Synthesis and evaluation of colorimetric chemosensors for monitoring sodium and potassium ions in the intracellular concentration range

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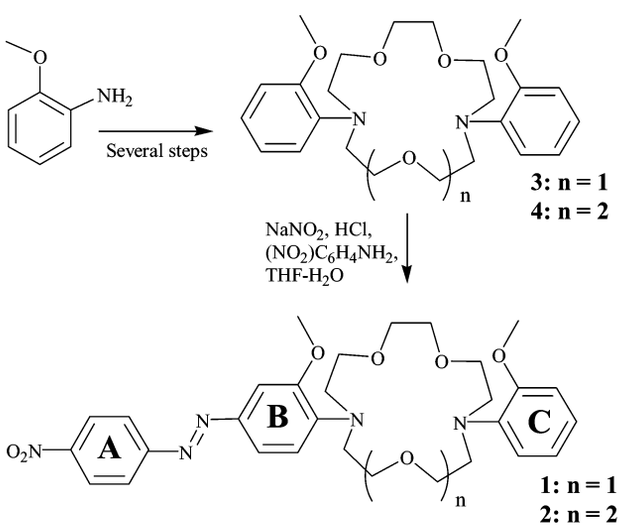
The azo-dye based colorimetric chemosensors **1** and **2** were designed for the detection of intracellular concentrations of Na^+ and K^+ respectively. The two sensors were synthesised from the *o*-anisidine based receptors **3** and **4** in a one step synthesis. Both displayed good selectivity and sensitivity for the relevant group I cations in the appropriate physiological conditions, with **1** displaying large changes in the absorption spectra upon Na^+ recognition with λ_{max} being shifted from 488 nm to 377 nm in the absorption spectra when measured in 50 : 50 MeOH–H_2O buffered pH 7.4 solutions and in the presence of high ionic strength. Similarly, **2** showed both a hypsochromic shift and hypochromic effect upon titration with K^+. These changes were clearly visible; with red to yellow colour changes taking place upon ion recognition.

Introduction

The recognition and targeting of ions and small molecules is an active area of research within the field of supramolecular chemistry.[1] Recently, fluorescent and luminescent sensors have been developed for the selective recognition of cations,[2] anions[3] and neutral molecules.[4] Concurrently, the use of colorimetric sensors or chromoionophores has been somewhat less investigated.[5,6] Like fluorescent chemosensors, the detection can be non-invasive, real-time and on a molecular scale.[1] However, the real advantage is that the recognition event, is visible to the naked eye, has reliable calibration stability, as well as being immune to biofouling such as autofluorescence and light scattering, as well as electrical interferences. Because of this, several researchers have recently developed optically based chemosensory systems for the recognition and monitoring of biologically relevant substrates with the aim of replacing the more conventional sensory devices such as electrochemical or flame spectroscopy.[7] Such devices have also potential use as probes for determining synthetic intermediates, for use in chiral recognition, for screening combinatorial libraries, *etc*.[8] Consequently, colorimetric sensors are being developed for use on solid supports, such as on gold nanoparticles for observing biomolecular interactions, as monolayers on glass surfaces, in polymeric matrices as films and in ion selective optodes.[9]

We have been interested in the development of luminescent[10,11] and colorimetric sensors that emit or absorb at long wavelengths (in the red and near infrared), and we have recently used simple azo-dyes as reporters for the potential recognition of sodium in serum, where good selectivity for Na^+ over K^+ in the Na^+ extracellular concentration range (133–145 mM for blood sodium *vs*. 3.5–4.8 mM for K^+) was observed.[12] Consequently, our intention was to expand this research work and to develop chromogenic chemosensors for the potential monitoring of cytosolic concentrations of Na^+ (10–40 mM for animal cells) and K^+ (upper limits of 120 mM).[13] In our earlier work we used an *o*-anisidine derived 15-crown-5-ether receptor, where the 2-methoxy group was shown by ^{1}H NMR studies to induce a lariat ether effect by directly coordinating to the alkali ion giving an extra coordination site.[12] Inspired by these results and those of Tsien *et. al.*[14] who developed fluorescent indicators for determining cytosolic sodium concentration (by measuring the changes in the fluorescence excitation spectra at *ca.* 350 nm), and de Silva *et. al.*[15] who developed photoinduced electron transfer (PET) sensors for Na^+, we synthesized the two bis *o*-anisidine based receptors **3** and **4**[16] (Scheme 1) with the aim that the two *o*-anisidine moieties would give rise to enhanced selectivity and sensitivity to allow for discrimination between Na^+ and K^+ for **3** and the reverse for **4** at cytosolic concentration ranges. Our preliminary X-ray crystal structure analysis of **3** and **4**, showed indeed that both formed complexes with Na^+ and K^+, yielding **3**·Na and **4**·K respectively.[16] In these, the ions were coordinating to the oxygens and the two nitrogens of the ring, but additionally both coordinated to the two-methoxy groups. This gave rise to dramatic structural changes in the receptor since the two aromatic rings became



Scheme 1 Synthesis of the two colorimetric chemosensors **1** and **2** from the corresponding bis *o*-anisidine (2-methoxyaniline) crown ethers **3** and **4**. **A**, **B** and **C** are numbering systems for the three aromatic rings (see Results and discussion).

