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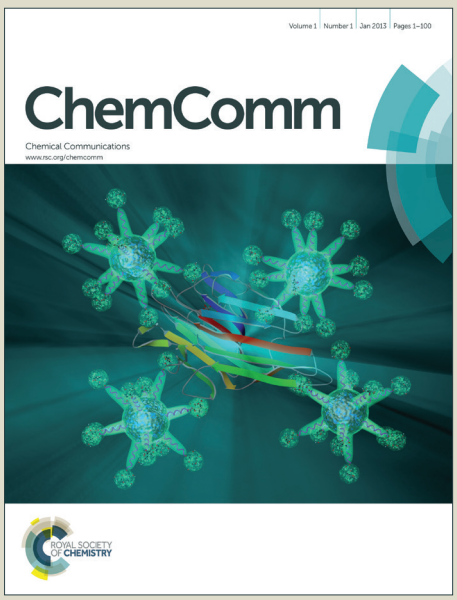

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ARTICLE TYPE

Fluorescence Lifetime-based Sensing of Sodium by an Optode

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We report a 1,2,3-triazol-fluoroionophore for Na^+ that shows *in vitro* a Na^+-induced fluorescence intensity and decay time enhancement. The Na^+-selective molecule 1 (Scheme 1) was incorporated into a hydrogel as a part of a fiber optical sensor. This sensor allows the direct determination of Na^+ in the range of 1–10 mM by reversible fluorescence decay time changes.

Sodium plays a crucial role in many physiological processes and the Na^+ concentration largely differs in intra- and extracellular compartments. For intracellular free Na^+ a range of 5-30 mM is found, whereas that of extracellular is more than 100 mM.[1] For the determination and visualisation of intra- or extracellular Na^+ levels, Na^+-responsive fluorescent probes are suitable tools. Minta and Tsien designed the first fluorescent indicators for cytosolic Na^+ (~30 mM) with dissociations constants K_d's <50 mM.[1] Several Na^+-sensitive fluorescent probes are commercially available e.g. SBFI (short for sodium-binding benzofuran isophthalate) (K_d ~11 mM),[1,2] Sodium Green (K_d ~21 mM),[2] CoroNa Green (K_d ~80 mM)[2,3a] and CoroNa Red (K_d ~200 mM)[2,3b]. Furthermore, photoinduced electron transfer (PET)-type fluoroionophores have proven successful as highly selective sodium sensing molecules.[4-6] For the analysis of sodium in serum and whole blood (~140 mM) He *et al.* designed a PET-fluoroionophore which consists of a *N*-(*o*-methoxyphenyl)aza-15-crown-5 ionophore (K_d ~80 mM) and a 4-aminonaphthalimid fluorophore.[4] An application of this fluoroionophore is the use in clinical diagnosis as a part of optical sensors for analytes such as Na^+ in whole blood.[7] For the continuous sensing of Na^+ several optodes based on fluorescence intensity changes were developed (e.g., to be used in films or beads).[8a-n] Many of those optode layouts are based on a generell concept, in which the detection of the targeted analyte and the optical signal generation are separated. The analyte (here Na^+) is bound and induces an ion exchange which subsequently is detected by a pH-sensitive dye. The advantage of such approach is that the optical part of the sensor can be applied to many different analytes. However, the optodes can be cross-sensitive to pH changes in the samples. Up to now, there is no fiber-optical sensor for direct Na^+ sensing (e.g. on the basis of a PET-fluoroionophore) incorporated in a hydrogel using fluorescence decay time changes as detection principle.

