CHEMISTRY
A EUROPEAN JOURNAL

FULL PAPER

DOI: 10.1002/chem.201302350

A Highly K^+-Selective Phenylaza-[18]crown-6-Lariat-Ether-Based Fluoroionophore and Its Application in the Sensing of K^+ Ions with an Optical Sensor Film and in Cells

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Abstract: Herein, we report the synthesis of two phenylaza-[18]crown-6 lariat ethers with a coumarin fluorophore (**1** and **2**) and we reveal that compound **1** is an excellent probe for K^+ ions under simulated physiological conditions. The presence of a 2-methoxyethoxy lariat group at the *ortho* position of the anilino moiety is crucial to the substantially increased stability of compounds **1** and **2** over their lariat-free phenylaza-[18]crown-6 ether analogues. Probe **1** shows a high K^+/Na^+ selectivity and a 2.5-fold fluorescence enhancement was observed in the presence of 100 mM K^+ ions. A fluorescent membrane sensor, which was prepared by incorporating probe **1** into a hydrogel, showed a fully reversible response, a response time of 150 s, and a signal change of 7.8% per 1 mM K^+ within the range 1–10 mM K^+. The membrane was easily fabricated (only a single sensing layer on a solid polyester support), yet no leaching was observed. Moreover, compound **1** rapidly permeated into cells, was cytocompatible, and was suitable for the fluorescent imaging of K^+ ions on both the extracellular and intracellular levels.

Keywords: crown compounds · fluorescence · gels · potassium · sensors

Introduction

Fluorescent molecular sensors for the recognition of alkali and alkaline-earth metal ions have attracted extensive attention over the past few years[1,2] because of the increasing interest in in vivo monitoring and in vitro diagnostics in rapid-screening systems.[3] The measurement of K^+ ions in blood samples is of particular interest because of the challenge of selectively distinguishing between extracellular K^+ (4 mM) and Na^+ ions (140 mM) with a resolution of <0.1 mM.[3] Further desirable features of a probe for K^+ ions are its stability and fast response, long-wavelength absorption (>400 nm) and emission maxima (>490 nm), and no interference by changes in pH value. A highly selective fluorescent molecular probe for potassium ions based on a calix[4]bisazacrown that contained a boron-dipyrromethene fluorophore was introduced by Valeur and co-workers, although this fluoroionophore only worked in EtOH/water mixtures.[4] PBFI (potassium-binding benzofuranisophthalate) is the most-popular indicator for intracellular K^+ ions.[5,6] PBFI incorporates a diaza-[18]crown-6 ether as the recognition unit and, though it lacks Na^+/K^+ selectivity, this probe is the only currently commercially available K^+ indicator.[7]

More-recent probes have overcome this problem of poor K^+/Na^+ selectivity by employing a [2.2.3]-triazacryptand (TAC) as a receptor unit.[8–14] The main drawback of these extracellular probes is their extensive multistep syntheses. Hence, there is still a demand for a simple, yet sensitive and selective, probe for monitoring extracellular concentrations of K^+ ions (about 4 mM) against intracellular levels (about 150 mM).

Recently, we showed that the electronic conjugation of 1,2,3-triazol fluoroionophore within the signal-transduction chain of phenylaza-[18]crown-6-1,2,3-triazol-1,4-diyl-coumarin yielded selective fluorescent probes for K^+ ions under simulated physiological conditions.[15–17] Unlike other probes for K^+ ions,[3,8–14] these probes were easily accessible in only

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201302350.

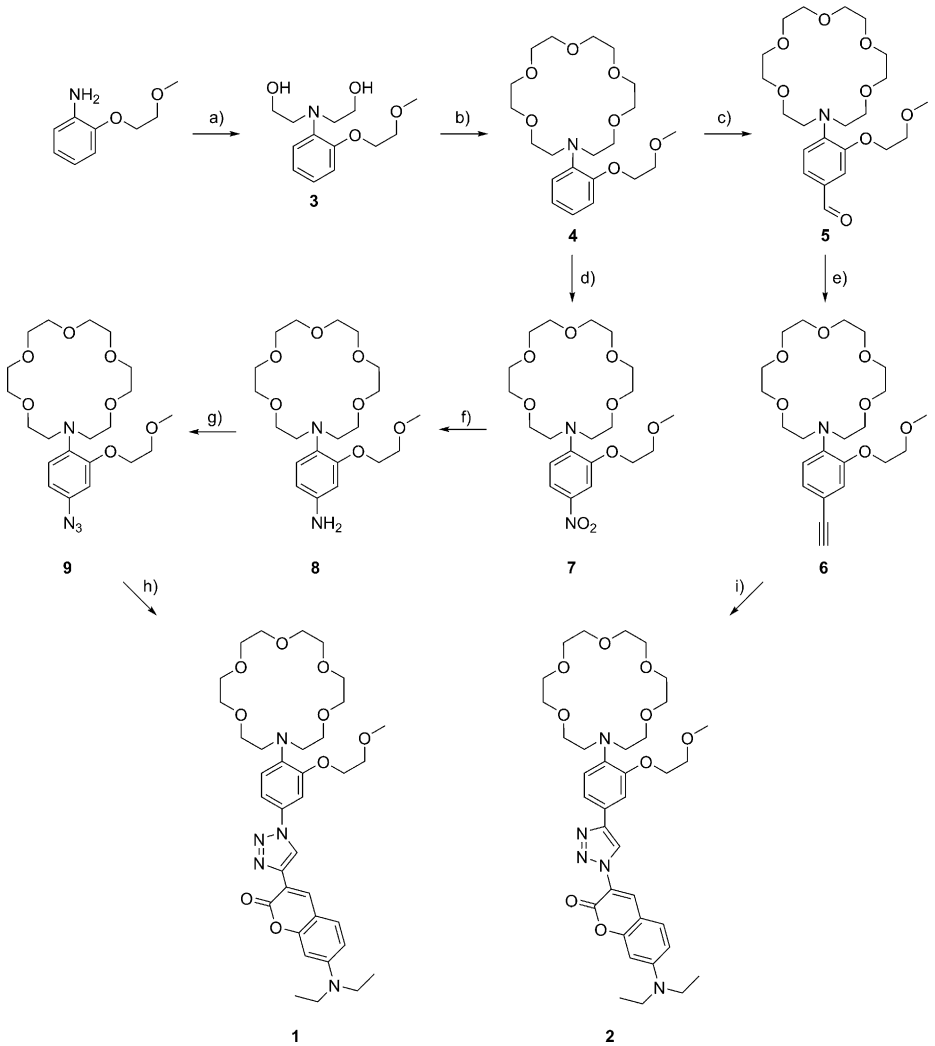
four steps. The estimated K_d values of these probes for K^+ ions are about 260 mM, which is not suitable for monitoring the physiological concentrations of K^+ ions.