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almost orthogonal to the nitrogens of the crown ether. With this in mind we set out to incorporate these receptors into colorimetric chemosensors. We predicted that since the ion recognition would lead to substantial reduction in conjugation between the lone pairs of the aromatic ring and the amine, any ICT (Internal Charge Transfer) based chemosensors would be expected to give rise to large colour changes upon ion recognition, since the ICT character should be diminished. Consequently **1** and **2** were designed and synthesised for the selective sensing of Na^+ and K^+ respectively. Here we give a full account of our results.

Results and discussion

The synthesis of **1** and **2** is shown in Scheme 1, from **3** and **4** respectively. We have recently reported the synthesis of **3** and **4**, which were made from *o*-anisidine in several high yielding steps, and the X-ray crystal structures of the corresponding **3**·Na and **4**·K complexes.[16] We found that it was necessary to develop a new synthetic strategy for these receptors since the direct incorporation of 2-methoxybenzene into diaza-15-crown-5 and 18-crown-6-ethers was not possible. The two chemosensors **1** and **2** were formed in 49 and 44% yield respectively after column chromatography on neutral alumina (70 : 30 hexane–ethyl acetate), as deep red solids, by reacting either **3** or **4** with *p*-nitroaniline (in an equimolar amount) by a method described by Vögel *et. al*.[5] **1** and **2** were characterised by using conventional methods. For instance the electrospray mass spectra (ESMS) for **1** showed a single peak at 580.23 for **1**·H^+. ^1H NMR of **1** also indicated that the C_{2V} symmetry previously observed in the ^1H NMR of **3** was not present, but two distinguishable singlets for the aromatic methoxy groups were observed at 3.91 and 3.83 ppm. Similar results were observed for **4** (see Experimental section). The formation of the symmetrical azo-dye species, *i.e.* the introduction of two azobenzene chromophores into **3** or **4**, was only observed in a minimal amount under these reaction conditions. We have however, not yet investigated their photophysical properties. In the remainder of this paper the results of the ability of **1** and **2** to act as selective chemosensors for Na^+ and K^+ respectively, in the appropriate cytosolic concentration ranges, of these ions will be discussed. These measurements were carried out in 50 : 50 MeOH–H_2O at pH 7.4 (0.1 M TRIS buffer) and in the presence of 0.1 tetramethylammonium chloride (TMACl) to maintain constant ionic strength. †

Evaluating the pK_a and the binding of group I and II alkali ions to the receptors 3 and 4

For use in physiological environment it is essential that the chemosensors are independent of pH above 6.5. Both **1** and **2** have two aromatic amino moieties (the two aza-crown amino moieties) that are susceptible to protonation (aromatic units **B** and **C** in Scheme 1). One could argue that these should have quite distinguishable pK_as since one of these is in conjugation with the aza-dye. The aza-dye has a strong internal charge transfer (ICT) ground state transition that is due to the electron withdrawing nitrogen moiety and the electron donating amine, giving rise to an internal charge transfer excited state (ICT). This ICT effect should substantially reduce the pK_a of amine in the **B** aromatic moiety. With this in mind we first set out to determine the pK_a of the two receptors. All binding values (and other physical parameters) discussed in this section are summarised in Table 1.

For **3**, the pK_as were determined spectrophotometrically in 50 : 50 MeOH–H_2O by UV–VIS absorption titration. The absorption spectra of both **3** and **4** in alkaline solution were very similar. **3** showed an absorption band at 253 nm (ε = 3.42) and a shoulder at 277 nm (ε = 1.79) in the free (or uncomplexed) form. The pK_a was determined from the changes in the 253 nm band upon acidification, by plotting the absorption changes as a function of pH (Abs. *vs.* pH profile). It was not possible to accurately determine two independent pK_as from these changes, but an average pK_a of 4.7 was determined. For **4**, with absorption bands at 250 and two minor bands at 273, and 277 nm, two pK_as of 7.0 (±0.1) and 4.9 (±0.1) were more easily determined, most likely since the charge repulsion between the two aromatic ammonium moieties is smaller than that seen in **3**. However, it is also possible that for **3** we are seeing an average pK_a value for the protonation of the two nitrogen moieties.

† UV–VIS spectra were recorded on a Shimadzu UV-2401 PC UV–VIS spectrometer (See Experimental section).

