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Sodium Green as a Potential Probe for Intracellular Sodium Imaging Based on Fluorescence Lifetime

Henryk Szmecinski, Joseph R. Lakowicz¹

Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 725 West Lombard Street, Baltimore, Maryland 21201

Abstract

We characterized the use of the fluorescent probe Sodium Green for measurements of intracellular free sodium using frequency-domain, phase-modulation fluorometry. The intensity decays were found to be strongly Na^+ dependent, with mean lifetime increasing from 1.13 ns in the absence of Na^+ to 2.39 ns in the presence of 140 mM Na^+ . Detailed analysis of the intensity decays in the presence of Na^+ and K^+ in the concentration range from 0 to 500 mM is provided. Sodium sensing using data measured at a single modulation frequency is described. Phase and modulation data showed high sensitivity to Na^+ and substantially lower sensitivity to K^+ . Additionally, exposure of Sodium Green to intense illumination indicated that Sodium Green is much more photostable than its precursor, fluorescein. These results indicate that lifetime-based measurements with Sodium Green can be used for imaging of intracellular free $[\text{Na}^+]$ in the range from about 0.5 to 50 mM with high accuracy.

The alkali ions Na^+ and K^+ play an important role in many fundamental biological processes (1). Indicators for intracellular Na^+ would be useful, for example, in studies of Na^+ exchange in smooth muscle (2) and degranulation of cells (3) and to study the major mechanisms of monovalent cation transport (4, 5). Concentration gradients of Na^+ and K^+ across the plasma membrane are partly responsible for membrane potential, epithelial transport, and regulation of other intracellular ions. The Na^+ gradient between extracellular (more than 120 mM) and intracellular (from 4 to 20 mM) concentrations is important for understanding cellular energy metabolism (6). Fluorescent indicators for Na^+ are valuable for nondestructive monitoring of spatial and temporal resolution of Na^+ in cells. However, only a few fluorescent Na^+ indicators, such as FCryp-2 (7), SBFI,² SBFO, and SBFP (8), F221 (9), and Sodium Green (10), have been developed. The requirement for wavelength shift in the excitation or emission spectrum of the probe for quantitative sodium measurements has been one of the reasons that the only fluorescent probe used for Na^+ cellular applications has been SBFI. It has been shown that SBFI is superior to other excitation wavelength-ratiometric probe SBFP (11).

¹To whom correspondence should be addressed.

²Abbreviations used: TMA(Cl), tetramethylammonium (chloride); SBFI, sodium-binding benzofuran isophthalate; SBFO, sodium-binding benzofuran oxazole; SBFP, sodium-binding benzofuran phthalate.

A newly available Na^+ -sensitive probe, Sodium Green (Scheme I), uses a new molecular design which has already resulted in the calcium probes, Calcium Green, Orange, and Crimson (12, 13) and the magnesium probes, Magnesium Green and Magnesium Orange (10, 14, 15). The new design of Sodium Green uses a high-quantum-yield fluorescein linked to a Na^+ -selective crown ether. This approach is versatile since a large number of dyes with desired fluorescence properties are known, and the fluorescence properties of dye are usually retained upon conjugation to ion binding moieties (12, 13, 15). These conjugated probes display long excitation and emission wavelengths, excellent changes of quantum yield, but no spectral shifts upon ion binding.

Recent reports (16, 17) have already shown the necessity of using Sodium Green for flow cytometric analysis of intracellular Na^+ concentration and for studying the effects of amiloride on Na^+ transport. Amiloride is strongly fluorescent when excited at the wavelength range for SBFI, making flow cytometric measurements difficult between 340 and 380 nm.

The spectral advantages of Sodium Green can be exploited using fluorescence lifetime imaging microscopy or lifetime-based sensing, which does not require ratiometric probes and is mostly independent of probe concentration (18). Lifetime-based sensing or imaging requires that both forms of indicator (free and ion-bound) be fluorescent but with different lifetimes. The measured lifetime is mostly independent of the total intensity of the probe, but reflects the fractional contribution of the Na^+ -free and Na^+ -bound forms of the probe and thus the Na^+ concentration. Regardless of the complexity of intensity decay of the probe, a single modulation frequency can be used to measure ion concentration with frequency-domain fluorometry (18). Fluorescent lifetime indicators have been characterized for Ca^{2+} (13, 19, 20), Mg^{2+} (15), and H^+ (21–24). We reported also that the K^+ probe, PBFI, changes its lifetime upon ion binding (18). The detailed lifetime characterization of SBFI and PBFI will be described elsewhere (25). The purpose of this paper is to provide the fluorescence properties and demonstrate Na^+ sensing based on the intensity decays of Sodium Green in the range of intracellular free Na^+ concentrations.

