Fluorescent PET(Photoinduced Electron Transfer) Sensors for Calcium Ions. Extension to Multiple Fluorophores and Virtual Spacers

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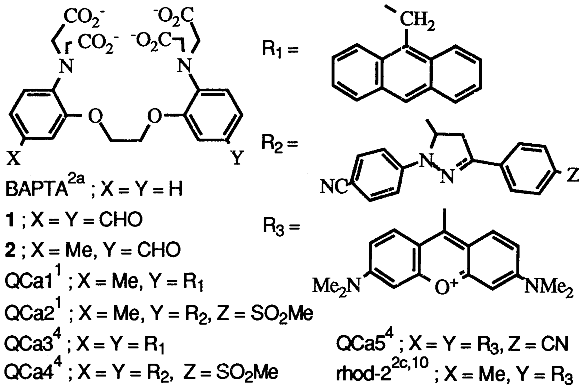
New bifluorophoric sensors QCa3 and QCa4 show larger Ca^{2+}-induced fluorescence enhancements and (for QCa4 only) lower Ca^{2+} binding constants than their monofluorophoric counterparts due to statistics and remote steric effects respectively. The PET sensor principle is extended to twisted biaryl systems which can give rise to twisted internal charge transfer (TICT) excited states by the re-examination of Tsien's rhod-2.



BAPTA[2a] ; X = Y = H
1 ; X = Y = CHO
2 ; X = Me, Y = CHO
QCa1[1] ; X = Me, Y = R_1
QCa2[1] ; X = Me, Y = R_2, Z = SO_2Me
QCa3[4] ; X = Y = R_1
QCa4[4] ; X = Y = R_2, Z = SO_2Me
QCa5[4] ; X = Y = R_3, Z = CN
rhod-2[2c,10] ; X = Me, Y = R_3

We recently developed the fluorescent sensors QCan(n=1,2)[1] selective for Ca^{2+} versus Mg^{2+} and H^+ to complement those available from Tsien's fine work.[2] The simple 'fluorophore - spacer - receptor' logic of our fluorescent PET sensor approach[3] now allows us to make two rational extensions. First, sensors with two fluorophores QCa3-5[4] are examined. Second, orthogonally twisted 'fluorophore - receptor' assemblies e.g. rhod-2[2c] are shown to be similar to PET systems as regards Ca^{2+} sensing. Some notable points emerge from the data on QCa3-5 in Table 1. **a**) QCa4&5 display the largest known Ca^{2+}-induced fluorescence enhancements ($FE_{Ca^{2+}}$) at pH7.3. The largest $FE_{Ca^{2+}}$ value known previously was 92 for QCa2.[1] Similarly, QCa3 has $FE_{Ca^{2+}}$ = 21 whereas QCa1 had 16.[1] This significant increase of $FE_{Ca^{2+}}$ compared to the monofluorophoric counterpart (31 and 96%), is due to a larger lowering of the fluorescence quantum yield Φ_{Fmin} in the Ca^{2+}-free sensor than Φ_{Fmax} in the Ca^{2+} -bound form. This can be understood on the basis of a statistical enhancement effect on the PET rate which competes with fluorescence. The presence of two identical fluorophores within the sensor structure allows the excitation to reside in either of two locations, if energy migration[5] is suitably efficient. This creates two potential sites for accepting the electron transferred

Table 1. Electronic spectral and ion-binding parameters for sensors QCa3-5 and rhod-2[a]

Parameter	QCa3	QCa4	QCa5	rhod-2
λ_{Abs}, S_0->S_1(nm), (ε_{max}(mol^{-1} dm^3 cm^{-1}))	388(13700), 368(15200), 350(10200)	391(63000)	384(64000)	547(87000)[g]
λ_{Flu}(nm)(Φ_{Fmin})	440, 417, 394(0.0003)	495(0.0003)	493(0.0006)	575(0.007)
$\Phi_{FmaxCa^{2+}}$($FE_{Ca^{2+}}$)	0.0060(21)	0.045(180)	0.056(100)	0.17(24)
$\Phi_{FmaxMg^{2+}}$($FE_{Mg^{2+}}$)	0.0003(1.1)[d]	0.0004(1.2)[d]	0.0008(1.3)[d]	0.009(1.3)[d]
Φ_{FmaxH^+}(FE_{H^+})	0.0015(5)[e], 0.008(26)[f]	0.0012(4)[e], 0.0036(12)[f]	0.0016(2.6)	0.025(3.5)
log $\beta_{Ca^{2+}}$[b]	6.8	5.2	5.5	6.1
log β_{H^+}[b,c]	-, 5.5, 3.1	-, 5.7, 3.2	-, 6.1, -	-, 5.8, -

