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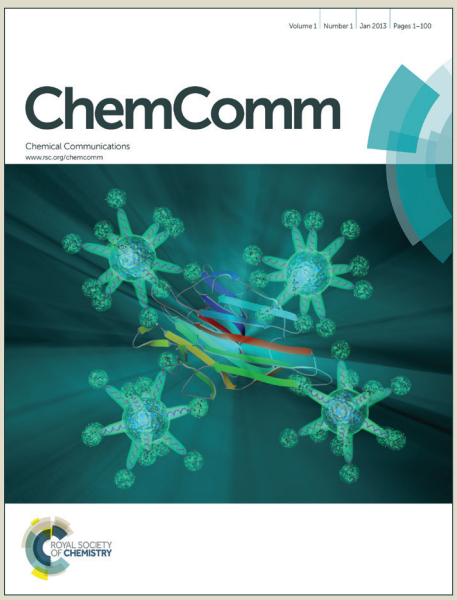

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A red-emitting ratiometric fluorescent probe based on a benzophosphole *P*-oxide scaffold for the detection of intracellular sodium ion

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We disclose the development of a ratiometric fluorescent probe based on a benzophosphole *P*-oxide and its application for the detection of intracellular Na^+ ions. Excitation by visible light induced a red emission from this probe in water, which was subject to a hypsochromic shift upon complexation with Na^+. Based on this change, a ratiometric analysis enabled us to visualise changes of the Na^+ concentration in living mammalian cells.

Ratiometric fluorescent probes are powerful diagnostic tools for the quantitative detection of metal ions in living systems. To date, a number of fascinating examples have been reported both for biologically relevant metal ions such as Ca^{2+} and Zn^{2+},[1,2] as well as for toxic ions such as Cd^{2+} and Hg^{2+}.[3,4] In this context, π-conjugated molecules consisting of an electron-donating (D) moiety and an electron-accepting (A) moiety represent a useful compound class.[5] Such D-π-A systems usually exhibit an emission from an excited state with intramolecular charge transfer (ICT) character, which is sensitive to changes of the electron-donating ability of the donor moiety. Once the donor moiety interacts with a target metal ion, the ICT character is reduced and hypsochromic shifts are observed for both the absorption and emission maxima.

Recently, we have developed the new D-π-A fluorophore **1** (Fig. 1), containing triphenylamine and benzophosphole *P*-oxide as the electron-donating and -accepting moieties, respectively.[6] A notable feature of **1** is its drastic change of fluorescence colour as a function of the polarity of the surrounding environment. Its photostability is also noteworthy, as it is higher than those of fluorescein and BODIPY, both of which are widely used in biological fluorescence imaging. Taking advantage of these features, **1** was successfully employed to discriminate hydrophobic oil droplets from other subcellular domains in adipocytes on the basis of different fluorescence colours.

As benzophosphole is a heavier analogue of indole,[7] **1** is reminiscent of a family of Indo fluorescent probes such as Indo-1 and mag-Indo, which are commonly used for the detection of Ca^{2+} and Mg^{2+}, respectively. Both show excellent ratiometric behaviour, *i.e.* a significant hypsochromic shift of the emission wavelength ($\Delta\lambda_{em}$ = *ca.* 75 nm) upon complexation to metal ions, while simultaneously maintaining high fluorescence quantum yields ($\Phi_F \sim 0.50$).[8] However, they require excitation by UV light (λ_{ex} = 330–365 nm) for optical imaging, which may inflict serious damage on biological samples. In addition, these Indo probes undergo rapid photobleaching during the observation.[9] We envisioned that the use of an electron-accepting benzophosphole *P*-oxide may overcome these drawbacks, as it should impart the probe with a more red-shifted absorption, suitable for an excitation at 405 nm, which is a wavelength commonly used in fluorescence microscopy. Moreover, previous studies suggested high levels of photostability for this compound class.