MATERIALS AND METHODS

Sodium Green tetra(tetramethylammonium) salt (cell impermeant) was obtained from Molecular Probes, Inc. (Eugene, OR). Its chemical structure is shown in Scheme I. Absorption and emission spectra were measured using a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer and a SLM 8000 photon-counting spectrofluorometer, respectively. The samples were freshly prepared in 5 mM Hepes buffer, pH 7.2, with 100 mM TMA(Cl) and were carried out at room temperature (20°C). The ionic strength of the buffer was not adjusted for increased concentrations of NaCl and KCl. Fluorescence intensity decays were measured with frequency-domain instrumentation previously described (26). Excitation was provided by a mode-locked argon ion laser (514 nm) with a repetition rate of 75.9 MHz. The emission was observed through a long-wave pass filter above 530 nm. The frequency-domain data were used to determine the intensity decay law using the multiexponential model (27, 28)

$$I(t) = \sum_{i=1}^n \alpha_i e^{-t/\tau_i},$$

[1]

where α_i are the preexponential factors (amplitudes), τ_i are the decay times, and n is the number of exponential components. The mean decay time $\bar{\tau}$ and fractional intensities f_i of each component are given by

$$\bar{\tau} = \sum_i \alpha_i \tau_i^2 / \sum_j \alpha_j \tau_j; f_i = \alpha_i \tau_i / \sum_i \alpha_i \tau_i.$$

[2]

The intensity decays of Sodium Green were also fitted to a global model in which the decay times were assumed to be independent of Na^+ or K^+ concentration, but the amplitudes of decay times reflect changes in the fractional amounts of each species for various concentrations of Na^+ or K^+ (free and ion-bound forms). Such analyses have been described previously for calcium (13, 20, 29) and magnesium probes (15). For the intensity decay measurements, we used magic angle conditions to eliminate the effects of Brownian rotation.

The apparent dissociation constants (K_D^X) were calculated with the assumption that the stoichiometry of binding is 1:1,

$$K_D^X = \frac{X_{\max} - X}{X - X_{\min}} [\text{M}^+],$$

[3]

where $[\text{M}^+]$ is the ion concentration and X is the value of the measured (or calculated) ion-dependent parameter. The apparent dissociation constant is equal to the ion concentration at the midpoint of Na^+ - or K^+ -dependent plots using the respective measurable parameters. It should be noted that only the ion-dependent intensity results in a true dissociation constant reflecting the equilibrium between free and ion-bound forms of Sodium Green. The dissociation constants calculated from the ion-dependent, time-resolved data (phase angle, modulation, or mean decay time) will usually result in values higher or lower than the true value because time-dependent parameters are not proportional to the concentration of either the free or the ion-bound form. However, the accessible range of ion concentrations that can

be measured with the chosen parameter is within a factor of 0.1 to 10 of the respective apparent dissociation constant.

RESULTS AND DISCUSSION

Absorption and Emission Spectra

Absorption spectra of Sodium Green with and without Na^+ are shown in Fig. 1. The probe concentration was $1.2 \mu\text{M}$ based on the extinction coefficient of $92,000 \text{ M}^{-1} \text{ cm}^{-1}$ for free form (Molecular Probes' data). The absorption spectrum of the free form (0 NaCl) displays only a modest change due to binding of Na^+ with a maximum at 507 nm. The relative emission spectra (excitation at 507 nm) are shown in Fig. 2. The maximum intensity is at 530 nm for the free and 532 nm for the Na^+ -bound form. The intensity of the Na^+ -bound form is about 6.7-fold higher than that for the free form. Sodium Green displays a K_d of 8.4 mM (Fig. 2, inset). These values are close to the characteristics provided by Molecular Probes: 7.3-fold increase in intensity and dissociation constant of 6 mM (10). The spectral properties of Sodium Green are similar to those of the Magnesium Green probe (15) and the Calcium Green probe (13), since all these probes contain the same FLuorescein derivative, 2',7'-dichloro-5-carboxyFLuorescein (Scheme 1).

