Density functional study of fluorescent indicators for the intracellular determination of Ca^{2+} and Mg^{2+} †

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Fluorescent indicators for the intracellular determination of Ca^{2+} and Mg^{2+} have been studied by means of theoretical calculations, based on Density Functional Theory (DFT). The indicators consist of the podant *o*-aminophenol-*N*,*N*,*O*-triacetic acid (APTRA), linked to an arylthiophene fluorophore, substituted in the *para* position with donor or acceptor groups. The interaction of Ca^{2+} and Mg^{2+} with the indicators was studied both in the gas phase and in solvent. In the gas phase, these cations both have a five-fold coordination. Binding with the cation results in a change in the hybridisation state of the nitrogen from sp^2 to sp^3; the nitrogen lone pair is no longer part of the conjugated system. The metal–nitrogen interaction is given up in solvent; the structure relaxes so that the nitrogen lone pair can again participate in the conjugated system of the fluorophore. The effect of the electron-withdrawing or -donating substituents on the cation–indicator interaction was investigated. Two effects determine the nature of the complexation in solvent. Firstly, there is the inherent binding energy of the indicator with the metal, which is favored by electron-donating substituents and weakened by electron-withdrawing groups. Secondly, there is a stabilising effect of the solvent on the free indicators; due to their smaller dipole moment, free indicators with electron-withdrawing groups are stabilised less by the solvent. For various substituents, these two effects evolve in opposite ways. This results in a small overall variation of complexation energies.

1 Introduction

The determination of intracellular ion concentrations by means of fluorescent indicators has become a powerful method for investigating the physiological role of cytosolic cations.[1,2] A wide range of fluorescent indicators for Na^+ and K^+ as well as Ca^{2+} and Mg^{2+} is commercially available. As they each suffer from particular shortcomings, it is of great interest and importance to develop and characterise new fluorescent indicators with improved characteristics. Recently, a new series of fluorescent indicators for the determination of Mg^{2+} and Ca^{2+} has been developed and characterised;[3] their structure is shown in Fig. 1. They consist of the podant *o*-aminophenol-*N*,*N*,*O*-triacetic acid (APTRA), linked to an arylthiophene fluorophore. Binding of the indicator to the cations results in a hypsochromic shift in both the excitation and emission spectra, allowing ratiometric measurements to be performed.

By modifying the substituents at the fluorophore end of the molecule, a series of indicators with absorption wavelengths ranging from 330 to 370 nm and emission wavelengths ranging between 400 and 580 nm are obtained.[3] The dissociation constants of the complexes with Mg^{2+} and Ca^{2+} are reported in Table 1 for indicators with a -CN, -H and $-OCH_3$ substituent, which are named Thio-CN, Thio-H and Thio-OCH_3, respectively. The calcium dissociation constants are much smaller than the dissociation constants for magnesium. The substituent effect on the complexation of Mg^{2+} is small, but complexation is enhanced by electron-releasing substituents. Ca^{2+} binding, however, is promoted by electron-withdrawing substituents. Consequently, this series contains an indicator with optimal selectivity for Ca^{2+} over Mg^{2+}, while the indicator at the other extreme of the series has optimal complexing abilities for Mg^{2+}. The effect of the substituent on the binding properties of the cations is usually explained by considering nitrogen as the link between the conjugated system and the cation binding site.[4] Its lone pair of electrons participates both in complexation and delocalisation over the conjugated system. The electronic effect of the substituent should be transmitted to nitrogen through the conjugated system, and hence influences not only the complexing ability of the nitrogen atom but also the position of the spectra.

In this work, a theoretical study of the indicators Thio-CN, Thio-H and Thio-OCH_3, and their binding with Ca^{2+} and

Table 1 Experimental values for the dissociation constants as a function of the substituent

	CN	H	OCH_3
Mg^{2+}/mM	4.96	4.57	3.71
Ca^{2+}/μM	32	56	126

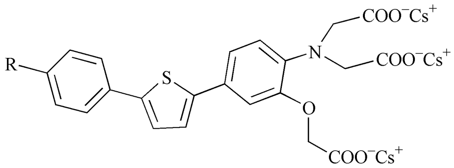

Fig. 1 Structure of the indicators (Thio-H: R = H; Thio-CN: R = CN; Thio-OCH_3: R = OCH_3).

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† Structural parameters of negatively charged clusters are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b007538h/

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Mg^{2+}, is presented. Density Functional Theory (DFT) has, over the last decade, emerged as a powerful method for electronic structure calculations of large-size systems, including electron correlation. The main difficulty in applying a theoretical approach to the study of the indicators of Ca^{2+} and Mg^{2+} is the modeling of the solvent effect. Whereas all experiments are performed in aqueous solution, standard DFT calculations are performed on isolated clusters (gas phase). As it is known that the solvent can influence the properties of solutes and chemical equilibria very substantially,[5] a proper description of the solvent effect is necessary. Two approaches for including solvent effects have been used in this study: the addition of water molecules into the DFT calculations and a continuum solvation model for the calculation of hydration free energies.

2 Computational details

Geometry optimisations, based on Density Functional Theory (DFT) were performed by means of the Turbomole code.[6] The B3LYP functional was applied, and the basis sets chosen are the Gaussian type basis sets of Schäfer *et al.*[7] For C, H, N, S, and O, a double-ζ basis set was used, while the double-ζ basis set was extended with one polarisation function in the case of Ca and Mg. Charges were obtained from Mulliken population analysis.

Gas phase geometry optimisations were performed on the free indicators (uncomplexed) and the indicators complexed with the cations Mg^{2+} and Ca^{2+}. To avoid working with highly charged systems as much as possible, the complexed indicators are represented by neutral M–IH clusters, where $M^{2+} = Mg^{2+}$ or Ca^{2+}, $I^{3-} = [C_{22}H_{15}NO_7S(R)]^{3-}$ (represented in Fig. 1 and 2), and a proton is added to keep the system neutral. Three possibilities exist for the position of the proton. Since it is not clear which one of the three carboxy groups will be protonated preferentially in solution, we have optimised firstly structures with a proton on the carboxy group of the ether arm of the podant. Secondly, the carboxy group on one of the amino arms was neutralized. We expect that the structures of models in which the other carboxy group of the amino arm is protonated will be similar to the latter clusters. The structures of these complexed indicators were compared to the uncomplexed IH_3 clusters, in which all carboxy groups are neutralized.