Herein, we would like to report the design of a ratiometric fluorescent probe based on a benzophosphole *P*-oxide scaffold for the detection of Na^+ ions. Despite the direct correlation of Na^+ to physiologically and pathologically important processes such as the control of membrane electrical potentials, calcium regulation, and the transport of neurotransmitters, only few probes for the visualisation of intracellular Na^+ behaviour have been reported.[10] Even though quantitative ratiometric Na^+ imaging can be carried out using SBFI, a commercially available indicator for Na^+, its applications to biological samples remain limited, due to the requirement of excitation with UV light. The development of a ratiometric probe that can be excited by visible light represents accordingly a highly important research target in the context of Na^+ sensing. Our new ratiometric Na^+ probe, **NaGY**, contains an aza-crown ether moiety, which serves both as the Na^+ binding site and as the electron-donating moiety (Fig. 1). In order to avoid specific accumulation on hydrophobic organelle, a carboxyl group was

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† Electronic Supplementary Information (ESI) available: Experimental details, Scheme S1, Figs. S1–S5, and NMR spectra. See DOI: 10.1039/x0xx00000x

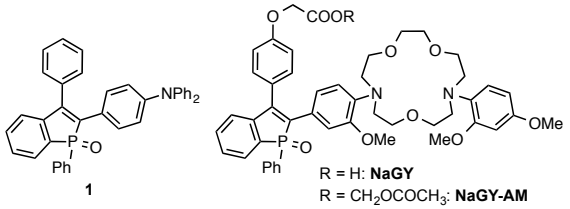
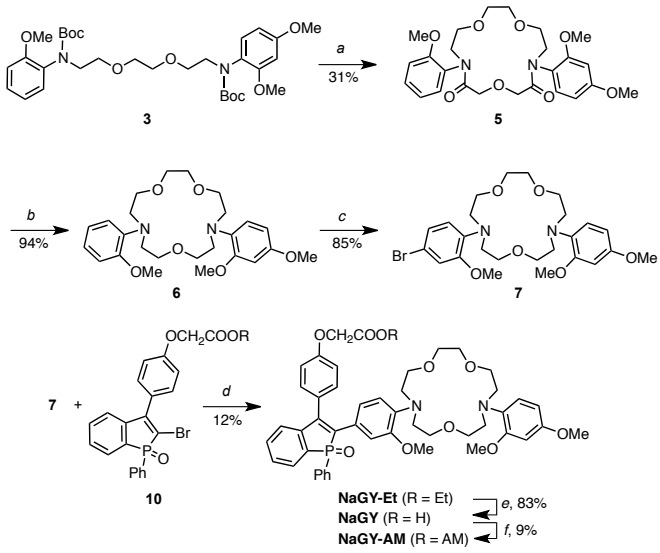


Fig. 1 Structures of fluorescent probes based on a benzophosphole *P*-oxide scaffold. Left: environment-sensitive probe **1**, right: ratiometric Na^+ probe **NaGY** and its membrane-permeable form **NaGY-AM**.



Scheme 1 Synthetic scheme of **NaGY** and **NaGY-AM**. *Reagents and conditions*: *a*, i) 1:6 CF_3CO_2H/CH_2Cl_2, 1.5 h; ii) diglycolyl chloride, pyridine, toluene, 100 °C, 3 d; *b*, $BF_3{\cdot}OEt_2$, $NaBH_4$, THF, reflux, 4 h; *c*, NBS, CH_3CN, –30 °C, 16 h, 85%; *d*, i) bis(pinacolato)diboron, Pd(dppf)Cl_2, KOAc, 1,4-dioxane, 100 °C, 24 h; ii) **10**, Pd(dppf)Cl_2, K_3PO_4, 1,4-dioxane, 100 °C, 17 h; *e*, LiOH·H_2O, H_2O, MeOH, 1.5 h; *f*, bromomethyl acetate, Et(*i*Pr)$_2$N, CH_2Cl_2, 18 h.

attached onto the 3-phenyl group of the phosphole ring. This probe exhibited an absorption band in the visible region for both the Na^+-free and the Na^+-bound forms, and displayed different emission colours in the red region of the visible spectrum depending on the Na^+ binding situation. Thus, the concentration of Na^+ in living cells could be successfully estimated based on the ratio of signal intensities for these two species.