To decrease the K_d value of the receptor, we took advantage of the fact that the attachment of a lariat group significantly increased the stability of complexes of monoazacrown ethers with alkali-metal ions whilst retaining the flexibility of the ionophores.[18] Further investigations on aza-[18]crown-6 lariat ethers found that the strongest coordination of K^+ ions was achieved by seven oxygen-donor atoms.[19] This result indicated that the introduction of a pendant 2-methoxyethoxy lariat group *ortho* to the trigger anilino donor of the phenylaza-[18]crown-6 K^+ receptor should lead to a substantial decrease in the K_d value. The 2-methoxyethoxy lariat unit is well-known. It was first introduced by the group of Gokel[18] for the N-substitution of aza-[18]crown-6 ether. Recently, the same lariat group was used for the functionalisation of a [2.2.3] cryptand.[8] However, the combination of the 2-methoxyethoxy lariat group with a simple phenlyaza-[18]crown-6 receptor for the selective complexation of K^+ ions has not been reported before.

Herein, we report the synthesis of a new K^+-ion receptor, *o*-(2-methoxyethoxy)phenylaza-[18]crown-6 (**4**), its ethynyl and azido derivatives (**6** and **9**, respectively), and two phenylaza-[18]crown-6-lariat-ether-based fluoroionophores (**1** and **2**, Scheme 1). Probes **1** and **2** are 1,4-disubstituted 1,2,3-triazol constitutional isomers. Our recent studies[15–17] revealed that constitutional isomerism had a fundamental influence on the stability of K^+ complexes and on the K^+/Na^+ selectivity of 1,2,3-triazol fluoroionophores. The stabilities of the K^+ complexes of probes **1** and **2** are about tenfold higher than those of their lariat-free analogues. Probe **1** is highly K^+/Na^+ selective and allows the detection of K^+ ions within the concentration range 2–100 mM. Furthermore, probe **1** was also embedded within a polymer matrix to demonstrate its application as a continuous sensor. Fluorescence microscopy experiments revealed that probe **1** rapidly permeated into cells, was cytocompatible, and was able to differentiate between extracellular (about 5 mM) and intracellular levels of K^+ ions (about 150 mM).

Results and Discussion

Synthesis of molecular probes 1 and 2: The synthesis of the new *o*-(2-methoxyethoxy)phenylaza-[18]crown-6 lariat ether (**4**) started from commercially available 2-(2-methoxyethoxy)aniline (Scheme 1); conversion into dihydroxy-aniline derivative **3**, followed by a macrocyclization step, yielded the new K^+ receptor **4**. Benzaldehyde **5** served as a precursor of alkyne **6** and was prepared through a Vilsmeyer reaction.[20] The transformation of the aldehyde unit in compound **5** into the terminal ethynyl unit in compound **6** was achieved by



Scheme 1. Synthesis of probes **1** and **2**: a) 2-chlorethanol, $CaCO_3$, water, 60 °C, 90 % yield; b) NaH, 1,17-ditosyl-3,6,9,12,15-pentaoxaheptadecane, MeCN, reflux, 20 % yield; c) $POCl_3$, DMF, 27 % yield; d) $NaNO_2$, water, glacial AcOH, RT, 23 % yield; e) dimethyl-1-diazo-2-oxopropylphosphonate,[21] K_2CO_3, MeOH, 61 % yield; f) H_2, 10 % Pd/C, MeOH, 75 bar, >99 %; g) $NaNO_2$, NaN_3, HCl, water, 0 °C to RT, 61 % yield; h) $CuSO_4$/Na ascorbate, 3-ethynyl-7-diethylaminocoumarin,[25] THF/water, 60 °C, 73 % yield; i) $CuSO_4$/Na ascorbate, 3-azido-7-diethylaminocoumarin,[24] THF/water, 60 °C, 93 % yield.

