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A Squaraine-based Near IR Fluorescent Chemosensor for Calcium.

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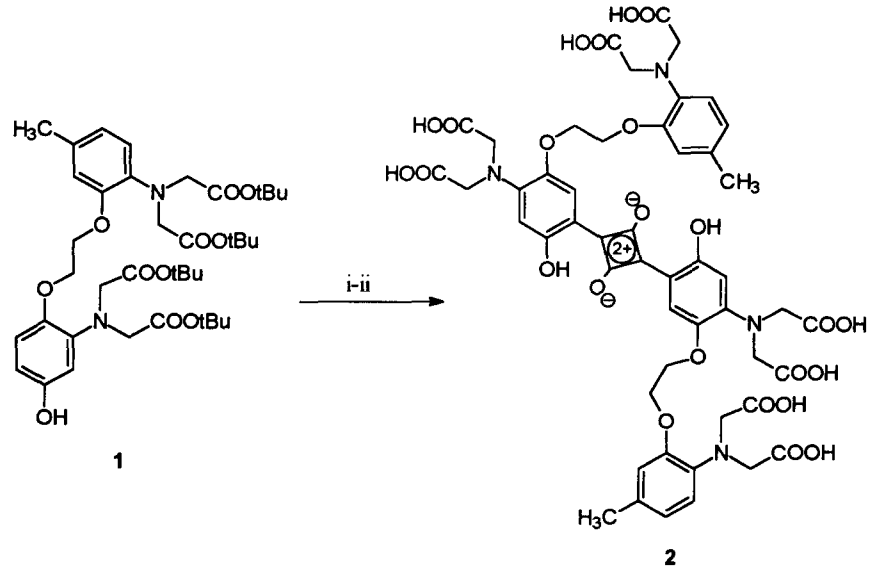
Abstract: A red to NIR emitting, highly Ca^{2+}-specific fluorescent chemosensor has been synthesized. In pH 7.2 aqueous buffers, the chemosensor signals Ca^{2+} by a decrease in emission intensity, whereas large excess of Mg^{2+} ions have no effect on either the absorption or the emission spectrum. The chemosensor is likely to be the prototype of a new generation of laser-diode excitable fluorescent chemosensors for Calcium. © 1997 Elsevier Science Ltd.

In recent years, a number of fluorescent chemosensors for both charged and neutral species have been reported.[1] Although for niche applications requirements may vary, for most applications an ideal chemosensor should sense in water. Spectral characteristics like high quantum yield (ϕ_f) large extinction coefficient (ε) and "brightness" (defined[2] as $\phi_f.\varepsilon$) are all important, but most interesting chemosensors are those with useful selectivities. Especially sensor molecules which can report micromolar levels of Calcium (in the presence of thousand-fold excess of Mg^{2+}) have been useful chemosensors,[2,3,4] not only because they provide excellent illustrations of the design principles, but also because they have been practical successes:[5] Intracellular Ca^{2+} signals are responsible for the initiation or otherwise regulation of a number of biological processes and there is a great interest in visualizing such Ca^{2+} spikes in real-time with the help of such fluorescent chemosensors. Real-time visualization of intracellular calcium fluxes requires sensor molecules with high affinities for Ca^{2+} and a distinct emission profile from that of the endogenous fluorophores. Despite their success, the earlier examples of fluorescent Ca^{2+} sensors suffered from one important disadvantage: the requirement for short wavelength excitation. Such excitation creates a significant background fluorescence (autofluorescence) and is highly damaging to the cell, complicating the study of *in vivo* processes. Short wavelength excitation also necessitates the use of expensive quartz optical components. In addition, laser-diode excitable chemosensors offer a number of advantages like potential for miniaturization, compatibility with fiber-optics applications, transdermal sensing, etc. So, there is a great impetus for the design of "long-wavelength" chemosensors.

"Squaraines" or "squarylium" compounds have been reported to have interesting applications in the areas of photoreceptors,[6] organic solar cells,[7] optical recording media.[8] Their fluorescence properties are reported,[9] some are known to be highly fluorescent in organic solvents. We have targeted squaraines as the core fluorophore for the sensor molecules because of the fact that similar structured-squaraines have reportedly[10] useful spectral characteristics such as long wavelength absorption (635-690 nm) and emission (650-700 nm), and very high extinction coefficient (ε > 300,000). It is surprising that these remarkable properties of squaraine compounds have not been studied in relation to chemosensor design.

We are here reporting the design, a straight-forward synthesis and spectral characterization of a long wavelength chemosensor based on a squaraine fluorophore. The sensor is designed to have two highly Calcium-selective BAPTA[11] chelator units fused to a squaryl moiety, thus forming a fluorescent



Scheme 1. Synthesis of the Ca^{2+}-specific fluorescent chemosensor: i) Squaric acid/n-BuOH-Toluene, Δ. ii) CH_2Cl_2/TFA.

squaraine system which is very sensitive to Ca^{2+} concentrations in the micromolar range. The signal results from the loss of conjugation of donor alkyl-amino functions on Ca^{2+} chelation, decreasing molar extinction coefficient at 698 nm from 300,000 to 50,000.