Sensor endurance is also a major challenge in general.[9] Concepts often rely on the ratiometric measurements of intensities or are based on fluorescence decay time determination. These two sensing schemes are independent on probe concentration and on aging effects of the optode (e.g., due to photobleaching). For monovalent ions such as sodium and potassium it is difficult to develop ratiometric fluoroionophores,[10] and only a few fluorescence decay time based probes for Na^+ are available, such as Sodium Green.[11]

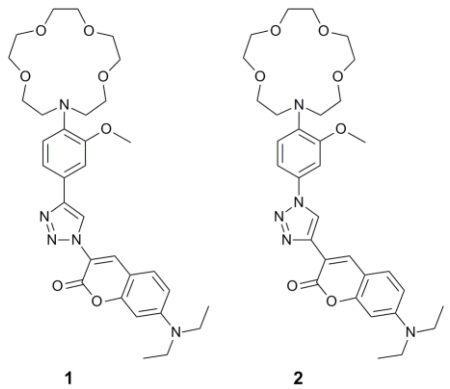
There is a fast increasing demand for tailored fluorescence probes (with respect to e.g., spectral range, analyte, photostability) in life sciences. Here, among others sodium is one of the top analytes and the development of a novel fluorescence probe based on the detection of changes in the fluorescence decay time is desireable. Developing a sensing scheme for the direct detection of sodium based on fluorescence decay time measurements will not only minimize the cross-sensitivity for pH and increase the overall performance of the sensor with respect to aging effects but it will also add another dimension (decay time in addtion to spectral parameters) to the analysis, which will be benificial for the development of multianalyte sensing schemes. Overall, the development of new optical sensor devices with (a) suitable K_d values for extracellular/blood or intracellular sodium measurements, (b) an excellent selectivity over physiological potassium concentrations, (c) no pH interference in the physiological pH range, (d) a fast response time, (e) a reversible signal change, and (f) a change of the fluorescence decay time are in the current focus of attention.

Recently, we found that in CuAAC (short for Cu(I)-catalyzed 1,3-dipolar azide alkyne cycloaddition reaction) generated fluoroionophores, the electronic conjugation of a *N*-phenylaza-18-crown-6[12a] or of a *N*-(2-methoxyethoxyphenyl) aza-18-crown-6[12b] ionophore and a 7-diethylaminocoumarin fluorophore through a 1,2,3-triazol-1,4-diyl π-linker results in a perfect signal transduction chain for the exclusive sensing of K^+ under simulated physiological conditions.[12a,b] Thereby, we prepared a fluorescent membrane sensor by incorporating the *N*-(2-methoxyethoxyphenyl)aza-18-crown-6-fluoroionophore into a hydrogel, which showed fast and fully reversible fluorescence intensity changes in the range of 1–10 mM potassium.[12b]

In this communication, we have maintained the signal transduction chain: aniline-triazole-coumarin in the construction of Na^+-sensitive PET-fluoroionophores **1** and **2**

(Scheme 1). Thus, for selective Na^+ binding the *N*-(*o*-methoxyphenyl)aza-15-crown-5 ionophore[4,6d] was selected owing to its excellent selectivity for Na^+ over K^+ in the range of 100–180 mM. Herein, in Tris buffer Na^+ induces a fluorescence intensity and decay time enhancement of **1** or **2**. When **1** was embedded in the hydrogel poly(2-hydroxypropyl)methacrylate (PHPMA), we found a reversible change in decay-time and intensity by fiber-optical fluorescence measurements. To the best of our knowledge, it is the first fiber-optical sensor for Na^+ sensing based on a combination of a PET-fluoroionophore and of the hydrogel PHPMA using fluorescence decay time changes as the fundamental detection principle.

The CuAAC of the ethynyl-fuctionalized *N*-(*o*-methoxyphenyl)aza-15-crown-5 with 3-azido-7-diethyl-aminocoumarin[13a] afforded 1,2,3-triazol-fluoroionophore **1**. The corresponding constitutional isomer **2** was obtained by the reaction of the azido-functionalized *N*-(*o*-methoxyphenyl)aza-15-crown-5 with 3-ethynyl-7-diethylaminocoumarin[13b] (see ESI†).



Scheme 1 Regioisomeric 1,2,3-triazol-fluoroionophores **1** and **2**.