The gas phase binding energy (BE) of Ca^{2+} and Mg^{2+} to the indicator was calculated as given by eqn. (1).

$$\mathrm{BE} = E(\mathrm{M{-}IH}) - E(\mathrm{M}^{2+}) - E(\mathrm{IH}^{2-}) \qquad (1)$$

The energy of IH^{2-} was calculated by performing a geometry optimisation on the indicator in which only one of the carboxy groups is protonated (the same carboxy group as in the M–IH under consideration); as such, these systems carry a two-fold negative charge. The structural parameters of these negatively charged clusters will not be discussed; they are available as supplementary material.

2.1. Addition of water molecules into DFT calculations

One way to consider the solvent effect in quantum chemical calculations is by adding interacting solvent molecules to the system.[8] In our case, this approach has been applied to investigate how the coordination environment of the metal ion in the indicator is influenced by aqueous solution. Starting with one, up to eight water molecules were considered. The effect of these solvent molecules on the strength of the binding of the metal ions with the indicator was examined. The complexation energy, $\Delta E_{\mathrm{compl}}$, of Ca^{2+} with the indicator was then calculated [eqn. (3)] as the reaction energy of the equilibrium (2).

$$[\mathrm{Ca(H_2O)_8}]^{2+} + \mathrm{IH}^{2-} \rightleftharpoons \mathrm{Ca{-}IH(H_2O)}_x + (8 - x)\mathrm{H_2O} \qquad (2)$$

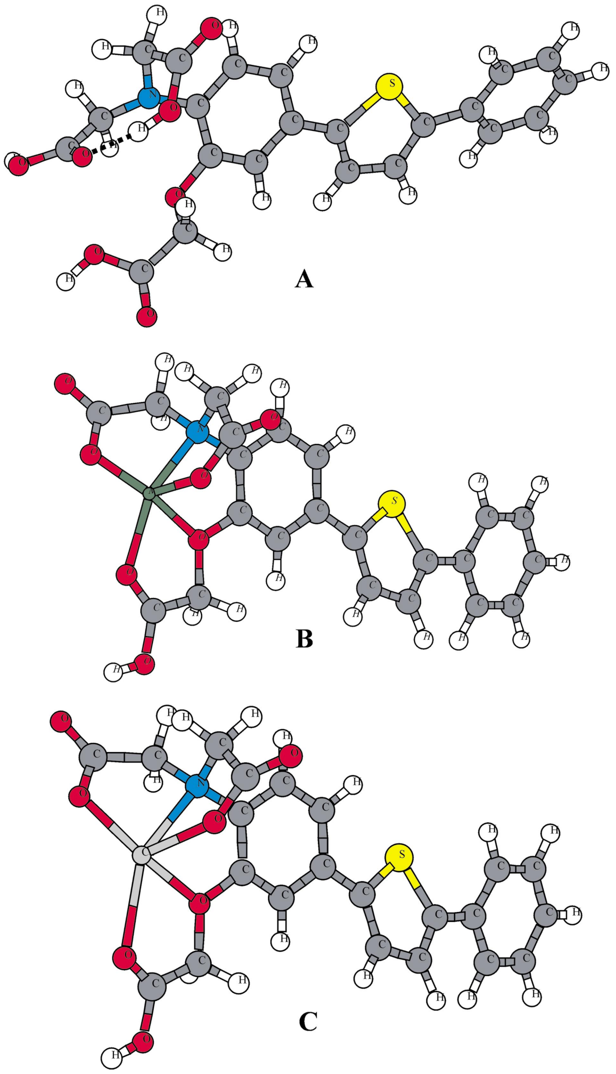

Fig. 2 Illustration of the optimised clusters of the free Thio-H (A), Thio-H complexed with Mg^{2+} (B) and Thio-H complexed with Ca^{2+} (C).

$$\Delta E_{\mathrm{compl}} = E[\mathrm{Ca{-}IH(H_2O)}_x] + (8 - x) \times E(\mathrm{H_2O}) - E[\mathrm{Ca(H_2O)_8}]^{2+} - E[\mathrm{IH}^{2-}] \qquad (3)$$

Here, x is the number of water molecules bound to the metal–indicator system (1–4). It has been shown that in aqueous solution, Ca^{2+} is surrounded by eight water ligands in its first solvation shell.[9,10] The energy of the $[Ca(H_2O)_8]^{2+}$ cluster was calculated by performing a geometry optimisation in C_s symmetry. In the case of Mg^{2+}, the complexation energy given by [eqn. (5)] was calculated for equilibrium (4), since the first

$$[\mathrm{Mg(H_2O)_6}]^{2+} + \mathrm{IH}^{2-} \rightleftharpoons \mathrm{Mg{-}IH(H_2O)}_x + (6 - x)\mathrm{H_2O} \qquad (4)$$

$$\Delta E_{\mathrm{compl}} = E[\mathrm{Mg{-}IH(H_2O)}_x] + (6 - x)\cdot E(\mathrm{H_2O}) - E[\mathrm{Mg(H_2O)_6}]^{2+} - E[\mathrm{IH}^{2-}] \qquad (5)$$

hydration shell of Mg^{2+} contains six water molecules.[9,10] The geometry of $[Mg(H_2O)_6]^{2+}$ was optimised in the D_{2d} point group. It should be noted that only relative values of the complexation energy are meaningful, since only part of the solvent effect is taken into account. The stabilisation and further relaxation of the species by the bulk solution are neglected, since all clusters are still considered as gas phase systems. Therefore, only the substituent effect on the complexation energies will be considered here. We also remark that the entropic effect, which can play a very important role in the complexation process, is not considered here. The replacement of the coordination shell of the cation by the multidentate indicator results in a decrease of the free energy of the system due to the increase in translational entropy of the displaced water molecules.[11] However, the entropy effect should be less important when comparing the complexation properties of one cation with indicators with different substituents,[11] since we expect that entropy effects will be about the same for indicators with different substituents.