Table 1 The various physical parameters measured for **1**–**4**

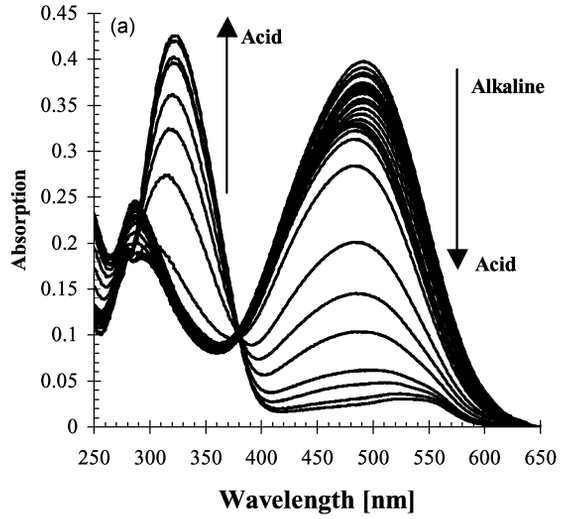
	3	**4**	**1**	**2**
λ_{max}/nm	253(3.42)	250(3.4)	491(4.36)	473(4.32)
(ε/cm^{-1} M^{-1})	277(1.79)	273(1.80)	287	2.82
pK_a	~4.7	7.0(±0.1)	6.1(±0.1)	6.3(±0.1)
		4.9(±0.1)	3.0(±0.1)	2.9(±0.1)
pNa	3.1(±0.1)	4.0(±0.1)	2.3(±0.1)	2.5(±0.1)
pK	<2.3[a]	3.9(±0.1)	1.6(±0.1)	2.1(±0.1)
pLi	2.5(±0.1)	<1[a]	1.8(±0.1)	<1[a]
pMg	<1[a]	<1[a]	<1[a]	<1[a]
pCa	<1[a]	<1[a]	<1[a]	<1[a]

[a] We were unable to determine these accurately.

The ability of **3** and **4** to selectively bind Na^+ and K^+ respectively was also investigated by carrying out UV–VIS spectroscopic titration. For **3**, a good selectivity was observed for Na^+ over that of K^+ with log β (binding constants) being 3.1 (±0.1) and <2.3 for Na^+ and K^+ respectively. The binding of other alkali group I and II cations was also tested, and for **3** these gave rather poor binding, with log β < 1 for both Mg^{2+} and Ca^{2+} but for Li^+, log β of 2.5 was determined. However, the affinity for Li^+ is an order of magnitude lower than for Na^+. A reverse trend was seen for **4**, the K^+ being slightly selective over that of Na^+. Furthermore, smaller absorption changes were observed for **4** upon addition of the above ions, with logβ = 4.0 (±0.1) for K^+ and 3.9 (±0.1) for Na^+. For Li^+, Mg^{2+} and Ca^{2+}, the changes were too small for accurate binding constant determination.

pK_a determination of chemosensors 1 and 2

As described above, the pK_a for **1** and **2** was also determined using spectrophotometric titration. In alkaline solution, **1** gave a strong absorption band with λ_{max} at 490 nm and a smaller band at 289 nm (Fig. 1a). Upon addition of acid the absorption at 490 gradually decreased, and a new band centred at 321 nm appeared, with a clear isosbestic point at 379 nm. The absorption at 279 nm was also affected upon acidification being slightly red shifted. From these changes two pK_as were determined as 6.1 (±0.15) and 3.0 (±0.1) for **1**. Of these the second pK_a of 3.0 was easily determined since large changes were observed in the absorption spectra between 4.1 and 1.5, with the absorption at 488 nm giving rise to *ca.* 95% of the total changes in the absorption spectra from pH 10–1.5. This can be seen in Fig. 1b. The two pK_as were assigned to the two nitrogen crown ether moieties (assigned as aromatic rings **B** and **C** in Scheme 1), the former to the methoxyaniline unit in ring **C** (Scheme 1), whereas the second one is due to the protonation of the aza dye nitrogen moiety, ring **B**. Here the strong electron withdrawing nitrogen moiety causes the electron density at the amino moiety to be substantially reduced and hence protonation is more difficult. Similar results were observed for **2**, as seen in Fig. 1b when measured at 473 nm. However, here the changes were somewhat clearer with stepwise protonations being observed with pK_as of 6.3 (±0.1) (for nitrogen moiety of **C**) and 2.9 (±0.1) (for **B**). This indicates that for sensor **1** only minor interferences, or pollution, could be expected in