using dimethyl-1-diazo-2-oxopropylphosphonate.[21] Functionalisation of compound **4** with an azide unit was carried out through a mild nitration reaction. Subsequent reduction into amine **8** and diazotization gave compound **9**, ready for the well-established Cu^I-catalyzed azide–alkyne cycloaddition (CuAAC) reaction.[22,23] Coupling of compound **6** and **9** with the respective coumarin-azide[24] or coumarine-alkyne[25] gave molecular probes **1** and **2**, respectively, in good yields. Hence, the introduction of the lariat ether to amend the K_d value of the iononphore could be achieved in a small number (5 or 6) of synthetic steps.

Photophysical investigation under simulated physiological conditions: The fluorescence-emission spectra of probe **1** under simulated physiological conditions were acquired as described in the Supporting Information at an excitation wavelength of 420 nm. Uncomplexed **1** displayed an emission peak at 493 nm and a low quantum yield ($\Phi_f = 0.062$). On increasing the K^+-ion concentration within the physiological range (2–160 mM), a 2.5-fold fluorescence enhancement was observed (Figure 1a and Figure 2). The maximum signal change was observed at 100 mM K^+, with a Φ_f value of 0.184. In the low-K^+-concentration range (1–10 mM), the signal intensity increased by 3.5% per 1 mM K^+.

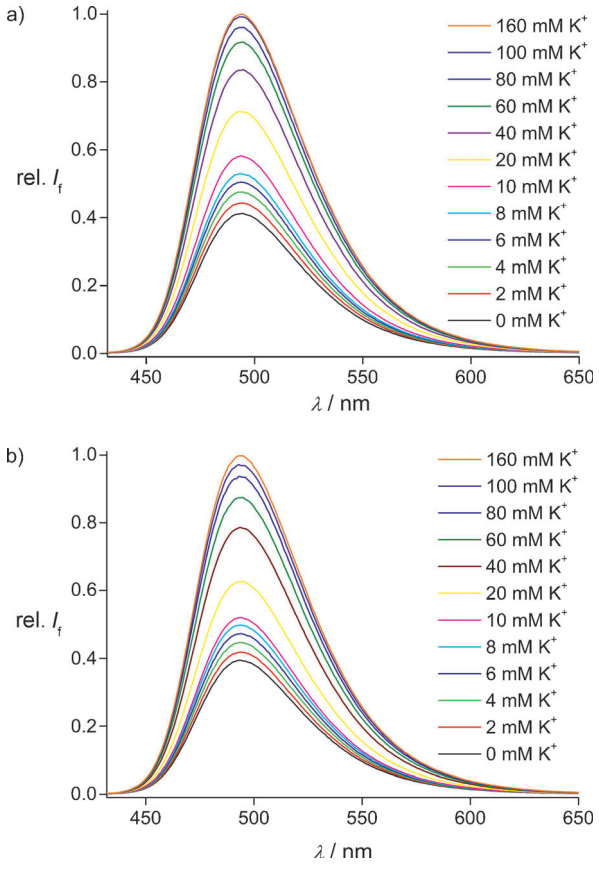


Figure 1. Normalized fluorescence spectra of probe **1** (10 μM) to K^+ in the presence of various concentrations of ions under simulated physiological conditions (10 mM Tris-buffer, pH 7.2); the ionic strength was maintained at 160 mM with a) choline chloride and b) various concentrations of Na^+ ions in combined K^+/Na^+ solutions.

The exposure of probe **1** to K^+-free solutions that contained various concentrations of Na^+ ions (0–160 mM) showed that the fluorescence of probe **1** remained almost unaffected by the competing metal ion ($\Phi_f = 0.062$ at 160 mM Na^+, Figure 2). This high K^+/Na^+ selectivity was further verified by using solutions that contained both K^+ and Na^+ ions (Figure 1b and the Supporting Information, Figure S2). The enhancement by a factor of 2.4 at 100 mM K^+ was only slightly lower than that in the experiment without Na^+ ions. The signal increased by a factor of 1.3 within the clinical concentration range (1–10 mM K^+), with a signal change of 2.4% per 1 mM K^+. Hence, the presence of Na^+ ions has a negligible effect on the performance of probe **1** under simulated physiological conditions.

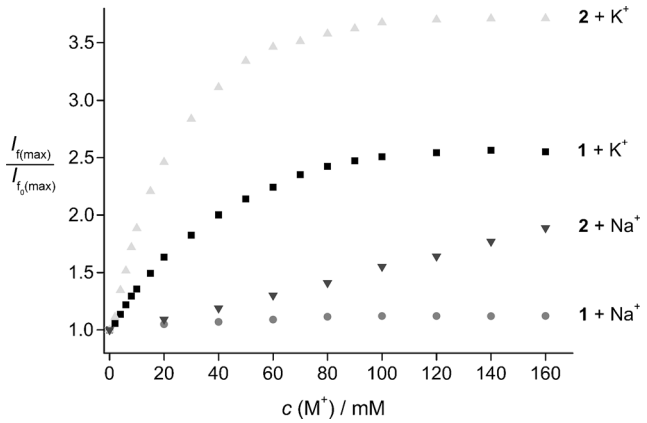


Figure 2. Fluorescence-enhancement factors ($I_{f(max)}/I_{f0(max)}$) for probes **1** and **2** (10 μM) under simulated physiological conditions (10 mM Tris-buffer, pH 7.2) in the presence of various concentrations of K^+ or Na^+ ions; the ionic strength was maintained at 160 mM with choline chloride.