2.2. Continuum solvation model

A second way to describe the solvent effect by means of quantum chemical calculations is provided by the use of continuum solvation models.[12] These models mimic the bulk solvent effect. The solute is placed in a cavity inside a dielectric continuum of permittivity ε that characterises the solvent. Continuum solvation models efficiently calculate the hydration free energy of a solute. Hydration free energies of the uncomplexed indicator systems were calculated with the continuum solvation model COSMO (conductor-like screening model)[13,14] implemented in GAUSSIAN 98,[15] called CPCM, and secondly also with PCM (polarisable continuum model) using GAUSSIAN 98. The UAHF (united atom model for Hartree–Fock) model was used to construct the solute cavity, which is of molecular form.[16] Calculations were performed at the HF/6-31G* level, because the parameters needed for the construction of the molecular cavity were obtained at this level of theory by fitting with experimental hydration free energies.[16] Single point energy calculations were performed on the gas phase B3LYP structures. The free energy of hydration, ΔG_{hydr}, can be expressed as eqn. (6), where ΔG_{el} is the electrostatic component of the total

$$\Delta G_{\text{hydr}} = \Delta G_{\text{el}} + \Delta G_{\text{cav}} + \Delta G_{\text{dis-rep}} \qquad (6)$$

hydration free energy. ΔG_{cav} is a positive term, corresponding to the work spent to form a cavity of the appropriate shape and volume in the liquid, in the absence of solute–solvent interactions. $\Delta G_{\text{dis-rep}}$ includes the solute–solvent dispersion contribution and a repulsion term. These dispersion–repulsion energy terms are obtained by the procedure described in ref. 16.

3 Results and discussion

3.1. Calculations for clusters in the gas phase

A first indication of possible differences between the interaction of Ca^{2+} and Mg^{2+} with the indicators can be obtained by comparing the structures of the uncomplexed indicators (IH_3) with the complexed ones (M–IH). A picture of the DFT-optimised free and complexed Thio-H indicators with a protonated CO^3O^- group is shown in Fig. 2. In Fig. 3(I), the geometric parameters of the free indicators are shown. Fig. 4 shows the geometrical parameters for the complexed indicators with a protonated CO^3O^- group. The labeling of the atoms and the definition of the dihedral angles in the indicators is indicated in the figures. The structural parameters of the complexed indicators with a protonated CO^2O^- group (of the amino arm) are presented in Table 2.

The fluorophore of all indicators (uncomplexed and complexed) is nearly but not completely planar, as can be seen from the values of the T_1 and T_2 dihedral angles (Fig. 3(I) and 4). These angles are small, but significantly different from zero.

Ca^{2+} and Mg^{2+} both obtain a five-fold coordination environment upon complexation with the indicator. The metal ions coordinate with one oxygen of each carboxy group, with the amino nitrogen atom and with the ether oxygen of the podant. Since Mg^{2+} is a smaller ion than Ca^{2+}, we systematically observe smaller bond distances in the case of Mg^{2+}. The shortest bond distances for the cations are obtained with the oxygens of the negatively charged carboxy functions. These

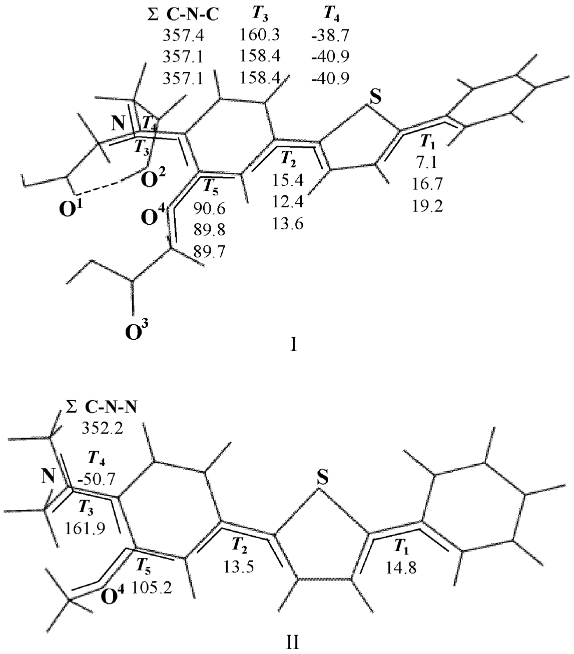

Fig. 3 (I) Structural parameters of the free indicator IH_3, with different substituents: R = CN (upper value), R = H (middle value), R = OCH_3 (lower value); (II) structural parameters of molecule A, obtained by replacing the carboxy groups of the indicator Thio-H with hydrogens (R = H). Bond distances are given in Å, angles in degrees. The labeling of the atoms and definition of the dihedral angles in the indicators are also shown.