For the preparation of **NaGY**, Na^+ binding site precursor **7** and benzophosphole *P*-oxide moiety **10** were synthesized separately, before being combined by a Suzuki-Miyaura cross-coupling reaction (Scheme 1). This synthetic procedure should be generally applicable, and thus allow the development of a variety of benzophosphole *P*-oxide-based ratiometric probes. The Na^+ binding site was synthesized according to a modified literature procedure.[11] Boc-protected mono- and dimethoxyanilines were treated consecutively with 1,2-bis(2-chloroethoxy)ethane to afford unsymmetrical **3**. The deprotection of **3**, followed by macrocyclisation with oxydiacetyl dichloride under high dilution conditions, furnished diamide 15-crown-5 derivative **5**. The Na^+-binding moiety **6**

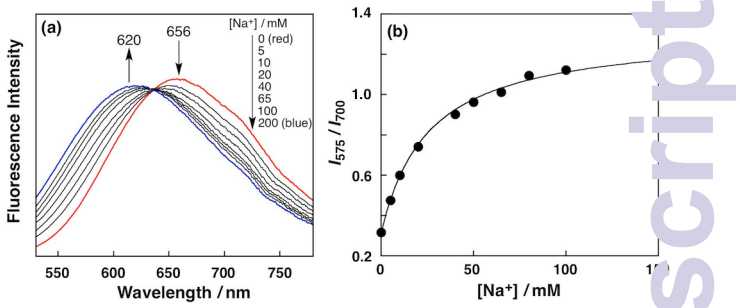


Fig. 2 (a) Spectral emission change of **NaGY** (25 μM) in 50 mM HEPES (pH 7.4) at an excitation of 405 nm upon addition of NaCl (0, 5, 10, 20, 40, 65, 100, or 200 mM). (b) Fluorescence intensity ratio between 575 and 700 nm (I_{575}/I_{700}) as a function of the Na^+ concentration with a best curve fitting for K_d = 16.0 ± 1.2 mM.

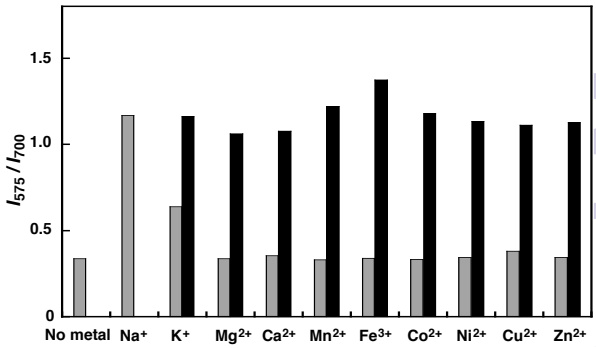


Fig. 3 Metal ion-selectivity of **NaGY** in response to the addition of various metal ions. Gray bars represent the fluorescence intensity ratio of **NaGY** (25 μM) between 575 and 700 nm (I_{575}/I_{700}) in the presence of either 150 mM of Na^+ or K^+, 10 mM of Ca^{2+} or Mg^{2+}, or 0.1 mM of Mn^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}. Black bars represent measurements after the addition of 200 mM Na^+ to the solution.

was obtained from a reduction with diborane generated *in situ* using $NaBH_4$ and $BF_3{\cdot}OEt_2$. Brominated **7** was subjected to Miyaura borylation conditions, which allowed a subsequent coupling with benzophosphole moiety **10**. Finally, the hydrolysis of the ethyl ester moiety in **10** afforded the ratiometric Na^+ probe **NaGY**, from which the membrane-permeable AM-ester form **NaGY-AM** (AM = acetoxymethyl) was prepared.

Spectroscopic measurements on **NaGY** were carried out in 50 mM HEPES (pH 7.4) containing 1% DMSO as a co-solvent. For **NaGY**, a broad absorption band centred on a maximum at 394 nm (ε = 5.63 × 10^3 M^{-1} cm^{-1}) was observed. Upon increasing the concentration of Na^+, the absorption maximum shifted hypsochromically to 371 nm, while maintain a molar absorption coefficient (Fig. S1, ESI†). During the titration, two distinct isosbestic points were observed at 337 and 374 nm, indicative of a single equilibrium between the Na^+-free and the Na^+-bound form of **NaGY** in solution. The hypsochromic shift in the absorption spectrum was assigned to the coordination of a Na^+ ion to the donor nitrogen atoms, which should reduce the ICT character of **NaGY**. Plotting the absorbance at 410 nm against the Na^+ concentration allowed the determination of the dissociation constant for Na^+ (K_d = 14.0 ± 0.1 mM; Fig. S2 ESI†). It should be noted that both species can be excited with visible light, *e.g.* by using a 405 nm diode laser, which is frequently used as an excitation source in confocal microscopy.