Frequency-Domain Intensity Decays

We measured the frequency-domain intensity decays of Sodium Green with various Na^+ and K^+ concentrations from 0 to 500 mM. The Na^+ -dependent intensity decays are shown in Fig. 3. The fluorescence intensity decays of Sodium Green were found to be multiexponential at all sodium concentrations. However, analysis of samples with various sodium concentrations resulted in three decay times which were independent of sodium concentration. Therefore, we used global multiexponential intensity decay analysis to determine changes in the decay time amplitudes of Sodium Green with increased sodium concentration. During the global analyses, the three decay times were global and amplitudes for each Na^+ concentration were fitted. The results of this global analysis are summarized in Table 1. The three decay times were found to be 0.09, 0.40, and 2.53 ns with amplitudes (α_i) and fractional intensities (f_i) strongly dependent on the sodium concentration (Table 1). The components with $\tau_1 = 0.09$ and $\tau_2 = 0.40$ ns appear to be associated with the sodium free form because their amplitudes and fractional intensities decreased as the Na^+ concentration increased. The component with a decay time of $\tau_3 = 2.53$ ns can be associated with Na^+ -bound form. The presence of the long component (2.53 ns) in the absence of sodium is similar to that previously observed for Calcium Green in the absence of Ca^{2+} (3.7 ns) (13) and Magnesium Green in the absence of Mg^{2+} (3.6 ns) (15). Since Sodium Green consists of two fluorescein molecules, energy transfer (homotransfer) between these two dyes is possible. The intensity decay analyses at various Na^+ concentration are valuable in the characterization of Sodium Green, but such detailed information is not required for fluorescence lifetime imaging or lifetime-based sensing. Intensity decays of Sodium Green require multiexponential intensity decay analyses, which in turn require variable frequency lifetime imaging and time-consuming analysis (27, 28). Such detailed time-resolved data obtained for well-controlled samples are important for comparison with decay time parameters obtained for the probe inside the cell. The comparison of such data will be a source of information about the behavior of a probe

when it is loaded into a cell and its interactions with other intracellular ions and proteins. However, for imaging or cellular applications, the Na^+ concentration can be determined from phase or modulation data measured at a single modulation frequency regardless of the complexity of the intensity decay. The mean lifetime of Sodium Green increases from 1.13 ns (free form) to 2.39 ns (Na^+ -bound form), which produces an excellent increase of phase angle and a decrease of modulation in a wide range of modulation frequencies (Fig. 3). For instance, at a modulation frequency of 151.8 MHz, the phase angle increased 36° and modulation decreased 32% due to sodium binding. These are the values that classify Sodium Green as an excellent lifetime-sensitive Na^+ probe.

Discrimination against Potassium

For an ideal Na^+ probe for intracellular applications, one expects about 20-fold higher K_d for K^+ (150 mM) than for Na^+ (8). The reported selectivity of Sodium Green based on intensity measurements is about 41-fold higher for Na^+ than for K^+ (10). The selectivity of Sodium Green is almost 2-fold better than that for the sodium probe SBFI, while the ion binding site, diazacrown ether, is the same for both probes (8). We measured intensity decays of Sodium Green at various KCl concentrations to understand the effects of K^+ binding. The intensity decays in the presence of K^+ were found to be more complex than those in the presence of Na^+ . The values of three decay times and their amplitudes were dependent on the K^+ concentration. It is known that complex intensity decays can be successfully described by the three-exponential model. However, in our case we expected an additional species as the K^+ -bound form. Assuming that the three components are present for the free form (see Table 1) we used the global four-exponential model to estimate the decay time of Sodium Green associated with the K^+ -bound form. During the global analysis one decay time was held fixed (0.09 ns), three decay times were global, and all (four) amplitudes were fitted. The fit to the four-exponential model resulted in an acceptable value of $\chi_R^2 = 2.9$ and reasonable changes in the values of amplitudes (and fractional intensities) with increased potassium concentration. Results of the detailed analysis of the global fit to the four-exponential model are given in Table 2. Interestingly, only two amplitudes (a_2 and a_3) and fractional intensities (f_2 and f_3) of decay times, 0.39 and 0.69 ns, display significant K^+ dependence (Table 2). The amplitude of the 0.69-ns decay time increased from 0.059 to 0.589 with increasing potassium concentration, indicating the K^+ -bound form of Sodium Green. It should be noted that more complex intensity decays of Sodium Green may exist in the presence of potassium. Nonetheless, K^+ -dependent amplitudes and fractional intensities of four decay times provide an explanation of why the fluorescence intensity and mean lifetime of Sodium Green increased only about 1.3-fold at 500 mM of KCl compared to the free form. One possible explanation is that K^+ is too large to fit fully inside a diazacrown ring, and possibly the binding on one side does not cause significant changes in the fluorescence properties (quantum yield and mean lifetime) of Sodium Green as that observed with Na^+ binding. The intensity and lifetime changes are modest since the difference between τ_2 and τ_3 is small and the decreased value of f_2 is compensated by the increased value of f_3 (see Table 2).

Apparent Dissociation Constants

The useful Na^+ concentration range that can be measured using phase-modulation data is related to the so-called apparent dissociation constant. The apparent dissociation constants were calculated from various Na^+ -dependent parameters using Eq. [3]. The value of an apparent dissociation constant is a useful parameter in lifetime-based sensing. It describes the analyte-sensitive concentration range associated with the measurable (or calculated) parameter. Its value defines the midpoint in a manner similar to that for intensity measurements, but may differ significantly from the true K_d (13, 15, 20, 21). Typically, analyte concentrations can be determined within a 10-fold range above and below the value of the apparent dissociation constant.