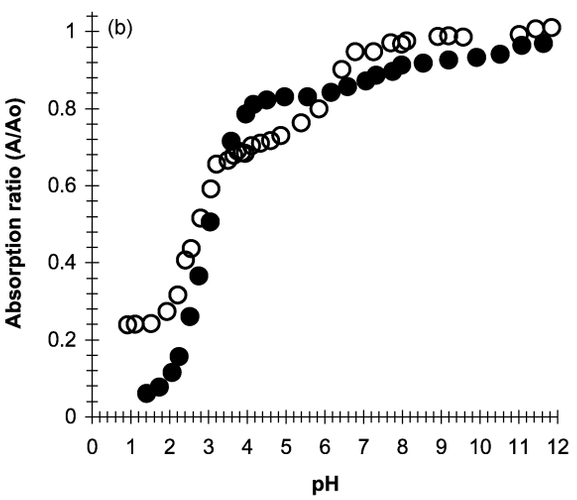


Fig. 1 (a) The changes in the absorption spectra of **1** as a function of pH. (b) A pH titration profile (Abs. ratio *vs.* pH) of **1** and **2** showing the stepwise protonations of the aniline nitrogen moieties in the receptor part of both **1** (●) and **2** (○) (in 50 : 50 MeOH–H_2O). Both were measured at λ_{max}.

the pH physiological range, but for sensor **2**, such interference would be somewhat greater below pH 6.5.

Evaluating the sensing ability of 1 and 2 towards Na^+ and K^+ respectively †

In 50 : 50 MeOH–H_2O at pH 7.4, the absorption spectra of **1** consisted of a strong band centred at 488 nm (λ_{max}, logε = 4.36), and a smaller band at 284 nm, Fig. 2, with an absorption minimal at 360 nm. This solution was deep red. Upon addition of Na^+ (from stock solutions of $NaCO_2CH_3$) the absorptions at 488 and 284 nm decreased dramatically in intensity, whereas the intensity at 360 nm increased, and was gradually shifted to 379 nm. An isosbestic point was observed at *ca.* 402 nm, but the absorption spectra of the receptor **3** showed a band tailing into this region, which made accurate determination difficult. After the addition of 100 mM of Na^+ the band at 488 nm had almost disappeared and the solution had changed colour from red to yellow, which was clearly visible, as shown in Fig. 3. The binding affinity of **1** towards Na^+ was determined from the changes in the absorption at 488 nm as a function of pNa ($-\log [Na^+]$), Fig. 4. Here the changes can be seen to take place over two pM units, being 'switched off' from *ca.* 3.6 to 1.5 pNa (or between 0.25 mM and 32 mM), which is an indication of simple equilibrium and 1 : 1 binding. From these changes a binding constant log β was determined as 2.3 (±0.1). A similar binding

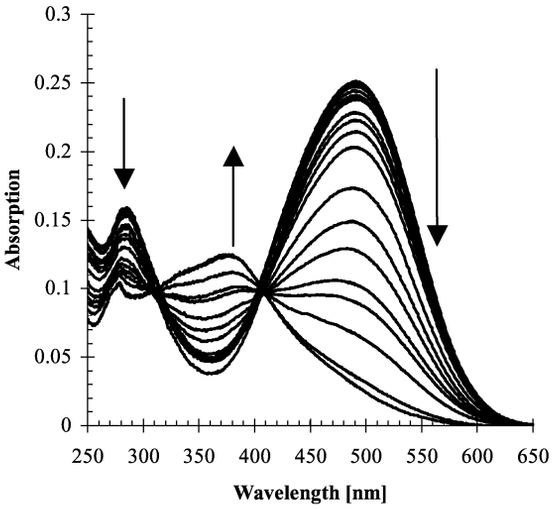


Fig. 2 The changes in the absorption spectra of **1** upon addition of Na^+ (as CH_3CO_2Na) in 50 : 50 MeOH–H_2O at pH 7.4 and 0.1 M TMACl. The absorption with λ_{max} at 488 nm decreases upon addition of Na^+ between $[Na^+] = 0 \longrightarrow 0.5$ M.

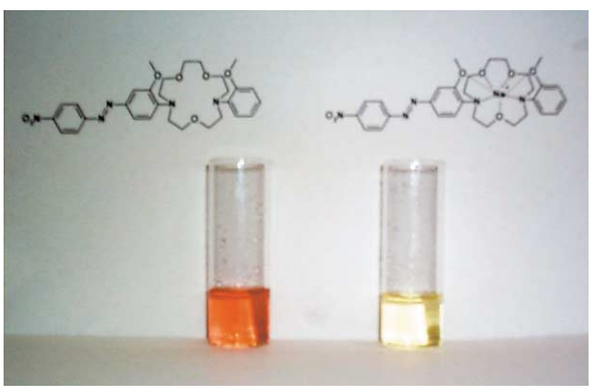


Fig. 3 The changes in the colour of the solution of **1** (red) upon addition of 100 mM of Na^+ (yellow).

