Pergamon

Tetrahedron Letters 41 (2000) 9185–9188

TETRAHEDRON LETTERS

Infrared fluorescence sensing of submicromolar calcium: pushing the limits of photoinduced electron transfer

Burcu Ozmen and Engin U. Akkaya*

Middle East Technical University, Department of Chemistry, TR-06531, Ankara, Turkey

Received 17 September 2000; accepted 26 September 2000

Abstract

A heptamethine cyanine–BAPTA conjugate was synthesized which signals calcium binding by an almost 4-fold increase in the emission intensity at the emission maximum of 782 nm. This significant enhancement factor opens new possibilities for the development of IR fluorescent chemosensors excitable with an inexpensive 780 nm laser diode and detectable with highly sensitive avalanche diodes. © 2000 Elsevier Science Ltd. All rights reserved.

Calcium is an important secondary messenger. A large number of biological processes are regulated by temporal and spatial fluctuations of calcium concentrations.[1–4] Fluorescent chemosensors signalling submicromolar calcium are of particular importance as the cellular resting concentration of this ion is about 100 nM.[5] As a matter of fact, previously unrecognized regulation phenomena were discovered by employing fluorescent probes for intracellular calcium.[5] Most, if not all of the practically useful Ca^{2+} sensors contain a bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) unit as the calcium receptor module. BAPTA is highly Ca^{2+} selective and the much larger concentration of intracellular Mg^{2+} has only negligible effect on the fluorescence signal.[6]

In fluorescence sensing methodologies, establishing a communication link at long wavelengths is very important. Long wavelength excitation and emission capability is a tremendous advantage: biological media are highly scattering and some are heavily pigmented, but in the near IR region fluorescence, detection of even a single molecule is possible,[7] mostly due to drastically reduced background fluorescence and scattering at these wavelengths. In addition, blue and green light used for exciting most sensors for Ca^{2+} can be damaging to the object of the study.[8] Therefore, there is considerable effort[9–13] to move excitation wavelengths towards the red end of the visible spectrum or preferably beyond. Yet, chemosensors that can be excited in the spectral regions, which are essentially free from background cellular fluorescence, remained elusive (Scheme 1).

* Corresponding author.

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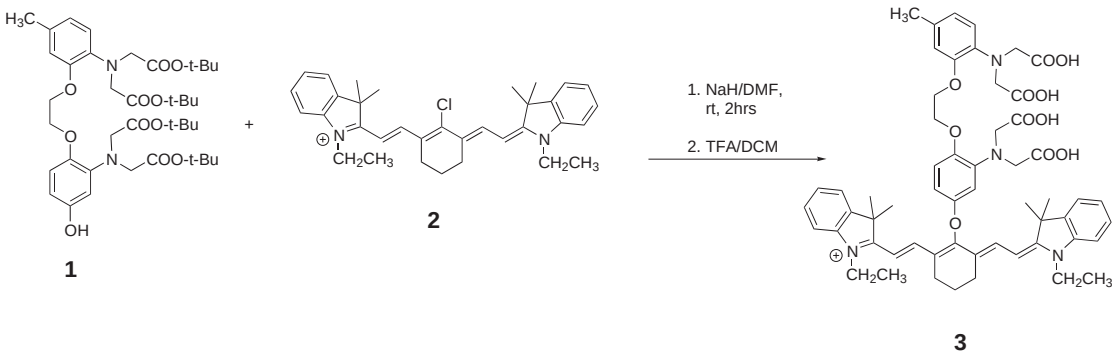
PII: S0040-4039(00)01662-2



Scheme 1. Synthesis of the IR fluorescent chemosensor **3**

We now report a heptamethine cyanine–BAPTA conjugate (**3**) which has a λ_{max} of 766 nm in aqueous solutions and emits at 782 nm. Submicromolar concentrations of calcium causes 3-fold increase in the emission intensity. With its high extinction coefficient (200 000) and moderate quantum yield (0.12 for the calcium-bound sensor), this new chemosensor seems to essentially combine all the desired characteristics of the ideal chemosensor. The signal originates from the ion modulation of photoinduced electron transfer (PET).[14–17] PET is recognized as one straightforward way of developing ion-sensing molecules. The requirements for a successful PET sensor are a fluorophore, an electron donor ligand/chelator, and a non-conjugated linker for these two units. A large number of examples[17] suggest that PET is very efficient when the HOMO–LUMO separation in the fluorophore is relatively wide. An inspection of the Weller equation[18] regarding PET processes clearly shows that as the S_0–S_1 energy separation decreases, finding a PET-active donor–fluorophore combination would be a challenge. The energy introduced into the system by excitation should be large enough to reduce the fluorophore and oxidize the donor. So, it seems efficient PET is less likely for long wavelength excitable fluorophores. An additional problem for such long wavelength sensing schemes is that the ultrafast radiationless deactivation of the excited state is very efficient,[19] thus reducing the quantum yield and chances for strong PET generated signals on ion binding.