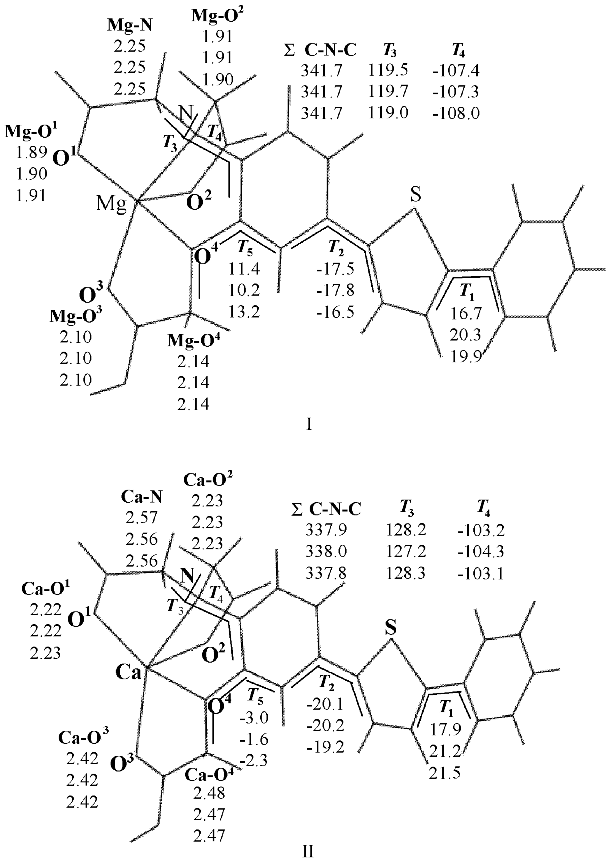

Fig. 4 Structural parameters for the complexed indicators with CO^3OH group (I: Mg^{2+} indicators, II: Ca^{2+} indicators) with different substituents: R = CN (upper value), R = H (middle value), R = OCH_3 (lower value). Bond distances are given in Å, angles in degrees. The labeling of the atoms and definition of the dihedral angles in the indicators are also shown.

Table 2 Structural parameters of the indicators with a CO^{2}OH group, complexed with Mg^{2+} and Ca^{2+}

	M–IH, M = Ca^{2+}			M–IH, M = Mg^{2+}		
t	CN	H	OCH$_3$	CN	H	OCH$_3$
M–O^1/Å	2.24	2.24	2.24	1.90	1.91	1.91
M–O^2/Å	2.40	2.40	2.40	2.06	2.07	2.07
M–O^3/Å	2.24	2.24	2.24	1.91	1.92	1.92
M–O^4/Å	2.43	2.43	2.43	2.10	2.10	2.10
M–N/Å	2.69	2.69	2.69	2.47	2.47	2.47
Σ C–N–C/°	337.6	337.7	337.7	343.1	342.9	343.1
T_1/°	14.9	18.9	16.9	14.4	19.0	17.7
T_2/°	−8.2	−7.3	−7.8	−13.9	−14.1	−10.8
T_3/°	126.8	126.8	126.8	123.5	123.4	123.7
T_4/°	−105.5	−105.3	−105.4	−102.4	−102.7	−102.1
T_5/°	0.0	0.0	−0.1	4.4	4.7	3.9

Ca–O distances are around 2.22 Å while the Mg–O bond lengths are around 1.90 Å.

The sum of the C–N–C angles around nitrogen in the indicator is a measure of the pyramidality (and hybridisation) of the nitrogen atom. In the uncomplexed indicator this sum is nearly 360 degrees, indicating that the amino nitrogen atom has sp^2 hybridisation. On the other hand, the sum of the C–N–C angles in the cation–indicator systems is significantly smaller than 360°. Complexation obviously results in a change in the hybridisation state of nitrogen from sp^2 to sp^3. The nitrogen lone pair no longer participates in the conjugated system of the fluorophore, but takes part in the binding with the cation instead, as can also be seen from the structures in Fig. 3. The binding of Ca^{2+} with the indicator results in a larger pyramidality of the amino nitrogen atom than does binding with Mg^{2+}. This indicates that the Ca–N interaction is stronger than the Mg–N interaction.

The T_3 and T_4 dihedral angles show how much the amino-group is rotated with respect to the fluorophore (see also Fig. 2). If T_3 were to be equal to 180 degrees and T_4 were to be zero degrees, then the amino group would have a completely planar aniline-like structure. In the uncomplexed indicators, the T_3 angle is around 160 degrees and T_4 is around −40 degrees. These values show that there is some distortion from the ideal aniline conformation. In the neutral form of the free indicator, an intramolecular hydrogen bond is observed, as can be seen in Fig. 2. To check if the distortion from planarity is due to this hydrogen bond, a comparison was made with molecule A. Molecule A is obtained by replacing with hydrogens the carboxy groups of the indicator Thio-H. The structure of this molecule was also optimised with B3LYP-DFT, and the resulting geometric parameters are reported in Fig. 3(II). A distortion from planarity is also observed, indicating that its cause is steric repulsion between the -CH$_3$ groups on nitrogen and the -OCH$_3$ group on the benzene ring.

As the distortion from an aniline-like conformation in molecule A and the uncomplexed indicators is relatively small, it can be expected that the nitrogen p lone-pair is still part of the conjugated system of the fluorophore. On the other hand, large structural distortions from aniline-planarity are observed for the nitrogen conformation when the indicators are complexed with Mg^{2+} or Ca^{2+}. In the complexed indicators, rotation around the C(aryl)–N bond is indeed possible because the nitrogen lone pair is involved in coordination with the cation rather than in conjugation with the fluorophore.

The structures of the M–IH clusters differing by the position of the acidic proton can also be compared. As is evident from the M–N distances in Fig. 4 and Table 2, the M–N interaction is weakest for systems with a negatively charged carboxy function of the ether arm. In this case, the cation is pulled towards the CO^{3}O$^-$ group and thus away from nitrogen.

As for the influence of the substituent on the structural parameters, no large changes in the structure of the podant are observed. Actually, the effect is most pronounced for the free indicator IH$_3$: the angle T_1, *i.e.* the dihedral angle between the thiophene and benzene ring with the substituent, is smaller if an electron-withdrawing substituent (-CN) is present, resulting in a more planar fluorophore. On the other hand, T_1 in IH$_3$ enlarges in the presence of an electron-donating group (-OCH$_3$).