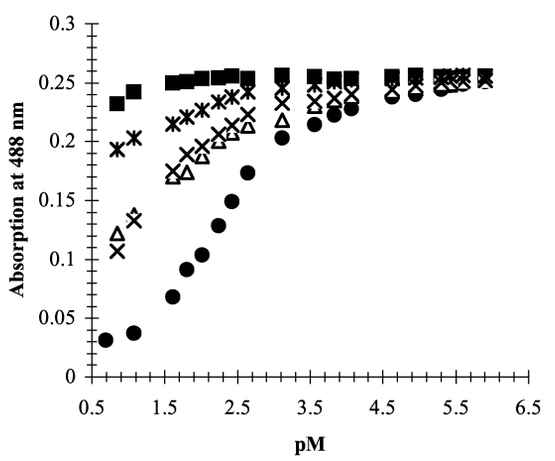


Fig. 4 The changes in the absorption spectra of **1** at 488 nm as a function of pM [pM = −log[M], M = Na^+ (●), K^+ (×), Li^+ (△), Mg^{2+} (■) and Ca^{2+} (✳)].

value was observed using NaCl, indicating that the counter anion was not affecting the absorption spectra.

The sensitivity and the selectivity of **1** was also investigated by titrating **1** using several biologically important group I and II cations (Fig. 4). In particular, we were interested in determining the affinity of **1** for K^+, which is the major group I competitor of Na^+. The changes in absorption spectra of **1** upon addition of K^+ (as KCO_2CH_3) can be seen in Fig. 5. As

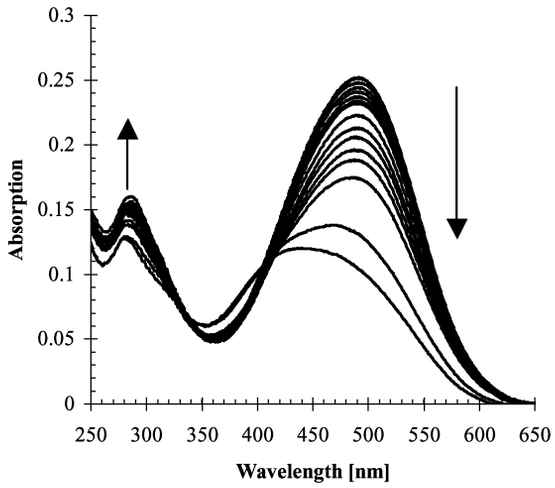


Fig. 5 Changes in the absorption spectra of **1** upon addition of K^+ (as CH_3CO_2K).

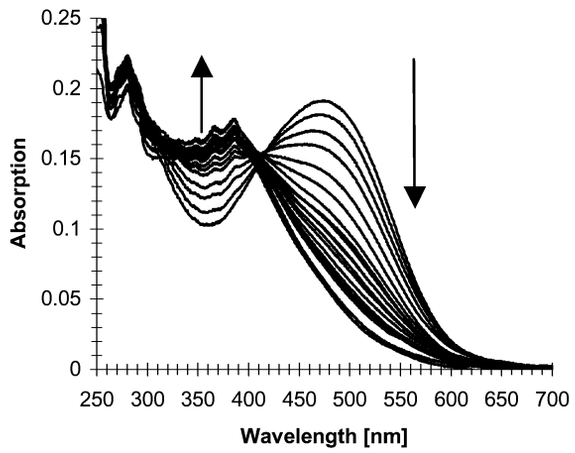


Fig. 6 The changes in the absorption spectra of **2** upon addition of K^+ (as CH_3CO_2K).

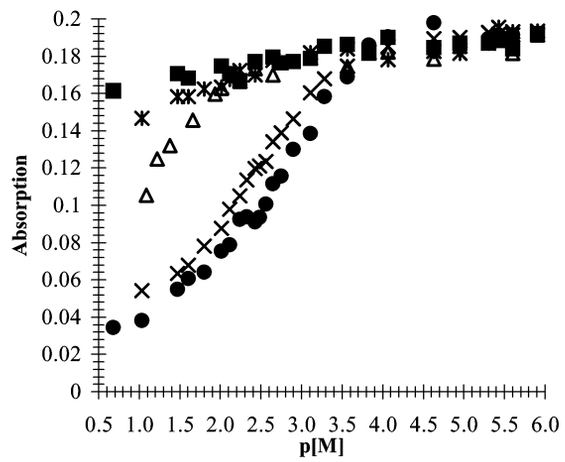


Fig. 7 The changes in the absorption spectra of **2** at 473 nm as a function of pM. pM [pM = −log[M], M = Na^+ (●), K^+ (×), Li^+ (△), Mg^{2+} (■) and Ca^{2+} (✳)].