Dissociation constants were calculated from plots of fluorescence intensities versus K^+-ion concentration (see the Supporting Information). The K_d value of probe **1** in Na^+-free solution was 26 mM and increased to about 29 mM (if approximated) for the combined K^+/Na^+ solutions, again demonstrating the high K^+/Na^+ selectivity of probe **1**.

The K^+/Na^+ selectivity of probe **2** was not as high as that observed for probe **1** (Figure 2).[26] Compound **2** complexed both K^+ and Na^+ ions more strongly than compound **1**. This result was further supported by the lower K_d values for compound **2** (compared to those for compound **1**) in solutions that contained either K^+ ions alone or both K^+ and Na^+ ions (see the Supporting Information, Figure S3c,d). In Na^+-free solutions, the signal intensity of probe **2** increased in the low-concentration range (1–10 mM) by 8.9% per 1 mM K^+ (see the Supporting Information, Figure S2c,d).

Hence, the appended 2-methoxyethoxy lariat group induced an approximately tenfold increase in the stability of the K^+ complexes of probes **1** and **2** over their lariat-free analogues.[15] The K_d values of the K^+ complexes of probes **1** and **2** in combined Na^+/K^+ solutions (about 29 mM) should be useful for the determination of K^+ concentrations within

the range 2–100 mM. The sensitivity of probes **1** and **2** towards change in pH value at various concentrations of K^+ ions within the pH range 6.8–7.8 (see the Supporting Information, Figure S4) was low, as shown by fluorescence increases that remained largely unaffected by pH value. Thus, probe **1** was selected for further imaging experiments in NRK cells and for studies on its application as a K^+-ion sensor because it showed higher K^+/Na^+ selectivity than probe **2** (see below).

The B3LYP/6-31G*-optimized geometries of compounds **1** and **2** (see the Supporting Information, Chapter 7, Figure S6) were similar to those of their lariat-free analogues.[15] Thus, we assumed that the sensing mechanism of probes **1** and **2** also involved photoinduced electron transfer (PET) processes on the basis of virtual spacers,[27] as discussed for their lariat free analogues.[15]

Imaging experiments in NRK cells: We also studied the uptake of probe **1** by normal rat kidney (NRK) cells that were grown in ordinary Petri dishes by using fluorescence microscopy. The probe was allowed to distribute over the cell body before the microscopic inspection. Figure 3, image B1 shows a fluorescence image of a confluent NRK monolayer after incubation with a solution of probe **1** for 30 min. The green fluorescence could be solely attributed to the K^+ complex with probe **1**, because fluorescence images without probe **1** did not show any cellular structures (Figure 3, image B3). The endomembranes of the cells fluoresced brightly, whereas the cell nuclei remained dark, thus indicating that the probe did not penetrate the nuclei. Fluorescence intensity was not homogeneously distributed within the cells, but was rather predominant within the hydrophobic membrane domains. A small band around the cell nucleus, possibly the endoplasmic reticulum (ER), showed higher fluorescence intensity compared to the more-peripheral endomembranes (for a magnified image of Figure 3, image B1, see the Supporting Information, Figure S5). The fluorescence image and the corresponding phase-contrast micrograph (Figure 3, image A1) did not reveal any changes in cell morphology, thus indicating that the probe was cytocompatible during the time period of incubation and observation.

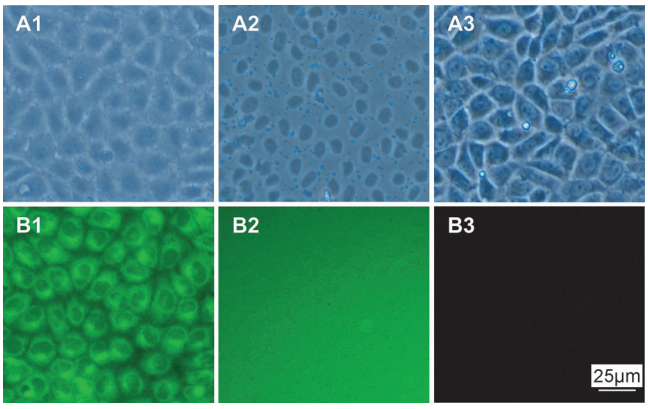


Figure 3. Images of a confluent NRK cell layer in buffer solution: A) Phase-contrast micrographs and B) fluorescence images (1) after incubation with probe **1** (30 min), (2) after incubation with probe **1** (30 min) and subsequent permeabilization with Triton X-100, and (3) after incubation with buffer only.