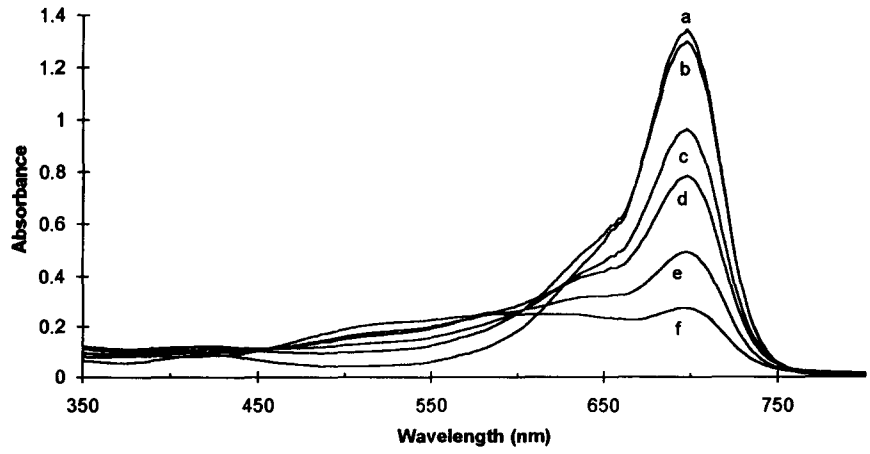


Figure 1. Absorbance spectrum of the chemosensor (5 μM) as a function of increasing Ca^{2+} concentrations in Ca-EGTA and MOPS buffered solutions at pH 7.2: (a) 0 μM; (b) 0.71 μM; (c) 1.89 μM; (d) 3.8 μM, (e) 38.9 mM; (f) 2500 μM.

The synthesis of the chemosensor starts from a t-Butyl ester analog of a known compound (Scheme 1). This tetra-t-butyl ester (**1**) was synthesized in five steps, essentially following a literature procedure for the tetraethyl ester,[3] only with an appropriate substitution, placing four t-butyl protective groups on the carboxyl functions. The tetra-t-butyl ester **1** was then reacted with squaric acid in a Dean-Stark apparatus, using dry toluene and butanol (50:50) as solvent, while water was removed azeotropically. An intense green solution was obtained, on cooling crystalline octa-t-butyl ester was obtained. t-Butyl groups were removed in CH_2Cl_2/TFA. Removal of the solvent gave the target chemosensor (**2**) in the form of a dark green solid with satisfactory analytical data. Unlike previously reported squaraines, this compound is highly soluble in both organic solvents and in water. Absorption spectrum (Figure 1) of the chemosensor in pH 7.2 aqueous MOPS[12] buffer solution shows one peak centered at 698 nm. Increasing concentrations of Ca^{2+} in buffered solutions cause a significant hypochromic effect, and some broadening of the peak. The spectra are somewhat complicated by the two separate binding events and the light sensitivity of the

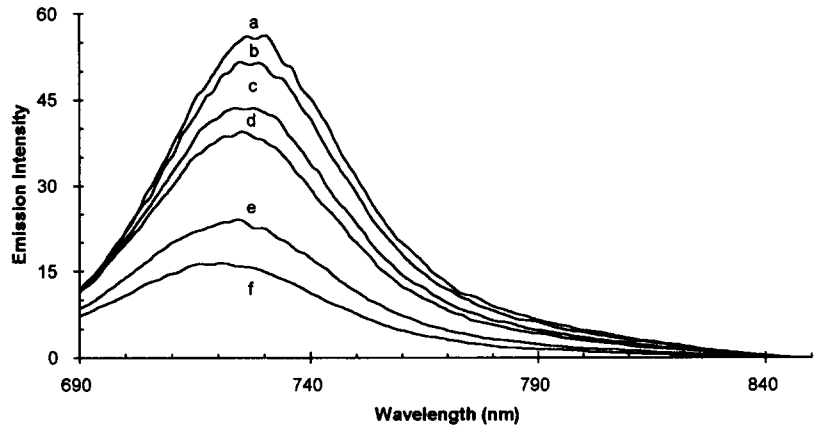


Figure 2. Emission spectrum of the chemosensor (0.5 μM) as a function of increasing Ca^{2+} concentrations in Ca-EGTA and MOPS buffered solutions at pH 7.2: (a) 0 μM; (b) 0.71 μM; (c) 1.89 μM; (d) 3.8 μM, (e) 38.9 mM; (f) 2500 μM. Excitation was at 670 nm.

Ca^{+2} -bound form, but the averaged dissociation constant of 3.6 μM seems to be in an ideal range for studying especially large ion spikes near the cellular membranes. Ca^{2+} in less then 2 μM concentration depresses the peak to 60 % of the original value, whereas 2 mM Mg^{2+} has no effect on the spectrum. We believe binding of Calcium while removing electron density from the donor group, increases the bulk of the substituent by organizing the chelator group near the squaryl moiety, resulting reduced conjugation. Also, partial neutralization of the carboxylate charges may allow aggregation of the fluorophore molecules, leading to broader peaks in absorption spectra and less than optimal isosbestic point. Anti-auxochromic effect is also reflected in the fluorescence emission spectra (emission maximum at 733 nm), with a small blue shift and decreased emission intensity (Figure 2).

The intense absorption at 698 nm suggests that the compound can be ideally excited with the 670 nm-laser diode. This would make this compound the first fluorescent chemosensor for Calcium that can be excited with such long wavelength sources.

It is very likely that this chemosensor **2** will open a new path for the development of better chemosensors based on squaraine system, and would offer considerable advantages by enabling laser diode excitation leading to the development of compact solid-state devices for cellular ion-flux studies and real-time imaging.

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11. BAPTA: 1,2-Bis(2'-aminophenoxy)ethanetetraacetic acid
12. MOPS: 4-Morpholinepropanesulfonic acid.

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