Table 3 Binding energy (kcal mol^{-1}) of Ca^{2+} and Mg^{2+} to the indicators in the gas phase. For the definition of the binding energy, see text

	Protonation on CO^{3}O$^-$ group			Protonation on CO^{2}O$^-$ group		
	CN	H	OCH$_3$	CN	H	OCH$_3$
Mg^{2+}	−589	−599	−600	−583	−593	−595
Ca^{2+}	−471	−481	−483	−471	−480	−482

Gas phase binding energies (calculated according to the definition given in section 2) of Mg^{2+} and Ca^{2+} with the indicators are reported in Table 3. Since both fragments carry an opposite and two-fold charge, and the complexation is considered in the gas phase, the calculated binding energies of course have very large negative values. When comparing the binding energies for Ca^{2+} and Mg^{2+}, considerably larger values are systematically found for the latter ion, the difference being greater than 100 kcal mol^{-1}. Due to the smaller radius of the Mg^{2+} ion, the positive charge density of this ion is much higher than that on Ca^{2+}, so that the electrostatic interaction between the indicator and Mg^{2+} is stronger.

The binding energies of indicators with the carboxylic protons in different positions can also be compared. It can be observed that the binding energy of Ca^{2+} does not depend much on the position of the proton: slightly lower binding energies are observed when the proton is placed at a carboxy group of the amino arm. In the case of Mg^{2+}, however, the difference is more pronounced, since the Mg–N interaction is weakened more when protonation occurs at the carboxy group of the amino arm. The difference in binding energy is about 5–6 kcal mol^{-1}.

The influence of the substituent on the cation–indicator binding energy is similar for Ca^{2+} and Mg^{2+}, and is also independent of the position of the carboxy proton, compared to the H-substituent. The presence of the electron-withdrawing -CN substituent decreases the binding energy of Ca^{2+} and Mg^{2+} by 9–10 kcal mol^{-1}, while in the case of the electron-donating -OCH$_3$ group, the binding energy increases by only 1–2 kcal mol^{-1}. This confirms that the electron-withdrawing or -donating effect of the substituent is transmitted through the conjugated π system of the fluorophore and, as such, influences the binding capacity of the podant.

The effect of the substituent on the binding energy is

Table 4 Charges, calculated by a Mulliken population analysis, in the free indicator

	Protonation on CO^3O^- group			Protonation on CO^2O^- group		
	CN	H	OCH_3	CN	H	OCH_3
COO^1	−0.9398	−0.9396	−0.9420	−0.9468	−0.9533	−0.9535
COO^2	−0.8790	−1.0119	−1.0121	−0.9178	−0.9246	−0.9268
COO^3	−0.9121	−0.9260	−0.9284	−0.9840	0.9897	−0.9903
O^4	−0.4837	−0.4847	−0.4835	−0.4934	−0.4945	−0.4947
N	−0.5978	−0.5953	−0.5971	−0.6195	−0.6204	−0.6229

reflected in the charges on the coordinating atoms. They are shown in Table 4 for the uncomplexed indicators IH^{2-}. Although the differences in the Mulliken charges are small, one can clearly observe a less-negative charge on the carboxylic oxygens if an electron-withdrawing substituent (-CN) is present. An opposite (but smaller) effect on the charges exists for the -OCH_3 substituent, in agreement with the smaller effect on the binding energy exerted by the -OCH_3 substituent. The effect of the substituent on the Mulliken charges is largest for the oxygen atoms in the carboxy group, although they are separated from the conjugated system by the CH_2 groups between the carboxy groups and either the amino nitrogen or the ether oxygen. The effect on the charges on nitrogen and the ether oxygen is small. Our calculations indicate that the effect of the substituent is not transmitted according to the mechanism usually assumed in literature;[4] since in the latter mechanism, only nitrogen is considered as the link between the conjugated system and the cation, the substituent can only influence the nitrogen–cation interaction. Such an assumption is obviously not confirmed by our calculations.

When comparing the calculated results to the experimental findings (the trends of the dissociation constants in Table 1), two discrepancies are found, which can both be attributed to the lack of solvent effects in the calculations. Firstly, the calculations predict that, intrinsically, Mg^{2+} binds more strongly to the indicator than Ca^{2+}. However, the dissociation constants obtained in solution indicate that complexation of Ca^{2+} with the indicator is stronger than complexation with Mg^{2+}. This means that not only the interaction of the cation with the indicator, but also the interaction with the solvent plays an important role in the stability and selectivity of complexation. Indeed, considering the differences in solvation energies between the two cations, the solvent may easily reverse the ordering of the cation–indicator binding energies.[11] Due to the smaller size, and the corresponding larger charge density of the Mg^{2+} ion, the hydration free energy of this ion (the experimental value is 454 kcal mol^{-1}) is much larger than for Ca^{2+} (380 kcal mol^{-1}).[17]

Secondly, the calculated substituent effects are different from what is observed experimentally in aqueous solution. In the gas phase calculations, electron-withdrawing substituents have a destabilising effect, while the experiments only show this trend for the Mg^{2+} ion, with an opposite trend found for Ca^{2+}. One should, however, bear in mind that the experimentally observed variation in equilibrium constants (K) corresponds to only a very small variation in the free energy of the reaction ($\Delta G^{\text{reaction}}$), according to eqn. (7).

$$\Delta G^{\text{reaction}} = -RT \ln K \qquad (7)$$

Using the values for the dissociation constants from Table 1, one calculates that the corresponding effect of the substituent (going from Thio-CN to Thio-OCH_3) on the free energy of dissociation is only 0.8 kcal mol^{-1} in the case of Ca^{2+}, while for the complexation reaction with Mg^{2+}, the effect is less than 0.2 kcal mol^{-1}. We cannot expect to accurately reproduce such small effects from our theoretical calculations. However, the calculated variations in the inherent binding energies are clearly considerably larger than those found from experiment. We therefore expect that including solvent effects in the calculations will result in a weakening of the calculated substituent effects on the binding energies.