In our design of an IR fluorescent Ca^{2+} probe, we had following considerations: (1) To improve the quantum yield by reducing emission loss due to non-radiative manifolds via *trans–cis* isomerization, the cyanine dye chosen was one in which three of the methine carbons were rigidified in a 6-membered ring system. (2) An electron rich BAPTA derivative was selected for conjugation, in effort to keep the K_d of the conjugate near the intracellular resting concentration of Ca^{2+}. (3) A representative of trimethylindolenine derived cyanine dyes, which are known[20] for higher water solubility and less tendency for aggregation, was chosen as the fluorophore module. Thus, 4-hydroxy-BAPTA (**1**) was synthesized in eight steps from commercially available materials by analogy to reported procedures[12] for the tetraethyl ester. The chloro-cyanine dye (**2**) was also synthesized according to a literature procedure.[21] The vinylic chlorine on the cyclohexane unit is reactive and can be substituted by phenolate and thiophenolate nucleophiles.[21] Sodium hydride in DMF was used to abstract the phenolic proton from the BAPTA derivative. The deprotonated compound **1**, reacted smoothly with the dye **2** yielding the tetra-*t*-butyl ester of the dye–chelator conjugate. The ester was purified by silica gel column chromatography ($CHCl_3$–MeOH 9:1). Deprotection of the carboxyl functions were carried out in DCM/TFA. Removal of the solvent resulted in an analytically pure sample of the target

compound. The absorption spectrum was obtained in 0 and 39.8 μM free Ca^{2+} solutions. There was no change in either the λ_{max} (766 nm) or the extinction coefficient ($\log \varepsilon = 5.30$). The emission spectra (Fig. 1) were acquired in solutions of varying free Ca^{2+} concentrations. The emission λ_{max} was at 782 nm.

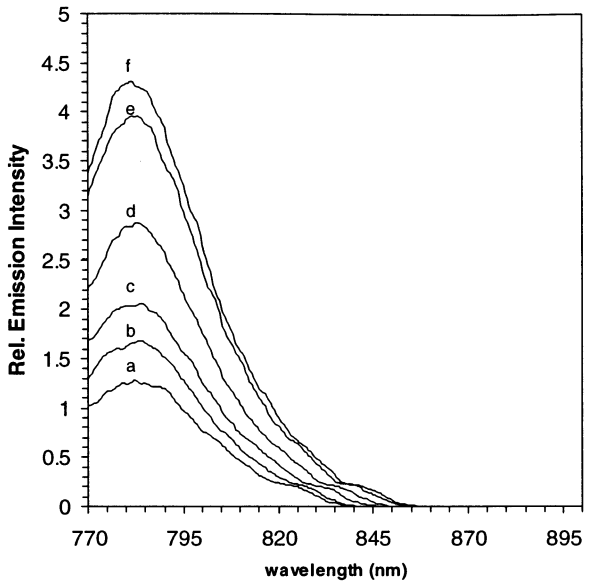


Figure 1. Fluorescence emission spectrum of compound **3** in solutions of varying free Ca^{2+} concentrations. (a), 0; (b) 75 nM; (c) 150 nM; (d) 300 nM; (e) 650 nM; (f) 39.8 μM. All solutions contained 100 mM KCl and were buffered at pH 7.2 using 100 mM MOPS. Free Ca^{2+} concentrations were adjusted by mixing 10 mM of CaEGTA and 10 mM EGTA in different ratios. The excitation wavelength was 760 nM, and both slits were set at 5 nM

The emission intensity as expected, was dependent on the free Ca^{2+} concentration. The change in the signal intensity is remarkable considering the long wavelength of excitation. The quantum yield was determined in reference to the NIR laser dye IR-125[22] and found to be 0.05 for the free chelator and 0.12 for the calcium-bound form. A Hill plot ($\log[(F-F_{min})/(F_{max}-F)]$ versus $\log[Ca^{2+}]$) of the emission data yielded a K_d of 240 nM which is close to the resting concentration of the intracellular Ca^{2+} concentration; thus the compound **3** provides an excellent dynamic range practical for applications.

Thus, our work demonstrates that effective PET-generated fluorescence signaling is possible even in the near IR. With much reduced Rayleigh and Raman scatterings and inexpensive diode laser operating at 780 nm, the chemosensor described here is likely to find practical applications. In addition, the cyanine structure is wide-open for structural modifications. Further optimization of excitation/emission wavelengths can be done with ease. Our work towards further refinement of the sensing capabilities of this class of chemosensors is in progress.

Acknowledgements

This work was supported by grants from the Middle East Technical University Research Funds (AFP-2000-01-03-05) and Scientific and Technical Research Council of Turkey (TBAG-1885).

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