[a] 5×10^{-6}mol dm^{-3} sensors in aerated water at pH7.3 (maintained with pH buffer (MOPS))with pCa and pMg buffers (EGTA and NTA).[2] pH variations done with pH buffers and EGTA(10^{-2}mol dm^{-3}). Φ_F values are obtained from corrected spectra after comparison with that of appropriate cases in the literature (QCa1 or QCa2[1] or Rhodamine B[12]). It is to be noted that, in contrast to QCa1&2, QCa3-5&rhod-2 show some noticeable cation - dependent alterations in absorption spectra. [b] Calculated according to the equation,[3a] $\log[(I_{Fmax} - I_F)/(I_F - I_{Fmin})] = pM - \log\beta$, from I_F (fluorescence intensity at λ_{max}) - pM profiles. Average gradient = 1.02, Average correlation coefficient = 0.986 (ave. n = 7). [c] No detectable inflection in the I_F - pH profile in the pH range 6-7. [d] Log $\beta_{Mg^{2+}}$ values cannot be quantitated in view of the small $FE_{Mg^{2+}}$ values measured at 0.05 mol dm^{-3} Mg^{2+}. [e] Refers to the fluorescence enhancement in the pH range 4-6. [f] Refers to the fluorescence enhancement in the pH range 2-4. [g] The precursor tetramethyl ester has ε_{max} = 95000 in methanol.

from (either of the two alkoxyaniline units of) the receptor module. Related statistical effects are partly responsible for the enhanced electron transfer quenching of aromatic hydrocarbon excited states by diamines[6a] and disulphides.[6b] In contrast, the bifluorophoric sensors are hardly affected by 1:1 Mg^{2+} binding which gives strong selection against Mg^{2+}. Their monofunctional versions gave small ϕ_F enhancements with Mg^{2+}.[1]

b) QCa4&5, but not QCa3, display lowered Ca^{2+} binding constants (log $\beta_{Ca^{2+}}$) relative to the value predicted from the parent BAPTA receptor (7.0[2a]) which is essentially attained by QCa1(6.6) and QCa2(6.5). These lowered log $\beta_{Ca^{2+}}$ values, which makes QCa4&5 useful for studying elevated intracellular Ca^{2+},[2d] are probably caused by inter - fluorophore steric effects (along with some dipole - dipole repulsion) which destabilize the Ca^{2+}-bound sensor relative to the Ca^{2+}-free form. Ca^{2+} binding rigidifies the sensor structure into a conformation where the two fluorophore modules are held *cis*. QCa4&5 possess rather rigidly connected 'fluorophore - spacer' assemblies which can make frequent van der Waals contact in the Ca^{2+}-bound state, according to CPK model examination. Conversely, the 9-anthracenyl methyl units of QCa3 have more rotational freedom and such destabilizing contacts are less probable. It is interesting that inter - fluorophore steric interactions remote from the Ca^{2+} binding site can influence log $\beta_{Ca^{2+}}$ values due to the rigidity of the Ca^{2+}-bound forms of QCa4&5. Remote influences concerning binding site activity are known in enzymology.[7]

Most fluorescent PET sensors, including QCa1-5, employ C$_1$ or C$_2$ spacers with sp^3 hybridization to insulate the π or n electron systems of the fluorophore and receptor modules. Another way of insulating the fluorophore and receptor modules is to employ a virtual or C$_0$ spacer in that the π systems of the fluorophore and receptor units are naturally orthogonal to a large extent due to the twisted biaryl system. We now note that Tsien's empirically designed sensor rhod-2[2c] fits this criterion. Related fluorescent pH sensors are known to be successful.[3,8] In such instances, the end result of excited state deactivation by a PET process *i.e.* the formation of a radical ion pair would appear indistinguishable from that found in a non - emissive twisted internal charge transfer (TICT) state.[8,9] Thus it was surprising that Ref. 2c reported $FE_{Ca^{2+}} = 3$, a rather low value. We have synthesized rhod-2 by a different route[10] and find that $FE_{Ca^{2+}} = 24$. This makes it useful for Ca^{2+} biomonitoring.[11]

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References and Notes

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4 QCa3 and QCa4&5 were synthesized using procedures from Ref. 1 starting with the tetra(methyl or ethyl) esters of BAPTA and **1**(prepared by analogy with **2**)[2b] respectively.

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9 The oxidation potential of the Ca^{2+}-free receptor BAPTA can be estimated as 0.82V(vs. SCE) which is the value for N,N-dimethyl 2-anisidine (H. Siegerman in N.L. Weinberg, "Technique of Electroorganic Synthesis, Part II," Wiley, New York (1975), p 667). This value is lower than that estimated for Rhodamine dyes (1.44 V) from data given in Ref. 3b, Part A, p.478. Therefore, TICT state evolution by electron transfer from the BAPTA unit to the photoproduced hole in the Rhodamine moiety is thermodynamically feasible.

10 **2** tetramethyl ester[2b] was condensed with bis(3-dimethylamino phenyl) ether (J.P. Critchley, A.P. Grattan , A.M. White, and J.S. Pippett, *J. Polymer Sci. A-1*, **10**, 1789 (1972)) and trifluoro acetic acid followed by propyl nitrite oxidation (procedure modified from R. Mohlau, and P. Koch, *Chem. Ber.*, **27**, 2887(1894); L.L. Woods, and J. Sapp, *J. Org. Chem.*, **27**, 3703(1962); P. Jakobsen, A.P. Andersen, H. Lyon, and S. Treppendahl, *Microsc. Acta*, **87**, 41(1983)) to give rhod-2 tetramethyl ester. Acid hydrolysis gives the tetracarboxylic acid and rhod-2 is the active form at pH7.3.

11 See, *Bioprobes*, **16**, 5(1992) for recent improvements.

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