Table 5 Geometrical parameters for Mg^{2+}–Thio-H as a function of the number of water molecules bound to the clusters

	H_2O molecules				
	0	1	2	3	4
Mg–O^1/Å	1.90	1.95	2.00	2.03	3.18
Mg–O^2/Å	1.91	1.99	2.02	2.01	1.92
Mg–O^3/Å	2.10	2.14	2.22	2.09	2.09
Mg–O^4/Å	2.14	2.26	2.23	3.72	3.93
Mg–N/Å	2.25	2.35	2.55	3.82	4.21
Mg–$O_{(\text{water1})}$/Å		2.05	2.12	2.06	1.95
Mg–$O_{(\text{water2})}$/Å			2.19	2.08	2.03
Mg–$O_{(\text{water3})}$/Å				2.21	2.16
Mg–$O_{(\text{water4})}$/Å					—
Σ C–N–C/°	341.7	340.9	344.4	360.0	359.8
T_1/°	20.3	21.4	21.9	15.1	19.5
T_2/°	−17.8	−19.1	−23.1	−28.1	−19.7
T_3/°	119.7	117.9	129.1	143.7	136.6
T_4/°	−107.3	−109.5	−94.6	−35.5	−38.2
T_5/°	10.2	16.6	−4.5	30.6	16.7

3.2. The solvent effect

The gas phase calculations showed that the metal ions acquire a five-fold coordination through complexation with the indicator. However, in most complexes of Mg^{2+} and Ca^{2+}, a higher coordination number can be obtained. For Mg^{2+}, an octahedral six-coordination is found to be dominant for oxygen donor ligands. In aqueous solution, the first hydration shell of Mg^{2+} contains six water molecules.[9,10] The most common coordination number for Ca^{2+} ranges between six and nine. In water this ion is surrounded by eight water ligands in its first solvation shell.[9,10] It is therefore likely that in the indicator complexes in solution, these metal ions will not only coordinate to the indicator, but also to additional water ligands. To examine these specific interactions with the solvent by means of the DFT calculations, we have gradually added water molecules to the complexed indicator systems. Since it was found that the effect of the substituent was independent of the position of the proton, only indicator systems in which the carboxy group of the ether arm is protonated are considered for these calculations, because these clusters show the largest inherent binding energy. The geometrical parameters for these systems are shown in Table 5 for Mg^{2+}, and in Table 6 for Ca^{2+}. The structures of the Mg^{2+} indicators with one, two and three water molecules are also shown in Fig. 5.

The addition of one or two water ligands to the cation–indicator system results in only a small effect on the coordination geometry of the metal ions in the indicator. Bond distances of the cations become slightly larger due to the larger coordination number. The effect on the metal–nitrogen bond distance is most pronounced. In the case of Mg^{2+} and Ca^{2+}, an elongation of 0.2–0.3 Å of the M–N bond distance is found upon addition of two water molecules, while the M–O distances

Table 6 Geometrical parameters for Ca^{2+}–Thio–H, as a function of the number of water molecules bound to the clusters

	H_2O molecules				
	0	1	2	3	4
Ca–O^1/Å	2.22	2.31	2.34	2.43	2.37
Ca–O^2/Å	2.23	2.25	2.33	2.32	2.29
Ca–O^3/Å	2.42	2.44	2.47	2.48	2.43
Ca–O^4/Å	2.47	2.54	2.57	2.83	3.79
Ca–N/Å	2.56	2.65	2.76	4.02	3.91
Ca–$O_{(water1)}$/Å		2.35	2.37	2.35	2.36
Ca–$O_{(water2)}$/Å			2.40	2.36	2.36
Ca–$O_{(water3)}$/Å				2.58	2.50
Ca–$O_{(water4)}$/Å					4.07
Σ C–N–C/°	338.0	337.9	338.8	359.7	359.7
T_1/°	21.2	21.3	21.7	15.1	16.7
T_2/°	−20.2	−20.2	−25.8	−0.3	−23.3
T_3/°	127.2	129.7	137.0	119.1	151.1
T_4/°	−104.3	−101.6	−93.4	−55.3	−34.8
T_5/°	−1.6	−3.9	−10.2	−44.2	13.2

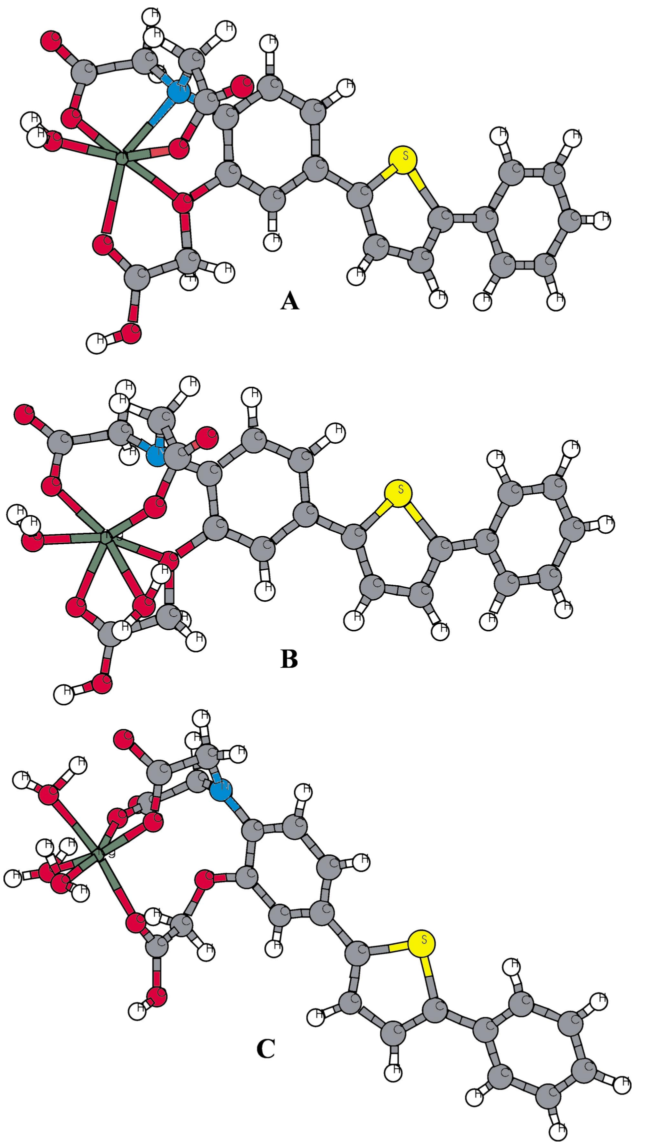

Fig. 5 Illustration of the optimised clusters of Thio-H complexed with Mg^{2+} in the presence of one (A), two (B) and three (C) water molecules.

are affected by only 0.1 Å. The sum of the three C–N–C angles remains smaller than 360°, indicating that the amino nitrogen atom keeps its sp^3 character. When two water molecules are added, the T_3 angle increases, while the T_4 angle becomes smaller. The effect on these angles is about 10 degrees. This shows that the distortions, induced in the indicator upon complexation with the cation, partially relax when one or two additional water molecules coordinate to the cation. However, the deviations of these angles from the values for the uncomplexed indicator are still large, demonstrating that the nitrogen lone pair remains involved in the coordination with the metal ion and is no part of the conjugated system.