After incubating the cells with Triton X-100, the cell membranes became permeabilized and dissolved (Figure 3, image A2), which finally led to cell death. Thus, the fluorescence image did not show any distinct cellular structures. Rather, a low background fluorescence was observed, because the ionophore had been released into the buffer (Figure 3, image B2). Even if the concentration of K^+ ions in the lysed cell sample was lowered to about 5 mM by dilution with Earle's balanced salt solution, fluorescence was still visible. Thus, one can clearly discriminate between intracellular and extracellular concentrations of K^+ ions by fluorescence microscopy.

Sensor applications: To use probe **1** in a sensor membrane for the continuous monitoring of K^+ concentration in flowing samples, such as blood, we incorporated it into a polymer matrix. We found that Hypan HN 80 hydrogel was a convenient polymer for making stable membrane sensors. Hypan is a polyacrylamide-co-polyacrylonitrile copolymer that contains hard (polyacrylonitrile domains, lipophilic) and soft blocks (polyacrylamide domains, hydrophilic).[28] Moreover, Hypan can take-up water in fractions up to 80% of its weight and has excellent ion permeability; this property would allow cations to rapidly reach probe **1**, which is mostly located in the hard blocks. Thin layers of a sensor cocktail (a solution of probe **1** and hydrogel in DMSO) were spread over 125 μm dust-free transparent slices of an ethylene-glycol-terephthalate polyester by knife coating. Unlike other optical membranes for sensing K^+ ions, this membrane required neither a multilayered structure[8] nor plasticisers or covalent binding of the indicator to one of the polymers.[8,29] Hence, both the preparation of the cocktail and the membrane sensor were significantly simplified. Circular sensor spots were punched out of the membrane and exposed to solutions with various concentrations of K^+ ions (Figure 4) in a flow-through cell. The cell was linked to a fluorimeter through a Y-shaped bifurcated optical fiber.

Embedding probe **1** into the membrane sensor shifted the absorbance maximum by 3 nm to 423 nm, whilst the emission maximum was shifted by only 3 nm to shorter wavelengths. This result is typical for a luminophore on embedding in a foil sensor with a more-lipophilic microenvironment.[29] A distinct increase in fluorescence was found (Figure 5) on increasing the concentration of K^+ ions in the sample flow (adjusted to an ionic strength of 150 mM with NaCl). This increase was reversible, as demonstrated by the time trace at decreasing concentrations of K^+. The forward response time (t_{90}) was less than 150 s over the full range and the backward response time (t_{90}) to lower concentrations of K^+ was 170 s. This behavior was even more rapid than found in earlier work for membranes based on co-ex-

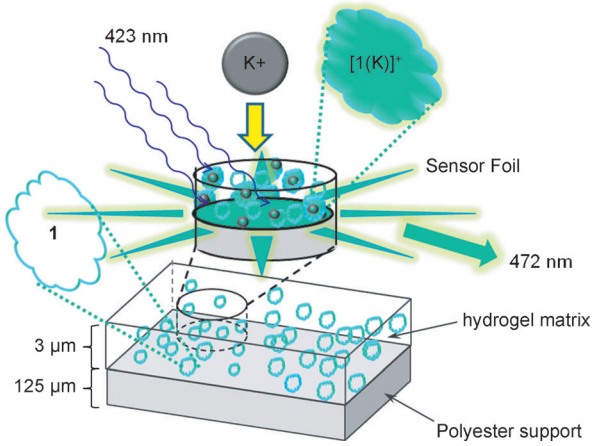


Figure 4. Application of probe **1** in a foil sensor.

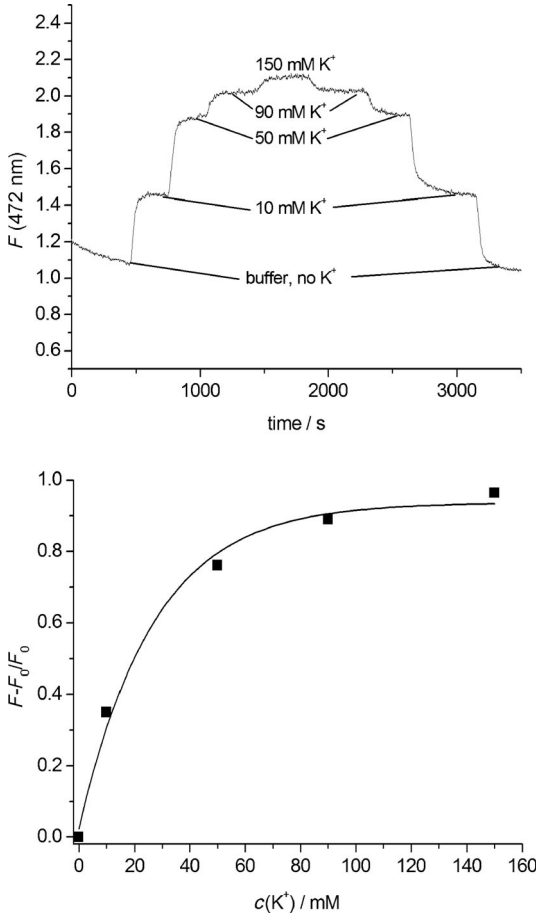


Figure 5. Top: Response time, signal change, and reversibility of the sensor membrane (that contained probe **1**) in the presence of various concentrations of K^+ ions (10 mM Tris-buffer, pH 7.3; the ionic strength was maintained at 150 mM with NaCl). Bottom: Calibration plot for the determination of K^+-ion concentration, as obtained from the sensor membrane (excitation/emission at 423/472 nm).

traction.[29] No leaching of probe **1** out of the membrane sensor was observed.