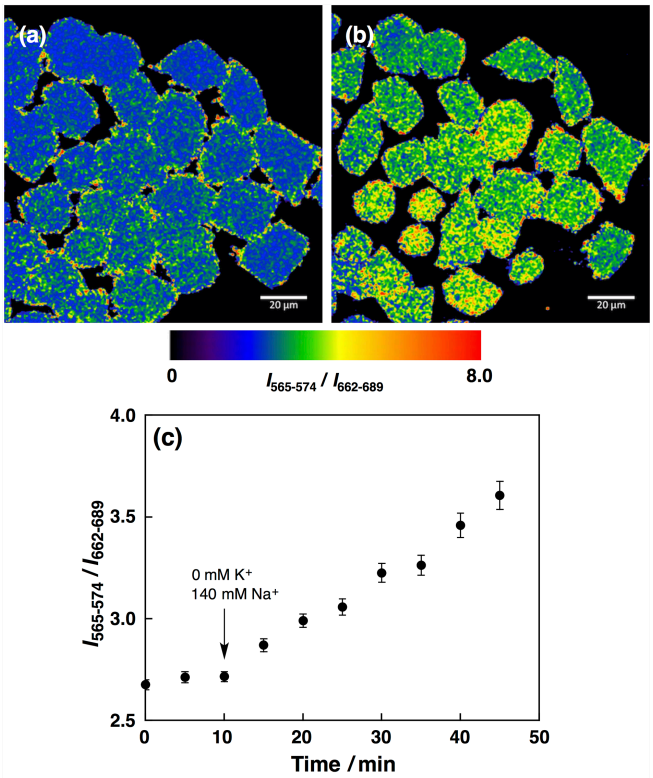


Fig. 4 Fluorescence intensity ratio images (λ_{ex} = 405 nm) of live HeLa cells with increases in cytosolic Na^+ levels visualized using **NaGY** (10 μM). Images in pseudocolor represent the ratio of emission intensities collected with channels of 565–574 nm ($I_{565-574}$) and 662–689 nm ($I_{662-689}$). Before staring acquisition, the cells were stained with **NaGY** (10 μM) for 30 min at 37 °C. (a) Cells incubated with basal DMEM medium (0 min). (b) Cells after incubation with K^+-free DMEM containing 140 mM of Na^+ (35 min). (c) Change of the average $I_{565-574}/I_{662-689}$ ratio of the cells ($N > 20$) during the incubation in the absence of extracellular K^+. The medium was replaced 10 min after the beginning of the measurements. Error bar = S. E.

Fig. 2a shows a set of emission spectra for **NaGY** in the presence of various concentrations of Na^+. To use the identical conditions for optical imaging of cells (*vide infra*), all spectra were recorded with an excitation wavelength of 405 nm. In the absence of Na^+, **NaGY** exhibited a broad emission band in the red region (λ_{em} = 656 nm; red line in Fig. 2a) concomitant with a very large Stokes shift (10100 cm^{-1}). This Stokes shift is even larger than that of **1** in ethanol (7100 cm^{-1})[6] and presumably arises from a more pronounced ICT character in the excited state. This would also partially account for the low fluorescence quantum yield of **NaGY** in the aqueous medium (Φ_F = 0.016). With increasing concentrations of Na^+, the emission spectra displayed hypochromic shifts, and at a Na^+ concentration of 200 mM the maximum emission wavelength reached 620 nm (blue line in Fig. 2a), where the quantum yield (Φ_F = 0.028) was comparable to that of Na^+-free **NaGY**. Because Na^+ binding caused the largest changes in fluorescence intensities at 575 nm and 700 nm upon excitation at 405 nm (Fig. S3, ESI†), we selected these wavelengths for the ratiometric analyses of **NaGY**. The plots of the fluorescence intensity ratios between 575 and 700 nm (I_{575}/I_{700}) against the Na^+ concentration furnished the dissociation constant for Na^+ (K_d = 16.0 ± 1.2 mM), which is in good agreement with the value obtained from the absorption titration (Fig. 2b). This nonlinear fitting analysis revealed that **NaGY** should be suitable for a detection of I.. concentrations between 5 – 60 mM, which is the optimal rang for a monitoring of intracellular Na^+ concentratio fluctuations.[12]