before for Na^+, the major changes occur in the 488 nm and 289 nm bands, but unlike that seen before, the changes at *ca.* 360–70 nm were smaller. However a clear isosbestic point was observed at 409 nm. Plotting the changes in the absorption spectra at 488 nm *vs.* p*K*, we estimate the p*K* to be *ca.* 1.6 (±0.1) but due to solubility problems we were unable to fully obtain a two unit sigmodal curve. However, as seen from Fig. 4, smaller absorption changes are observed than seen for Na^+, and the affinity for K^+ is at least an order of magnitude smaller. It is thus obvious that **1** is a highly selective chemosensor for Na^+ even at high K^+ concentrations. The affinity towards other physiologically active ions was rather poor, for Mg^{2+} and Ca^{2+} the absorption spectra only change slightly between pM = 2.4–1. These changes were too small for accurate binding constant determination. This is to be expected since it is well known that these ions are better complexed using EDTA type ligands. We also investigated the sensitivity of **1** towards Li^+, which is a known drug for mental disorder and as such is often found in both serum and in blood. As expected, due to its small size and high charge density, Li^+ did induce some changes in the absorption spectra of **1**, but only at higher concentrations (Fig. 4). From these changes we estimate the binding affinity to be similar to that observed for K^+, *ca.* 1.8 or so.

The complexation of Na^+ to **1** was also monitored by recording the ^{1}H NMR. Here the changes in the methoxy resonances and in the crown ether protons were very pronounced. Some changes were also observed in the aromatic regions for the protons assigned to the receptor site of **1** (rings **B** and **C** in Scheme 1), whereas the changes in those protons assigned to ring **C** were not noticeably shifted.

The sensitivity and selectivity of the potassium sensor **2** was evaluated in a similar manner to that described above. The effect in the absorption spectra upon adding K^+ (as acetate solution) to **2** can be seen in Fig. 6. Prior to the titration, an absorption spectrum similar to that seen for **1** was observed with λ_{max} at 473 nm (logε = 4.32), with a second band at 282 nm, and an absorption minimum at 354 nm. Upon titration with K^+, the absorption at 473 nm reduced substantially, and the absorption at 354 nm increased. An isosbestic point was observed at *ca.* 412 nm, but upon addition of high concentrations of K^+ (*ca.* 0.05 M) the absorption at this wavelength became slightly blue shifted (by $\Delta\lambda \longrightarrow 10$ nm). The band at 282 nm was also slightly affected. Unlike that seen for **1**, the absorption of **2** at 354 nm also developed three clear bands at 384, 365 and 347 nm. As before these changes were visible to the naked eye with the colour of the solution changing from red to yellow.

As shown above for **1**, the binding constant for K^+ was determined from the changes in the absorption spectra. The changes in the absorption spectra at 473 nm were plotted as a function of pK, Fig. 7. As before the profile was sigmodal, being 'switched off' between *ca.* 3.5 and 1 pK (between 100 mM and 3 mM). This is a rather larger switching range than was seen for the Na^+ profile of **1** (Fig. 4). However, we believe that it signifies a 1 : 1 binding and simple equilibrium. From these changes a logβ = 2.54 (±0.1) was determined. This is over an order of magnitude higher than the binding affinity seen for **1** upon K^+ recognition. This is to be expected since the larger 18-crown-6 cavity of the receptor can accommodate the ion preferentially over the smaller 15-crown-5 ether cavity of **1**. As before, the K^+ recognition of **2** was also monitored by ^{1}H NMR, where the major changes were seen for the two methoxy resonances and the crown ether protons.

We also evaluated the sensitivity of **2** towards other group I and II cations. To our surprise the affinity of **2** towards Na^+ was almost the same as that for K^+, Fig. 7. Here the absorption spectra of **2** was clearly 'switched off' between pNa = 1.2–3.5, almost overlapping with the affinity of K^+. From these changes we determined the binding constant for Na^+ to be 2.1 (±0.1) Concurrently, the affinity for Li^+, Mg^{2+} and Ca^{2+} was rather poor, only detecting Ca^{2+} above 2 pCa units. We were unable to measure the binding constant for any of these latter ions, but we estimate the affinity of **2** for Ca^{2+} to be *ca.* 1.2 (none of these ions shows a clear sigmodal curve over two pM units, indicating that the sensor response has not been fully achieved). Surprisingly, the affinity of **2** towards Li^+ was somewhat reduced compared to that seen for **1**, which could possibly be due to its small size. Within the large cavity of **2**, the ion can possibly reside closer to the aromatic ring **C**, since the amine

there is more able to coordinate to the ion than the one on ring **B** since it has higher charge density (as was demonstrated in Fig. 1).

