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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 Ca²⁺ and Zn²⁺, as well as for toxic ions such as Cd²⁺ and Hg²⁺. In this context, π-conjugated molecules consisting of an electron-donating (D) moiety and an electron-accepting (A) moiety represent a useful compound class. 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. 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, 1 reminiscent of a family of Indo fluorescent probes such as Indo 1 and mag-Indo, which are commonly used for the detection of Ca²⁺ and Mg²⁺, respectively. Both show excellent ratiometric behaviour, i.e. a significant hypsochromic shift of the emission wavelength (Δλem = ca. 75 nm) upon complexation to metal ions, while simultaneously maintaining high fluorescence quantum yields (Φf ~ 0.50). However, they require excitation by UV light (λex = 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. 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. 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...
diamide 15 and dimethoxyanilines were coupled reaction (Scheme 1). This synthetic procedure should be applicable.

Scheme 1 Synthetic scheme of NaGY and NaGY-AM. Reagents and conditions: a, i) 1:6 CF,CO,H/CHCl₂, 1.5 h; ii) diglycol chloride, pyridine, toluene, 100 °C, 3 d; b, BF₃·OEt₂, NaBH₄, THF, reflux, 4 h; c, NBS, CH₂CN, -30 °C, 16 h, 85%; d, i) bis(pincalato)diboron, Pd(dppf)Cl₂, KOAc, 1,4-dioxane, 100 °C, 24 h; ii) 10, Pd(dppf)Cl₂, K₂PO₄, 1,4-dioxane, 100 °C, 17 h; e, LiOH·H₂O, H₂O, MeOH, 1.5 h; f, bromomethyl acetate, Et(Pr)₃N, CH₂Cl₂, 18 h.

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.

was obtained from a reduction with diborane generated in situ, using NaBH₄ and BF₃·OEt₂. 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 = acetoxyethyl) 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 × 10³ 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⁺ (Kₛ = 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.
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revealed that NaGY should be suitable for a detection of Na+ concentrations between 5 – 60 mM, which is the optimal range for monitoring intracellular Na+ concentration fluctuations.12

To test the performance of NaGY in biological systems, we measured its emission spectrum in the presence of various biologically relevant metal ions (Fig. 3). Most importantly, NaGY showed a high selectivity towards Na+. A significant change in the fluorescent properties was neither observed upon addition of 10 mM of Mg2+, Ca2+, or Fe3+, Co2+, Ni2+, Cu2+, or Zn2+.14 Moreover, the presence of these metal ions did not interfere with the complexation of Na+. However, the addition of K+, which is the major intracellular cation present in the concentration of ca. 150 mM, resulted in a minor hypsochromic shift (Δλem = 7 nm) of the emission spectrum (Fig. S4, ESI†). Plotting the I757/I700 ratio against the concentration of K+ furnished the corresponding dissociation constant for K+ (Kd = 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 fluorescent 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 for the detection of Na+ concentration changes in living cells, HeLa cells were incubated for 30 min with NaGY-AM (10 μM), i.e. 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 times with DMEM, their fluorescent images were recorded using a confocal microscope (λex = 405 nm), equipped with a GaAsP multi-channel spectral detector. For the ratiometric analysis, integrated emission intensities in the range of 565–574 nm (I565–574) and 662–689 nm (I662–689), corresponding to the Na+-bound and Na+-free form, respectively, were collected.15 Figure 4a shows the change of pseudocolored ratio images upon inhibition of the Na+/K+ pump, i.e. by replacing the incubation medium to K+-free DMEM containing 140 mM of Na+ because Na+/K+ pump activity is sensitive to intracellular Na+ concentration.16 During the incubation period, the I565–574/I662–689 ratio increased linearly after blocking the Na+/K+ pump. This result is in good agreement with that reported by Despa et al.,13 where the Na+ concentration in HeLa cells increased linearly from 10 to 35 mM during a 40 min incubation period in 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 fluorophore. This probe can be excited using visible light (λex = 405 nm) and it exhibits a hypsochromic shift of its emission spectrum upon complexation with Na+. The ratiometric analysis delivered a
Na⁺ dissociation constant ($K_d = 16.0 \pm 1.2$ 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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Notes and references
15. The same fluorescence behavior was observed by using the ratio of $I_{585}/I_{488}$, see Fig. S8 in ESI.