However, when a third water ligand is added, the coordination environment of the cations changes more drastically. The metal ion remains coordinated to the three oxygens of the carboxy groups, while the metal–ether oxygen distance increases. The coordination of Mg^{2+} with the ether oxygen is completely lost, while in the case of Ca^{2+}, the bond distance to O^4 becomes longer, without losing its coordination. The coordination of both metal ions to the nitrogen atom is completely given up, the Mg–N distance becomes 3.82 Å, the Ca–N distance is 4.02 Å. The loss of the coordination of the metal with nitrogen is also reflected in the change in hybridisation state of the nitrogen atom. The sum of the three C–N–C angles becomes 360°, indicating that the hybridisation state of nitrogen is again sp^2. However, for the Ca^{2+} ion this does not mean that the nitrogen lone pair again participates in the conjugated system of the fluorophore. This can be seen from the values of the T_3 and T_4 dihedral angles in Table 6, which are still considerably different from the corresponding values for the free indicator (Fig. 3). On the other hand, in the indicator system with Mg^{2+}, the distortion of the indicator due to metal coordination is smaller (Table 5 as compared to Fig. 3), and the values of T_3 and T_4 remain much closer to those observed for the uncomplexed indicator. This shows that the presence of solvent molecules relaxes the distortions induced in the indicator by Ca^{2+} and Mg^{2+}, re-introducing the nitrogen lone pair in the conjugated system only in the case of Mg^{2+}.

For neither cation does the addition of a fourth water molecule to the indicator systems produce additional coordination to the metal. The water ligand instead forms hydrogen bonds with one of the uncomplexed oxygens of the carboxy groups and with one of the water ligands in the first hydration shell of the metal ion. Several structures are possible, with a different position of the fourth water molecule. The structural parameters of these systems only show small differences, and only the geometrical parameters of the most stable structure are given in Tables 5 and 6. Upon addition of a fourth water molecule, the T_3 and T_4 angles in the Ca^{2+} indicator become much closer to the values in the free indicator. This indicates that the distortions induced in the indicator due to complexation are also, in the case of Ca^{2+}, relaxed by the presence of the solvent. It is beyond the purpose of this study to examine the second solvation shell of the metal indicator systems, so we assume that in aqueous solution, the first coordination shell of the cation is approximately described by the clusters where three water ligands are included.

The complexation energy (calculated according to the definition given in section 2) shows the effect that bonding with additional water molecules has on the strength of the binding of the metal ions with the indicator. The complexation energy (reported in Table 7) takes into account the effect of the change in coordination of the metal ion in the presence of the first hydration shell, described by the added water molecules. The stabilisation and further relaxation of the species by the bulk solution is still neglected, since all species are still considered as gas phase systems. It was found that the more water molecules are considered in the metal–indicator system, the more negative becomes the complexation energy. However, the trend of adding water molecules to the metal–indicator system is the same for the different substituents, and also for Mg^{2+} as compared to Ca^{2+}. For Thio-CN, the complexation energy remains 8–9 kcal mol^{-1} smaller than for Thio-H, no matter how many water molecules are considered in the clusters. For Thio-OCH_3, the

Table 7 Complexation energies (kcal mol^{-1}) of the metal–indicator systems as a function of the number of water molecules present and the substituent. For the definition of the complexation energy, see text

	CN	H	OCH_3
CaI	−103	−112	−114
$CaI(H_2O)_2$	−174	−182	−185
$CaI(H_2O)_3$	−202	−210	−213
$CaI(H_2O)_4$	−228	−236	−238
MgI	−176	−186	−188
$MgI(H_2O)_3$	−255	−264	−265

Table 8 Hydration free energies (kcal mol^{-1}), calculated with CPCM at the HF/6-31G* level

	CN	H	OCH_3
Protonation on CO^3O^-	−146	−155	−154
Protonation on CO^2O^-	−156	−165	−164

complexation remains 2–3 kcal mol^{-1} stronger than for Thio-H. The calculations thus clearly indicate that the substituent effect is transmitted through the carboxy groups, since it is still observed when the cation–nitrogen interaction is lost by adding water molecules.

Finally, the effect of the bulk solution was considered by calculating the hydration free energy of the participating species, using continuum solvation models. Hydration free energies, calculated with CPCM, are reported in Table 8 for uncomplexed indicators (IH^{2-}). The hydration free energies obtained with PCM did not differ more than 1 kcal mol^{-1} from the CPCM values. The bulk solvent effect of the metal–indicator complexes[18] is not considered; we expect this effect to be much smaller than in the case of the free indicators, because the complexed indicators are neutral, whereas the uncomplexed indicators are charged.

As is evident from Table 8, the hydration free energy of the uncomplexed indicator (IH^{2-}) is dependent on the substituting group. Firstly, the electrostatic contribution to the hydration free energy of the free indicators depends on the character of the substituent. An electron donating substituent will increase the dipole moment of the system; therefore, the indicator is more stabilised by a polar solvent. However, ΔG_{cav} is also dependent on the substituent, because more voluminous substituents will give rise to larger cavitation energies (in the order $OCH_3 > CN > H$). The dispersion and repulsion terms do not vary significantly for the considered substituents. The sum of the above trends determines the trend of the total hydration free energy. The stabilisation of the free indicator by the solvent is largest for the unsubstituted indicator and Thio-OCH_3. For Thio-CN, the hydration free energy becomes smaller by 9 kcal mol^{-1}; this electron-withdrawing group obviously decreases the dipole moment of the indicator significantly.