The UV-Vis- absorption spectra of **1** and **2** in Tris buffer (10 mM, pH = 7.2) show the typical long-wavelength coumarin CT absorption band at λ_{max} = 422 nm (**1**) or 419 nm (**2**) (Figure S1a and S1b). The isomeric 1,2,3-triazol-1,4-diyl linkage has only a small effect on λ_{max}. In the UV-Vis-absorption spectra of **1** and **2** a second CT absorption at ~280 nm (**1**) or ~315 nm (**2**) is observed (Figure S1) which is typical for π-conjugated 1,2,3-triazol-1,4-diyl-fluoroionophores.[12a,b] The lowest energy coumarin absorption bands at 422 nm for **1** and 419 nm for **2** are essentially unchanged upon Na^+ or K^+ addition (Figure S1a,b). The coordination of Na^+ in the cavity of the aza-15-crown-5 unit can be seen from the short-wavelength CT transition. The binding of Na^+ reduces the electron donor character of the anilino unit which leads to a decrease of the CT absorption band at ~280 nm (**1**) or ~315 nm (**2**) (Figure S1).

As already observed for *N*-phenylaza-18-crown-6 and *N*-(2-methoxyethoxyphenyl)aza-18-crown-6 substituted 1,2,3-triazol-coumarin fluoroionophores[12a,b] the fluorescence of the coumarin unit is almost completely quenched. The fluorescence quantum yields of **1** and **2** in Tris buffer are 0.009 and 0.051, respectively. Probably, a reductive PET occurs from the anilino-triazole unit to the coumarin moiety (see ESI†).

We investigated the influence of Na^+ and K^+ on the fluorescence of **1** and **2** in the physiological interesting concentration range of 0–180 mM Na^+ or K^+ (see ESI† for K^+). Figure 1a shows the fluorescence enhancement of **1** in a buffered H_2O/DMSO mixture (99/1, v/v; 10 mM Tris; pH = 7.2) in the presence of 0–180 mM Na^+. We observed Na^+-induced fluorescence enhancements for **1** (FEF_{Na+} : 5.0 ± 0.1) and **2** (FEF_{Na+} : 2.5 ± 0.1). The fluorescence signal of **1** and **2** is slightly effected by K^+ (FEF_{K+} : 1.5 ± 0.1, Table S1). The *FEF* of **1** in the presence of 180 mM Na^+ is higher than for **2** (see Table S1). A Na^+-induced fluorescence enhancement of **1** (*FEF* : 4.7 ± 0.1) and **2** (*FEF* : 2.6 ± 0.1) is also observed in the presence of physiological concentrations of K^+ ($[K^+]+[Na^+]$ = 180 mM). Furthermore, Benesi-Hildebrand plots for Na^+ binding showed a linear relationship (R^2= 0.998 (**1**), Figure S5a and R^2= 0.9986 (**2**), Figure S5b), indicating 1:1 complexation between **1** or **2** and Na^+. The K_d values were calculated from the fluorescence titration curves. The K_d value of **1** for Na^+ is ~120 mM (Figure S5a and S7a) and of **2** for Na^+, we found a higher K_d value of ~260 mM (Figure S5b and S7b), suggesting that K_d is influenced by the isomeric triazole linkage.

The selectivity of **1** for Na^+ over K^+ in the physiological range is shown in Figure S3c. The K_d value of **1** for K^+ is ~276 mM and of **2** for K^+ is ~1060 mM (Figure S6a and S6b), indicating that **1** is ~3-fold more selective for Na^+ than for K^+ and **2** ~4-fold.

To investigate the pH-sensitivity of **1** and **2**, the fluorescence intensity was measured in H_2O at different pH values (see ESI†). The resulting pK_a of **1** is 4.9 and that of **2** is 4.6, respectively. **1** and **2** are unaffected in the physiological relevant pH range of 6–8 (Figure S8).