From the results above, it can be seen that **2** can detect intracellular concentrations of K^+ quite efficiently. However, the rather strong binding to Na^+ is somewhat a drawback. Despite this, and the fact that **2** responds to K^+ concentrations that mirror the intracellular concentration range, we conclude that **2** is capable of selectivity detecting K^+ accurately provided that Na^+ concentration levels are below 3 mM. These findings are in accordance with those published by Tsien *et al.* for their fluorescence excitation based radiometric sensors.[14b]

Conclusion

The synthesis of the two new colorimetric sensors **1** and **2** has been described. These can be easily synthesised in moderately good yield from the two *o*-anisidine based receptors **3** and **4**. The affinity of these colorimetric sensors towards group I and group II ions was investigated by UV–VIS spectroscopy and by ^{1}H NMR. Both sensors were found to be almost pH independent in the physiological range, with pK_a of 3.0(±0.1) and 2.9(±0.1) for the nitrogen moiety assigned to aromatic ring **B** (Scheme 1). Further pK_as at *ca.* 6.1 were also determined but these only contributed a small amount to the overall change in the absorption spectra and hence colour. Both sensors **1** and **2** changed colour from red to yellow upon Na^+ and K^+ recognition (similar colour changes were seen upon pH titration from alkaline to acidic solution). For **1**, it was found that the sensitivity towards Na^+ overlapped comfortably with the concentration range found for cytosolic Na^+. At the same time the sensitivity towards K^+ (and other potential group I and II competitors) was much less. We thus conclude that **1** is a potential candidate for colorimetric determination of cytosolic Na^+. Chemosensor **2** however, showed good affinity towards K^+. Unfortunately, the concurrent affinity to Na^+ was close to that of K^+. However, since the cytosolic concentration of Na^+ is so much lower than that of K^+, it will only affect the K^+ monitoring at low K^+ levels. This is still a drawback to our design and we are currently modifying our design to overcome this.

In summary, we have developed new colorimetric sensors for biologically important cations. To the best of our knowledge these are the first examples of azo-dye based chemosensors for determining cytosolic concentrations of Na^+ and K^+.

Experimental

Melting points were determined using a Electrothermal 1A9000 melting point apparatus. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrophotometer equipped with a Gateway 2000 4DX2–66 workstation. Solid samples were dispersed in KBr and recorded as clear pressed discs. ^{1}H NMR spectra were recorded at 400 MHz using a Bruker Spectrospin DPX-400 instrument. Tetramethylsilane (TMS) was used as an internal reference standard, with chemical shifts expressed in parts per million (ppm or δ) downfield from the standard. ^{13}C NMR were recorded at 100 MHz using a Bruker Spectrospin DPX-400 instrument. Mass spectra were determined by detection using Electrospray on a Micromass LCT spectrometer, using a Waters HPLC. The whole system was controlled by MassLynx 3.5 on a Compaq Deskpro workstation.

UV–VIS spectra were recorded on a Shimadzu UV-2401 PC UV–VIS spectrometer (see Experimental section). Typically a solution of the sensor was made up with optical density of 0.2–0.4 (10 μM on average, but for the pK_a measurements these were somewhat higher at 18 μM) in 50 : 50 MeOH–H_2O solvent systems containing the ionic strength. pH titrations were carried out by adjusting the pH of the above solution (10–20 ml) to high alkaline value (TBAOH or KOH) and aliquots of acid (HCl or TFA) were added. The titration using groups I and II alkali metals was carried out on 10 ml samples, after adjusting the pH to 7.4, aliquots (μL) of 3.0 M to 0.5 mM stock solutions were added. The absorption spectra were corrected for volume. Determination of pK_a and pM values was according to published procedure.[17]

1-{3-Methoxy-4-[13-(2-methoxyphenyl)-1,4,10-trioxa-7,13-diazacyclopentadecan-7-yl]phenyl}-2-(4-nitrophenyl)diazene (1)