To test the performance of **NaGY** in biological systems, w measured its emission spectrum in the presence of various biologically relevant metal ions (Fig. 3). Most importantl , **NaGY** showed a high selectivity towards Na^+. A significant change in the fluorescent properties was neither observed upo addition of 10 mM of Mg^{2+} or Ca^{2+}, which exist in the cytoplasm in the mM range,[13] nor upon addition of 0.1 mM of biological trace metals including Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}.[14] Moreover, the presence of these metal ions did n interfere with the complexation of Na^+. However, the additio of K^+, which is the major intracellular cation present in th concentration of ca. 150 mM, resulted in a minor hypsochrom shift ($\Delta\lambda_{em}$ = 7 nm) of the emission spectrum (Fig. S4, ESI†). Plotting the I_{575}/I_{700} ratio against the concentration of K furnished the corresponding dissociation constant for K^+ (K = 223 ± 7 mM; Fig. S5, ESI†), which was 16 times larger than that for Na^+, indicating that a potential physiological change of the K^+ concentration should not affect the ability of **NaGY** to detect Na^+ concentrations significantly. Unlike SBFI,[10a] the emission spectrum of **NaGY** remained constant over a wide range of pH values (pH 5–8), and therefore its fluorescence properties should not be affected by intracellular pH changes (Fig. S6, ESI†). Moreover, trypan blue assay revealed negligible cytotoxicity of **NaGY** (Fig. S7, ESI†).

In order to explore the potential application of **NaGY** fo the detection of Na^+ concentration changes in living cells, HeL cells were incubated for 30 min with **NaGY-AM** (10 μM), *i.* the membrane-permeable form of **NaGY**, in Dulbecco Modified Eagle's Medium (DMEM) that contained *ca*. 150 mM of Na^+ and 5 mM of K^+. After washing the cells three time with DMEM, their fluorescence images were recorded using a confocal microscope (λ_{ex} = 405 nm), equipped with a Ga.. multi-channel spectral detector. For the ratiometric analysis, integrated emission intensities in the range of 565–574 nm ($I_{565-574}$) and 662–689 nm ($I_{662-689}$), corresponding to the Na^+-boun and Na^+-free form, respectively, were collected.[15] Figure 4 shows the change of pseudocoloured ratio images upo inhibition of the Na^+/K^+ pump, *i.e*. by replacing the incubatio medium to K^+-free DMEM containing 140 mM of Na^+ becaus Na^+/K^+ pump activity is sensitive to intracellular Na concentration.[16] During the incubation period, the $I_{565-574}/I_{662-68}$ ratio increased linearly after blocking the Na^+/K^+ pump. Th result is in good agreement with that reported by Despa *et al*., where the Na^+ concentration in HeLa cells increased linearly from 10 to 35 mM during a 40 min incubation period in the same K^+-free medium,[17] suggesting that **NaGY** can probe the change of the Na^+ concentration within the physiologically relevant concentration range in living cells.

In conclusion, we have developed a ratiometric fluorescent probe for Na^+ based on a benzophosphole *P*-oxide fluorophor This probe can be excited using visible light (λ_{ex} = 405 nm) and it exhibits a hypsochromic shift of its emission spectrum upo complexation with Na^+. The ratiometric analysis delivered a

Na^+ dissociation constant (K_d = 16.0 ± 1.2 mM) that is suitable for monitoring the change of intracellular Na^+ concentrations. Using this probe, we have demonstrated a ratiometric visualisation of intracellular Na^+ dynamics caused by blocking the Na^+/K^+ pump in living mammalian cells. This probe should hence represent a promising diagnostic tool for the investigation of Na^+ dynamics in neuronal cells, *e.g.* in the context of potential-evoked Na^+ influx.

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