It is thus shown that the solvent will influence the complexation of the indicators with Ca^{2+} and Mg^{2+} significantly. The electron-withdrawing CN-substituent will decrease the inherent binding energy of the indicator with the cations by about 9 kcal mol^{-1}, but the bulk solvent will exert the opposite effect, since it stabilises the free indicator by the same amount of energy. Thus, these two opposite effects result indeed in an independence of the cation–indicator interaction for Thio-H and Thio-CN. The effect of the -OCH_3 substituent is much smaller, calculations predict both similar inherent binding energies and solvent effects as for Thio-H. A third effect is the solvent effect on the complexed indicators, which is expected to be small and not much dependent on the substituent, but might cause the experimentally observed differences in complexation behavior observed for Ca^{2+} and Mg^{2+}.

4 Summary

The theoretical calculations have provided more insight into the complexation of indicators with Mg^{2+} and Ca^{2+}. Gas phase calculations show that these metal ions reach a five-fold coordination upon complexation with the indicator. Both metals coordinate to one oxygen of each carboxy group, the amino nitrogen and the ether oxygen. Coordination to nitrogen results in a change of the hybridisation state of this atom from sp^2 to sp^3, because the nitrogen lone pair becomes involved in the coordination and cannot participate in the conjugated system.

The discrepancies between gas phase calculations and experimental observations are due to solvent effects, which play a significant role in the complexation. In solvent, the calculations predict that three water molecules are present in the first coordination sphere of the cations, when complexed with the indicator. By including these water molecules, the cation loses its coordination to the amino nitrogen. This results in a planarisation of this nitrogen atom and a relaxation of the strain imposed on the indicator by coordination with the metal ion, such that the nitrogen lone pair can again participate in the conjugated system.

The variation in dissociation constants as a function of the substituent is determined mainly by the following two factors. Firstly, there is the inherent binding energy of the indicator with the metal, which is favored by electron-donating substituents and weakened by electron-withdrawing groups. Secondly, there is a stabilising effect of the solvent on the free indicators; due to their smaller dipole moment, free indicators with electron-withdrawing groups are stabilised less by the solvent. Since these two effects on the complexation strength show a strong but opposite trend, both effects tend to cancel each other when the solvent is taken into consideration.

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References

1 G. Grynkiewicz, M. Poenie and R. Y. Tsien, *J. Biol. Chem.*, 1985, **260**, 3440.
2 R. Y. Tsien, *Annu. Rev. Neurosci.*, 1989, **12**, 227.
3 E. Cielen, PhD Thesis, K. U. Leuven, 1999.
4 S. Fery-Forgues, J. Bourson, L. Dallery and B. Valeur, *New J. Chem.*, 1990, **14**, 617.
5 C. Reinhardt, *Solvents and solvent effects in organic chemistry*, VCH, Weinheim, 1990.
6 R. Ahlrichs, M. Bär, M. Häser, H. Horn and C. Kölmel, *Chem. Phys. Lett.*, 1989, **162**, 165.
7 A. Schäfer, H. Horn and R. Aldrichs, *J. Chem. Phys.*, 1992, **97**, 2571.
8 A. Pullman, *Quantum theory of chemical reactions*, *Volume II*, ed. R. Daudel, A. Pullman, L. Salem and A. Veillard, Reidel Publishing Company, Dordrecht, 1980, p. 1.
9 F. M. Floris, M. Persico, A. Tani and J. Tomasi, *Chem. Phys. Lett.*, 1994, **227**, 126.
10 M. Pavlov, P. E. M. Siegbahn and M. Sandström, *J. Phys. Chem. A*, 1998, **102**, 219.
11 J. M. Lehn, *Alkali Metal Complexes with Organic Ligands, Structure and Bonding 16*, Springer Verlag, Berlin, 1973, p. 1.
12 J. Tomasi and M. Persico, *Chem. Rev.*, 1994, **94**, 2027.
13 V. Barone and M. Cossi, *J. Phys. Chem. A*, 1998, **102**, 1995.
14 A. Klamt and G. Schüürmann, *J. Chem. Soc.*, *Perkin Trans. 2*, 1993, 799.
15 *Gaussian 98* (Revision A. 5), M. J. Frisch, G. W. Trucks, H. B. Schlegel, P. M. W. Gill, B. G. Johnson, M. A. Robb, J. R. Cheeseman, T. A. Keith, G. A. Petersson, J. A. Montgomory, K. Raghavachari, M. A. Al-Laham, V. G. Zakrzewski, J. V. Ortiz, J. B. Foresman, J. Cioslowski, B. B. Stefanov, A. Nanayakkara,

M. Challacombe, C. Y. Peng, P. Y. Ayala, W. Chen, M. W. Wong, J. L. Andres, E. S. Replogle, R. Gomperts, R. L. Martin, D. J. Fox, J. S. Binkley, D. J. Defrees, J. Baker, J. P. Stewart, M. Head-Gordon, C. Gonzalez and J. A. Pople, Gaussian, Inc., Pittsburgh PA, 1998.

16 V. Barone, M. Cossi and J. Tomasi, *J. Chem. Phys.*, 1997, **107**, 3210.

17 W. E. Morf and W. Simon, *Helv. Chim. Acta*, 1971, **54**, 794.

18 The calculation of the hydration free energies of the complexed indicators using continuum solvation models is not straightforward. Firstly, the radii needed for the construction of the molecular cavity are not optimised in the UAHF model for Mg^{2+} and Ca^{2+}. Therefore, the Mg and Ca atomic radii were used. Secondly, for the complexed indicators, we observed large discrepancies between the hydration free energy calculated with PCM and CPCM, both for the absolute and relative values. We expect that these problems arise from the complex shape of the systems.