A calibration plot revealed that the membrane sensor covered the clinically relevant range (1–10 mM). Herein, the resolution, as determined by the equation: $(\Delta F - F_0)/F_0)/\Delta c(K^+)$, was the best reported so far and the signal change was about 7.8% per 1 mM K^+. Interestingly, this result was even better than that observed with more-complex membrane sensors (three layers and the probe covalently bound to the polymer)[8] and showed the improved performance of probe **1**. The detection limit (at 3σ) for the determination of K^+ ions with the membrane sensor was 800 µM K^+. Importantly, on the immobilization of probe **1** in the polymer matrix, a clear discrimination between the average extracellular (4 mM) and intracellular levels (150 mM) of K^+ was possible, thus rendering probe **1** an attractive fluoroionophore for applications in clinical diagnostic systems.

Conclusion

In conclusion, herein, we have reported the facile synthesis and application of K^+-selective 1,2,3-triazol-fluoroionophores **1** and **2**, which featured a new *o*-(2-methoxyethoxy)-phenylaza-[18]crown-6-lariat-ether receptor. Probe **1** performed excellently under simulated physiological conditions and exhibited high K^+ selectivity over Na^+. Probe **1** had a low K_d value of about 29 mM in combined K^+/Na^+ solutions and showed a 2.4% signal change per 1 mM K^+. The K^+/Na^+ selectivity of constitutional isomer **2** was not as high as that observed for probe **1**, but, at low Na^+ concentrations, K^+ was detected with a greater fluorescence enhancement (8.9% per 1 mM K^+ within the range 1–10 mM). Probe **1** was also incorporated into a membrane sensor that consisting of a polymethane hydrogel and, in this form, it enabled the continuous monitoring of physiological K^+ levels with a rapid response time. Moreover, cellular studies showed that probe **1** was not damaging to cells. In summary, probe **1** is well-suited for differentiating between extracellular (about 5 mM) and intracellular levels of K^+ ions (about 150 mM) in aqueous samples, if contained in foil sensors, or in cellular experiments by using fluorescence microscopy.

Experimental Section

General methods and reagents: All commercially available chemicals were used without further purification. Solvents were distilled prior use. ^1H and ^{13}C NMR spectra were recorded on 300 MHz and 600 MHz instruments, respectively; data are reported as follows: chemical shift (δ, in ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qu = quintet, sx = sextet, dd = doublet of doublets, m = multiplet), integration, coupling constant (in Hz). ESI spectra were recorded on a Micromass Q-TOF micro mass spectrometer in positive electrospray mode. IR spectra were recorded on a Thermo Nicolet Nexus FTIR instrument. Air/water-sensitive reactions were performed in oven-dried glassware under an argon atmosphere. Column chromatography was performed on silica gel (Merck; silica gel 60, 0.04–0.063 mesh).

***N*,*N*-Bis(2-hydroxyethyl)-2-methoxyethoxyaniline (3)**: A mixture of 2-methoxyethoxyaniline (21.9 g, 0.131 mol), 2-chloroethanol (52.77 g, 0.656 mol), and $CaCO_3$ (18.37 g, 0.184 mol) in water (300 mL) was stirred

for 6 days at 60 °C. After cooling to RT, Na_2CO_3 (75.0 g, 0.7 mol) was added and the mixture was stirred for 40 min at 60 °C. Then, the resulting solid was filtered off and the aqueous layer was saturated with NaCl and extracted with methyl-*tert*-butyl ether (3×500 mL). The combined organic phases were dried over K_2CO_3, concentrated in vacuo, and the residue was purified by column chromatography on silica gel (EtOAc) to give compound **3** as a red oil (30.1 g, 90 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.22 (dd, J = 7.8, 1.6 Hz, 1H), 7.14–7.08 (m, 1H), 7.01–6.95 (m, 1H), 6.91 (dd, J = 8.1, 1.2 Hz, 1H), 4.13–4.10 (m, 2H), 3.76–3.73 (m, 2H), 3.47 (t, J = 5.3 Hz, 4H), 3.43 (s, 3H), 3.15 ppm (t, J = 5.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 155.25, 139.28, 126.01, 125.51, 122.23, 113.33, 70.70, 67.80, 59.66, 58.93, 57.93 ppm; MS (ESI+): *m*/*z* calcd for $C_{13}H_{22}NO_4$: 256.15; found: 256.13.