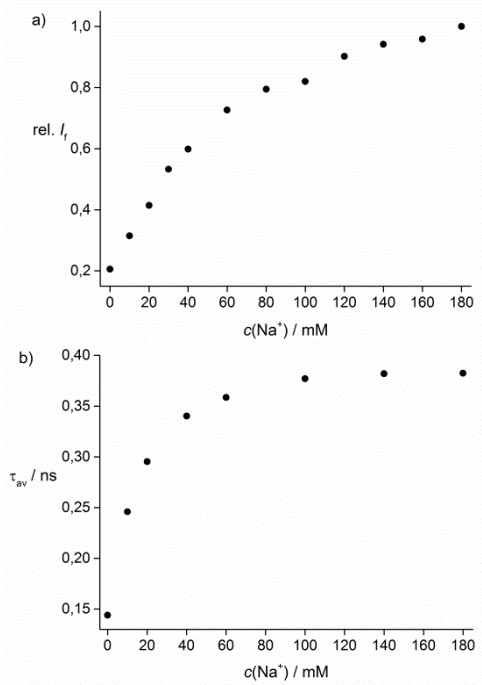


Fig. 1 Titration curves of **1** (c= 10^{-5} M, λ_{ex} = 422 nm) in 10 mM Tris buffer (pH = 7.2) + Na^+ based on a) fluorescence intensity at 500 nm and b) fluorescence decay time measurements (see ESI†).

Due to the underlying PET process in **1**, the observed fluorescence kinetics are complex. The fluorescence decay curves at λ_{em} = 500 nm could be reasonably well fitted using a bi-exponetial decay kinetics yielding main decay times of 0.08 ns (90%) for **1** and 0.39 ns (99%) for **1** + 180 mM Na^+ (Table S1). During complexation of Na^+ the fraction of the short fluorescence decay time (0.08 ns) component decreases and the averaged fluorescence decay time increases (Figure 1b) as the fraction of the longer fluorescence deacy time increases. The K_d value of **1** + Na^+ calculated based on the averaged fluorescence decay time is ~15 mM (see ESI†). The fluorescence decay times of **1** were only slighty affected by K^+ ions (see Table S1). Further, we observed for **2** + Na^+ a similar decay time behaviour as found for **1** + Na^+ (Table S1).

Probe **1** was selected for studies on its application as a Na^+-sensor because it showed higher Na^+/K^+ fluorescence response than probe **2**. To apply **1** in a sensor membrane for continuous monitoring of Na^+ concentrations in liquid streams, such as blood, we incorporated it into a polymer matrix. The hydrogel PHPMA is a convenient polymer for making stable membrane sensors.[14a,b] For immobilisation of **1** within the hydrogel, both are dissolved in EtOH. This homogenoues mixture was used for film and optode preparation (see ESI†). Firstly, we prepared films of the mixture on glass slides, which we placed in standard cuvettes. The concentration of **1** in this film is $3.75 \cdot 10^{-5}$ M. In order to avoid the inherent to fluorescence intensity based measurement difficulties (vide supra) we determined Na^+-induced fluorescence decay time changes by time-correlated single photon counting (TCSPC). In hydrogel we found for **1** a fluorescence decay time τ_f of 2.1 ns, which is distinctly longer than in solution (see Table S1). In solution the decay time is faster than in the fixed polymer, because the rate constant of quenching by radiationless processes is larger. The decay time τ_f increases from 2.1 ns in 10 mM Tris buffer to 2.7 ns in the presence of 140 mM Na^+. The K_d-value determined by TCSPC measurements of **1** for Na^+ is 42 mM in the polymer PHPMA (see Figure S12).

