ratio for Ca^{2+}/Mg^{2+} (Figure 3). The improvement of activation ratio for **MPFCP-2** over **MPFCP-1** was 8-fold. This certain observation indicates that **MPFCP**-2 has higher Ca^{2+}/Mg^{2+} selective than **MPFCP-1**. The findings also revealed that the pKa values of **MPFCP** were 5.3 for **MPFCP-1** and 6.4 for **MPFCP-2** (Figure S3 in supporting information). Low pKa value specifies that pH variation over a reasonable range of intracellular values hardly affects either the spectra of the Ca^{2+}-free or Ca^{2+}-bound species or the Ca^{2+}dissociation constant.[5a]

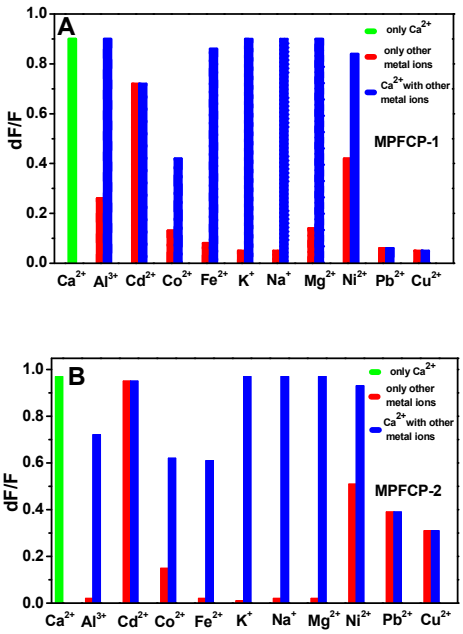


Figure 3 Relative fluorescence intensity of 5.0μM MPFCP in the presence of 50μM Mg^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} Ni^{2+}, Cu^{2+}, Pb^{2+}, Al^{3+}, N^{a+} and K^+(red bars) followed by addition of 50μM Ca^{2+}(blue bars).

Cell permeability is an essential requirement for probes to be used within the cellular environment. In general, however, **BAPTA**-based calcium ion probes cannot pass through the cell membranes of living cells, because of the nature of negative ion. Accordingly, whether the cell permeability of **BODIPY-BAPTA** probes can be improved by the branched PEG groups was examined. The uptake of **BODIPY-BAPTA** probes was quantitatively analyzed by flow cytometry.[25] The fluorescence intensity of the cells incubated with **BODIPY** probes (no PEG chains) was very weak. With more PEG chains, The mean fluorescence intensity in the cells incubated with **MPFCP-2** was about 10 times higher than that observed in the cells incubated with **MPFCP-1 (**Figure S4**)**. The results indicated that the cellular uptake of **BODIPY-BAPTA** probes was significantly

improved by PEG chains.

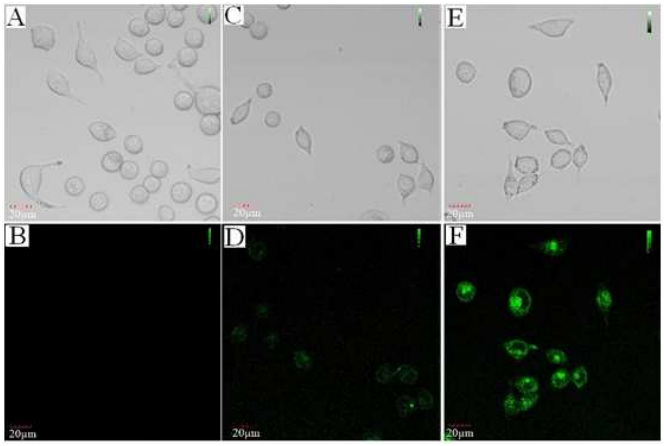


Figure 4 the bright-field images of Hela cells incubated by 20μM MPFCP-1 (A), MPFCP-2 (C) and the confocal images of HeLa cells incubated by 20μM MPFCP-1 (B), MPFCP-2 (D), the bright-field (E) and the confocal images (F) of HeLa cells incubated by 20μM MPFCP-2 with ionomycin calcium (5μM).