***N*-(2-Methoxyethoxyphenyl)aza-[18]crown-6 ether (4)**: NaH (80 %, 5.2 g) was added to a solution of compound **3** (17.86 g, 69.9 mmol) in dry MeCN (510 mL) over a period of 1 h under an argon atmosphere. This mixture was heated at reflux and a solution of 1,17-ditosyl-3,6,9,12,15-pentaoxaheptadecane (35.16 g, 69.9 mmol) in dry MeCN (250 mL) was added dropwise over a period of 4 h. Then, the mixture was heated at reflux for 11 h and the resulting precipitate was filtered off. The solvent was removed and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 95:5 v/v) to yield compound **4** as a brown oil (16.45 g, 57 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.12–6.82 (m, 4H), 4.10–4.07 (m, 2H), 3.73–3.45 (m, 26H), 3.39 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 152.04, 140.15, 121.96, 121.18, 121.14, 113.83, 71.02, 70.71, 70.59, 70.56, 70.27, 70.02, 67.54, 58.81, 52.63 ppm; MS (ESI+): *m*/*z* calcd for $C_{21}H_{36}NO_7$: 414.25; found: 414.16.

***N*-(4-Formyl-2-methoxyethoxyphenyl)aza-[18]crown-6 ether (5)**: $POCl_3$ (37 g, 241 mmol) was added to a solution of compound **4** (9.98 g, 24.1 mmol) in dry DMF (60 mL) at −10 °C. The mixture was stirred for 12 h at RT, then heated at 70 °C and stirred for 1 h. The solution was slowly poured over ice (250 mL), neutralized with Na_2CO_3, and extracted with CH_2Cl_2 (3×250 mL). The combined organic phases were dried over $MgSO_4$, concentrated in vacuo, and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 95/5 v/v) to yield compound **5** as a yellow-brown oil (2.87 g, 27 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 9.69 (s, 1H), 7.30 (dd, 1H, J = 8.3 Hz, 1.8 Hz), 7.26 (d, 1H, J = 1.8 Hz), 6.93 (d, 1H, J = 8.3 Hz), 4.11–4.08 (m, 2H), 3.71–3.55 (m, 26H), 3.36 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 190.14, 149.69, 146.08, 128.22, 126.73, 116.46, 111.01, 70.70, 70.61, 70.55, 70.48, 70.42, 69.95, 67.46, 58.65, 52.61 ppm; MS (ESI+): *m*/*z* calcd for $C_{22}H_{36}NO_8$: 442.24; found: 442.25.

***N*-(4-Ethynyl-2-methoxyethoxyphenyl)aza-[18]crown-6 ether (6)**: Dimethyl-1-diazo-2-oxopropylphosphonate[21] (522 mg, 2.7 mmol) was added to a solution of compound **5** (1 g, 2.3 mmol) and K_2CO_3 (626 mg, 4.6 mmol) in dry MeOH (20 mL) and the mixture was stirred for 10 h at RT. Then, the solvent was removed and the residue was diluted with water (50 mL) and CH_2Cl_2 (50 mL). The organic layer was washed with water (2×50 mL), dried over $MgSO_4$, concentrated in vacuo, and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 95/5 v/v) to yield compound **6** as a light-brown oil (603 mg, 61 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.02 (dd, J = 8.2 Hz, 1.8 Hz, 1H), 6.92–6.89 (m, 2H), 4.07–4.04 (m, 2H), 3.72–3.47 (m, 26H), 3.38 (s, 3H), 2.98 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 150.57, 141.23, 125.66, 119.37, 116.92, 113.83, 84.09, 75.57, 70.84, 70.75, 70.63, 70.59, 70.36, 69.97, 67.61, 58.78, 52.45 ppm; MS (ESI+): *m*/*z* calcd for $C_{23}H_{36}NO_7$: 438.25; found: 438.21.

***N*-(4-Nitro-2-methoxyethoxyphenyl)aza-[18]crown-6 ether (7)**: $NaNO_2$ (0.81, 11.7 mmol) was added to a solution of compound **4** (4.4 g, 10.65 mmol) in a mixture of water (340 mL) and glacial acetic acid (34 mL) over a period of 10 min. Then, the reaction mixture was stirred for 16 h at RT, neutralized with LiOH, and extracted with CH_2Cl_2 (3×100 mL). The combined organic phases were dried over $MgSO_4$, concentrated in vacuo, and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 95/5 v/v) to yield compound **7** as a yellow solid (1.1 g, 23 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.80 (dd, J = 9.1, 2.5 Hz, 1H), 7.64 (d, J = 2.5 Hz, 1H), 6.88 (d, J = 9.1 Hz, 1H), 4.15–4.12 (m, 2H), 3.75–3.58 (m, 26H), 3.40 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 148.39, 146.33, 139.08, 118.57, 115.65, 108.21, 70.77, 70.68, 70.64, 70.56, 70.52, 69.93, 67.92, 58.82, 52.83 ppm; MS (ESI+): *m*/*z* calcd for $C_{21}H_{35}N_2O_9$: 459.23; found: 459.26.

***N*-(4-Amino-2-methoxyethoxyphenyl)aza-[18]crown-6 ether (8)**: 10 % Pd/C (30 mg) was added to a solution of compound **7** (0.22 g, 0.48 mmol) in dry MeOH (30 mL). The mixture was hydrogenated in an autoclave for 16 h at 75 bar. The catalyst was removed by filtration through a bed of Celite and the solvent was removed in vacuo to yield a colorless oil. This compound quickly decomposed if exposed to air, thereby showing a color change to dark purple. Therefore, no further purification was attempted and compound **8** was directly used in the next step. Yield: 0.2 g, 97 %; MS (ESI+): *m*/*z* calcd for $C_{21}H_{37}N_2O_7$: 429.26; found: 429.20.