To a 50 ml round-bottomed flask (RBF), *p*-nitroaniline, 0.104 g (0.766 mmol) was added containing 10 ml of 1 : 1 THF–H_2O, and $NaNO_2$, 0.053 g (0.766 mmol). To the solution that was being stirred at 0 °C, HCl, 0.15 ml (12 M) was slowly added. This solution was added dropwise to a 100 ml single necked RBF which contained 7,13-bis(2-methoxyphenyl)-1,4,10-trioxa-7,13-diazacyclopentadecane (**3**), 0.300 g (0.696 mmol) in 20 ml of 1 : 1 THF–H_2O. This solution was stirred at 0 °C. This solution was left stirring overnight at room temperature. The resulting dark red solution was reduced down, dissolved into 60 ml of $CHCl_3$, and washed with water, 2 × 30 ml. The organic phase was dried over $MgSO_4$ and reduced to a deep red oil. The crude material was purified on an Alumina column using 70 : 30 hexane–ethyl acetate, to produce **1** as a deep red solid 0.199 g [49.27% Yield], mp 137–139 °C. Calculated for $C_{30}H_{37}N_5O_7\cdot(H_2O)_{3/2}$: C, 59.39; H, 6.65; N, 11.54. Found: C, 59.63; H, 6.37; N, 11.14%; Calculated for $C_{30}H_{38}N_5O_7$:[MH^+ peak] *m*/*z* = 580.2771, found: 580.2770; δ_H($CDCl_3$, 400 MHz) 8.34 (d, 2H, *J* = 9.0 Hz, Ar–*H*), 7.95 (d, 2H, *J* = 9.0 Hz, Ar–*H*), 7.61 (d,d, 1H, J_1 = 8.52 Hz, J_2 = 2.0 Hz Ar–*H*), 7.48 (d, 1H, *J*= 2.0 Hz, Ar–*H*), 7.02 (d, 2H, *J* = 8.52 Hz, Ar–*H*), 6.96 (t,d, 1H, J_1= 7.54 Hz, J_2 = 1.48 Hz, Ar–*H*), 6.86 (t,d, 2H, J_1 = 7.54 Hz, J_2 = 1.48 Hz, Ar–*H*), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.76 (t, 2H, *J* = 5.52 Hz, OCH_2 CH_2O), 3.74–3.67 (m, 6H, OCH_2 CH_2O), 3.66–3.64 (m, 6H, OCH_2 CH_2O), 3.49 (t, 4H, *J* = 4.04 Hz, OCH_2 CH_2O), 3.41 (t, 2H, *J* = 4.52 Hz, OCH_2 CH_2O); δ_C($CDCl_3$, 100 MHz) 156.53, 153.18, 151.36, 147.75, 146.30, 145.19, 140.54, 124.68, 123.01, 122.80, 122.50, 121.36, 120.77, 116.74, 111.96, 102.85, 71.49, 71.17, 70.94, 70.83, 69.82, 69.69, 55.59, 55.41, 53.68, 53.37, 53.09, 52.03; Mass Spec (MeCN, ES+) *m*/*z* Expected: 579.65, Found: 580.23 (M + H); IR υ_{max}(cm^{-1}) 2950, 2904, 2859, 1587, 1509, 1448, 1376, 1334, 1241, 1103, 1058, 1022, 860, 798, 768, 686, 615.

1-{3-Methoxy-4-[16-(2-methoxyphenyl)-1,4,10,13-tetraoxa-7,16-diazacyclooctadec-7-yl]phenyl}-2-(4-nitrophenyl)-diazene (2)

The synthesis was identical to that of **1**, using *p*-nitroaniline, 0.104 g (0.70 mmol) in 10 ml of 1 : 1 THF–H_2O, and $NaNO_2$, 0.053 g (0.766 mmol), 0.15 ml of HCl (12 M). This solution was added dropwise to a 100 ml single necked RBF which contained 7,16-bis(2-methoxyphenyl)-1,4,10,13-tetraoxa-7,16-diazacyclo-octadecane (**4**) 0.300 g (0.632 mmol) in 20 ml of 1 : 1 THF–H_2O. The crude material was purified on an alumina column using 70 : 30 hexane–ethyl acetate, to produce 0.175 g of **2** (44.41% yield) as a deep red solid, mp 95–97 °C. Calculated for $C_{32}H_{41}N_5O_8$: C, 61.62; H, 6.63; N, 11.23. Found: C, 61.89; H, 6.82; N, 10.84%; Calculated for $C_{32}H_{42}N_5O_8$: [MH^+ peak] *m*/*z* = 624.3047, found: 624.3041; δ_H($CDCl_3$, 400 MHz) 8.34 (d, 2H, *J* = 9.0 Hz, Ar–*H*), 7.96 (d, 2H, *J*= 8.52 Hz, Ar–*H*), 7.64 (d,d, 1H, J_1= 8.52 Hz, J_2= 2.0 Hz, Ar–*H*), 7.49 (s, 1H, Ar–*H*), 7.15 (d, 1H, *J*= 7.52 Hz, Ar–*H*), 7.10 (d, 1H, *J*= 8.02 Hz, Ar–*H*), 7.0 (t, 1H, *J*= 7.52 Hz, Ar–*H*), 6.91 (t, 1H, *J*= 7.52 Hz, Ar–*H*), 6.86 (d, 1H, *J*= 7.52 Hz, Ar–*H*), 3.93 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.75 (s, 8H, OCH_2 CH_2O), 3.63–3.58 (m, 12H, OCH_2 CH_2O), 3.55–3.52 (m, 4H, OCH_2 CH_2O); δ_C($CDCl_3$, 100 MHz) 156.45, 153.12, 151.55, 147.76, 146.54, 144.77, 139.38, 124.65, 122.80, 122.71, 122.57, 121.44, 120.72, 117.42, 111.85, 102.87, 70.75, 70.62, 70.09, 70.0, 55.57, 55.39, 52.78, 52.70; Mass Spec (MeCN, ES+) *m*/*z* Expected: 623.7, Found: 624.3 (M + H); IR

υ_{max}(cm^{-1}) 2929, 2871, 1587, 1519, 1500, 1457, 1394, 1344, 1251, 1139, 1093, 1027, 923, 862, 738, 688, 626.

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