HeLa cells were tested to demonstrate the applicability of **MPFCP** in intracellular Ca^{2+} imaging. The cells were incubated with **MPFCP** solution (20 uM) for 30 min and were then washed three times with PBS buffer. The excitation wavelength was fixed at 515 nm, and the fluorescent signals were collected from 520 nm to 600 nm. Figure 4B shows the confocal microscopy images of HeLa cells incubated with MPFCP-1, in which fluorescence was not detected. The absence of fluorescence in this condition indicates that **MPFCP-1** cannot pass through the cell membranes because of the lower wrapped extent of branched PEG. Meanwhile, Figure 4D illustrates the representative confocal images of HeLa cells incubated with **MPFCP-2**. In this case fluorescence was observed in living cells, suggesting that **MPFCP-2** could pass through the cell membranes more easily. The significantly hydrophilic and biocompatible PEG chains may become part of the encapsulation for BAPTA and may improve the cellular uptake of **MPFCP-2**. [26]

The usefulness of **MPFCP-2** for monitoring the changes in intracellular cytoplasmic Ca^{2+} concentration was subsequently evaluated. The **MPFCP-2** stained cells were placed into ionomycin to increase the cytosolic Ca^{2+}.[27] The cells were then stimulated with 5 μM ionomycin calcium salt whose fluorescence signal was then significantly increased (Figure 4F). The changes in the fluorescence intensity of **MPFCP-2** corresponded to the changes of calcium concentration within the cell. Stimulated with ionomycin calcium salt, the intracellular Ca^{2+} concentration increased. Such an increment can be attributed to both the intracellular Ca^{2+} mobilisation and the promotion of extracellular Ca^{2+} influx.[28] In the absence of extracellular Ca^{2+}, the addition of 100 mM ATP also remarkably increased the fluorescence emission of cells (Figure S5).[29] These results demonstrate that **MPFCP-2** can evidently visualise the intracellular Ca^{2+} concentration waves in living cells. To the authors' knowledge, **MPFCP-2 is** the first calcium ion probes with **BAPTA** acid that could pass through the cell membrane by itself and monitor the changes of intracellular Ca^{2+} signal.

Conclusions

In summary, new fluorescent Ca^{2+} probes are designed based on **BODIPY** fluorophore with branched PEG chains. **MPFCP-1,** one **BODIPY** fluorophore on BAPTA chelator, exhibits low molar absorption coefficient, low dissociation constant for Ca^{2+}, and limited cell load. Meanwhile, MPFCP-2, synthesised with two BODIPY fluorophores and one BAPTA chelator, is partly encapsulated with more PEG chains. **MPFCP-2** has high molar absorption coefficient, medium dissociation constant for Ca^{2+}, and improved sensitivity and selectivity for Ca^{2+} over other metal ions. Moreover, **MPFCP-2** with **BAPTA** acid can pass through the cell membrane by itself, and can monitor the changes of Ca^{2+} signal in intracellular cytoplasmic. Based on the research findings, **MPFCP-2** probes can be considered a powerful means for measuring the changes in the Ca^{2+} concentrations of living cells.

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Experimental

Materials and instrumentation

All the chemicals used in synthesis are analytical pure and were used as received. UV/Vis spectra were recorded on a Shimadzu WV-2550 spectrophotometer. Fluorescence spectra were recorded on a Shimadzu RF-5301 fluorescence spectrophotometer. The ^{1}H NMR spectra were recorded at 20°C on 600 MHz NMR spectrometer (Bruker). Mass spectra were carried out using MALDI-TOF/TOF matrix assisted laser desorption ionization mass spectrometry with autoflexIII smartbeam (Bruker Daltonics Inc). CLSM images were obtained using Olympus confocal laser scanning microscopy (Olympus Fluoview FV1000)

Cell culture and intracellular Ca^{2+} imaging

HeLa cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C in a 5% CO2/95% air incubator in a humidified atmosphere. For fluorescence imaging, HeLa cells were grown in DMEM on a 35mm glass bottom poly-D-lysine coated Petri-dish for at least 24h to enable adherence to the bottom.

The cells were loaded with MPFCP according to a following procedure. Briefly, the 200 μl of 100 μM Hanks' balanced salt solutions (HBSS) of MPFCP was added to the dish (final concentration of MPFCP is 20 μM), and then the cells were incubated for 30 min at 37°C. Afterward, the cells were washed

several times with PBS solutions for removing MPFCP. To stimulate cells to cause a calcium signal, 100 μM of ionomycin solution in HBSS was added to the dish with 5 μM final concentration.

Notes and references

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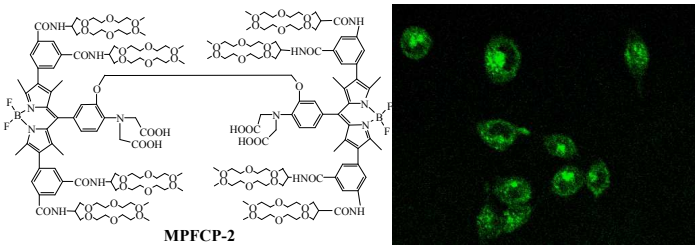
Graphical Abstract



MPFCP-2

The cellular uptake of **MPFCP-2** is improved by PEG encapsulation method, and then **MPFCP-2** could pass through the cell membrane by itself, and monitor the changes of intracellular Ca^{2+} signal.