***N*-(4-Azido-2-methoxyethoxyphenyl)aza-[18]crown-6 ether (9)**: A solution of compound **8** (0.2 g, 0.467 mmol) in 4 M HCl (3.6 mL) was cooled to 0 °C. A solution of $NaNO_2$ (32 mg, 0.467 mmol) in water (1.8 mL) was slowly added and the mixture was stirred at 0 °C for 10 min. Then, a solution of NaN_3 (0.45 mg, 0.7 mmol) in water (1.8 mL) was added at 0 °C and the mixture was stirred for 14 h at RT, neutralized with Li_2CO_3, and extracted with $CHCl_3$ (3×50 mL). The organic layers were combined, dried over $MgSO_4$, concentrated in vacuo, and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 95/5 v/v) to give compound **9** as a brown oil (0.13 g, 61 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.89–6.59 (m, 3H), 4.13–4.10 (m, 2H), 3.77–3.56 (m, 26H), 3.43 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 146.06, 138.63, 121.84, 116.44, 111.74, 110.21, 72.49, 71.07, 70.53, 70.31, 70.25, 69.75, 68.06, 61.64, 59.05 ppm; MS (ESI+): *m*/*z* calcd for $C_{21}H_{35}N_4O_7$: 455.25; found: 455.25.

7-(Diethylamino)-3-{1-[3-(2-methoxyethoxy)-4-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)phenyl]-1*H*-1,2,3-triazol-4-yl)}-2*H*-chromen-2-one (1): Compound **9** (228 mg, 0.5 mmol), 3-ethinyl-7-diethylamino-coumarin[25] (121 mg, 0.5 mmol), $CuSO_4$·5H_2O (6.3 mg, 5 mol %), and sodium ascorbate (9.9 mg, 10 mol %) were dissolved in THF/water (3:1 v/v, 15 mL). The reaction mixture was stirred at 60 °C for 24 h. Then, water (15 mL) was added and the aqueous layer was extracted with $CHCl_3$ (3×15 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 9:1 v/v) to give compound **1** as a yellow solid (254 mg, 73 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.60 (s, 1H), 8.58 (s, 1H), 7.35 (d, J = 8.9 Hz, 1H), 7.29 (d, J = 2.2 Hz, 1H), 7.21 (dd, J = 2.2, 8.6 Hz, 1H), 7.11 (d, J = 8.6 Hz, 1H), 6.57 (dd, J = 2.4, 8.9 Hz, 1H), 6.47 (d, J = 2.1 Hz, 1H), 4.17–4.14 (m, 2H), 3.75–3.72 (m, 2H), 3.70–3.31 (m, 31H), 1.16 ppm (t, J = 7.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 160.42, 155.85, 151.96, 150.62, 141.99, 140.36, 138.30, 130.83, 129.32, 120.51, 120.18, 112.72, 110.38, 109.20, 108.48, 106.10, 96.85, 70.68, 70.64, 70.51, 70.46, 70.25, 69.78, 67.83, 58.75, 52.50, 44.67, 12.29 ppm; HRMS (ESI+): *m*/*z* calcd for $C_{36}H_{50}N_5O_9$:696.3609; found: 696.3583.

7-(Diethylamino)-3-{4-[3-(2-methoxyethoxy)-4-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)phenyl]-1*H*-1,2,3-triazol-1-yl}-2*H*-chromen-2-one (2): Compound **6** (235 mg, 0.5 mmol), 3-azido-7-diethylamino-coumarin[24] (139 mg, 0.5 mmol), $CuSO_4$·5H_2O (6.7 mg, 5 mol %), and sodium ascorbate (10.6 mg, 10 mol %) were dissolved in THF/water (3:1 v/v, 15 mL). The reaction mixture was stirred at 60 °C for 24 h. Then, water (15 mL) was added and the aqueous layer was extracted with $CHCl_3$ (3×15 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 9:1 v/v) to give compound **2** as a yellow solid (349 mg, 93 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.69 (s, 1H), 8.38 (s, 1H), 7.43–7.34 (m, 3H), 7.08 (d, J = 8.2 Hz, 1H), 6.64 (dd, J = 2.5, 8.9 Hz, 1H), 6.51 (d, J = 2.2 Hz, 1H), 4.22–4.19 (m, 2H), 3.77–3.74 (m, 2H), 3.73–3.37 (m, 31H), 1.20 ppm (t, J = 7.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 156.82, 155.65, 151.97,151.44, 147.47, 140.18, 134.22, 129.86, 123.89, 120.62, 119.54, 118.78, 116.88, 111.01, 109.99, 107.03, 96.91, 70.96, 70.71, 70.56, 70.54, 70.32, 69.97, 67.64, 58.80, 52.70, 44.87, 12.32 ppm; HRMS (ESI+): *m*/*z* calcd for $C_{36}H_{50}N_5O_9$: 696.3609; found: 696.3448.

Acknowledgements

A.S. gratefully acknowledges a stipend from the DAAD within the Michail Lomonosov Programme.

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Received: June 19, 2013
Published online: September 17, 2013