In the next step we investigated the τ_f behaviour of **1** in a PHPMA film using a low-cost frequency-domain spectrometer (FD-S) suitable for *in-situ* applications. Therefore, we prepared a PHPMA-based film with a higher dye concentration of **1** ($c = 7.5 \cdot 10^{-4}$ M) which is needed for the FD-S set-up due to sensitivity requirements. We found decay times for **1** (τ_f = 2.2 ns) and **1** + 100 mM Na^+ (τ_f = 2.9 ns) in PHPMA by FD-S (see Figure S13) comparable to the TCSPC measurements but the K_d-value of **1** + Na^+ decreases to 1.6 mM. Due to the higher dye concentration in the polymer matrix the sensitivity to sodium seemed to increase. The K_d-value of **1** + K^+ in PHPMA is 6355 mM (Figure S13) indicating the highly improved Na^+/K^+ selectivity of the sensor film.

Finally, in order to develop this outstanding Na^+-selective film further into an optode for fiber-optical measurements (Figure S10 and S11), fluorescence intensity and decay time measurements were successfully carried out. The fluorescence intensity increases by dipping the optode in different concentrated Na^+ solutions until 50 mM (see Figure 2a) were present. The K_d value of **1** + Na^+ based on intensity changes determined by the optode is 6 mM. A comparable result was found by using FD-S. Here, the K_d value of **1** + Na^+ is 2.4 mM with this fiber-optical sensor (see Figure 2b). Consequently, this optode allows a determination of Na^+ by fluorescence decay time measurements in the range of 1–10 mM.

Further, for continous sensing of Na^+ in the range of 1–10 mM by fluorescence intensity or decay time changes of this optode we tested the reversibility. As a proof of principle our first results for continous Na^+ sensing are shown in Figure S17. The response time of the optode after switching the solution from 0 mM Na^+ to 10 mM Na^+ during a time period of 2 h shows a good reversible behaviour. The response time depends on the direction of change: by increasing sodium concentration the response is faster (< 8 min) than decreasing concentration (15 min). Here, the layout of the optode (thickness of the polymer, concentration of the probe) is a key parameter in this respect and will be further improved in order to reduce the response time.

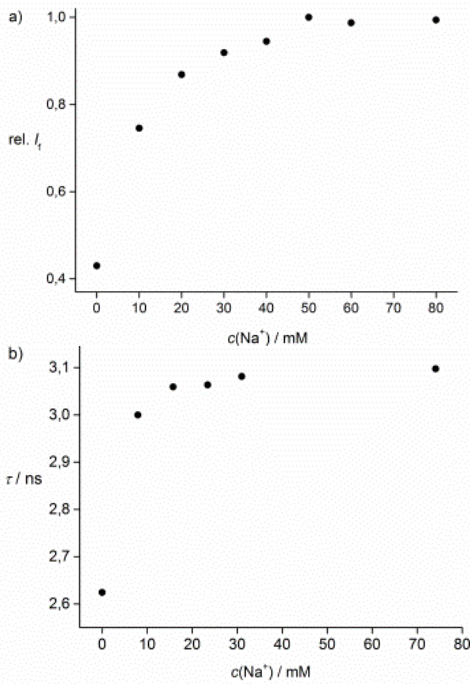


Fig. 2 Titration curves of **1** ($c = 7.5 \cdot 10^{-4}$ M) in PHPMA + Na^+ based on a) fluorescence intensity and b) FD-S measurements by an optode.

In summary, we have synthesised two regioisomeric fluoroionophores **1** and **2** for Na^+ by "click" chemistry. In solution they showed different K_d-values (**1** + Na^+ ~120 mM and **2** + Na^+ ~260 mM) under simulated physiological conditions. Probe **1** is a capable fluoroionophore for the selective determination of extracellular Na^+ levels by fluorescence intensity changes. Further, **1** was incorporated into a membrane sensor that consisting of a polymethacrylate hydrogel and, in this form, it enabled the continuous monitoring of Na^+ (1–10mM) with a reversible response based on fluorescence decay time measurements. Overall, we have shown the first reversible fiber-optical sensor for Na^+ ions utilizing fluorescence decay time changes. Further studies to improve the brightness, the photostability, the Na^+-induced overall fluorescence decay time changes and the response time of our optode are currently performed in our laboratories.

Notes and references

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† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

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