To demonstrate Na^+ sensing with the phase-modulation method we chose data at a single modulation frequency, 151.8 MHz. Figure 4 displays phase angle (top) and modulation (bottom) data for Sodium Green at various solution compositions. The experimental data were fitted to Eq. [3] and the lines represent fitted curves with midpoints equal to the calculated apparent dissociation constants. The apparent dissociation constants (Fig. 4) were determined with an accuracy of 5–15%. This indicates that phase and modulation data can be described by the sigmoidal curve as typically observed with intensity measurements.

In case A, where the buffer contained various concentrations of Na^+ (no K^+), phase and modulation display great sensitivity with high dynamic range. The determined apparent dissociation constants are 2.2 and 0.95 mM from phase angle and modulation, respectively. The change in phase angle is about 36° and modulation is about 32%. Examination of Na^+ -dependent phase angle and modulation at modulation frequencies higher than 151.8 MHz revealed a minor shift of the apparent dissociation constants toward higher values (about 10–15%). However, the optimal modulation frequencies (maximum changes in phase angle and modulation) are in the range from 200 to 400 MHz (see Fig. 3).

Case B, where the buffer contained only KCl (no Na^+), illustrates the discrimination against K^+ . The determined apparent dissociation constants for K^+ are 24.5 and 14.3 mM from phase and modulation, respectively. These values are about 11- to 15-fold higher than those for Na^+ and the apparent discrimination against K^+ is significantly lower than that reported from intensity measurements, 41-fold (10). However, the changes in phase and modulation due to K^+ binding to Sodium Green are markedly lower (11° and 7%, respectively) than those observed with Na^+ .

To mimic biological conditions we used a mixture of $[\text{K}^+]$ and $[\text{Na}^+]$, keeping the combined ionic concentrations of these two species at 135 mM. Na^+ -dependent phase angle and modulation for a mixture of two ions are shown in Fig. 4, case C. The apparent dissociation constants for Na^+ in the presence of potassium from phase and modulation are 8.3 and 4.3 mM, about 4-fold higher than those in the absence of K^+ . From the point of view of intracellular sodium imaging the most important fact is that the sensitivity to Na^+ is preserved in the presence of K^+ with phase and modulation measurements. The changes in phase and modulation between 0 Na^+ (135 mM K^+) and 135 mM Na^+ (0 K^+) are about 25° and 25%, respectively. This high sensitivity of Sodium Green may be sufficient to distinguish the differences in sodium concentration between the nuclear region and the rest

of the cell (2, 11). Currently, the best available excitation wavelength-ratiometric probe, SBFI, changes the 340/380 nm excitation ratio by only a factor of 2.8 within the same Na^+ concentration range ($\text{Na}^+ + \text{K}^+ = 135 \text{ mM}$) (8, 11). Therefore, one may expect significantly better resolution from Sodium Green with phase-modulation fluorometry than from SBFI with excitation ratio. However, the best way would be to use both probes within the same cells with combined methods, for SBFI (excitation ratio) using the observation below 500 nm (no overlap with emission of Sodium Green) and for Sodium Green (phase-modulation) using excitation longer than about 430 nm (SBFI does not absorb).

Data presented in Fig. 4 show that the expected typical concentration of free Na^+ in resting cells (4–20 mM) and for stimulated or perturbed cells (0–100 mM) (6) should be easily detected from phase angle measurements. The modulation of Sodium Green is more sensitive to Na^+ than phase angle and can be useful for measurements of sodium concentrations below 20 mM. It should be noted that according to Molecular Probes Inc. the dissociation constant values may considerably depend on the presence of other ions, temperature, pH, ionic strength, viscosity, protein binding, and other factors. The intracellular indicator on the investigated system should be calibrated using the pore-forming antibiotic gramicidin. Such calibration has been performed using Sodium Green in CHO IS1 cells (17).

Photobleaching of the Fluorescence of Sodium Green

A significant difficulty in wavelength-ratiometric or lifetime imaging is changes in the excitation spectra, emission spectra, or intensity decay due to the intense illumination present in a fluorescence microscope. In principle, if the probe photobleaches and becomes non-fluorescent, then the measured sodium concentration using phase-modulation or wavelength-ratio methods should be independent of the extent of photobleaching. However, in practice, one often observes not perfect photobleaching, but rather phototransformation to a different species with different spectra, different intensity decays, and sensitivity to analyte. Such changes in decay times with illumination have been observed for the calcium probe quin-2 (29), fura-2 (20), and magnesium probes (15). An additional difficulty is that free and ion-bound forms may photobleach differently due to different fluorescent properties which may significantly affect the calibration curves.

To understand the effects of photobleaching of Sodium Green on the decay times, we measured the intensity decays of two samples in the absence and presence of 135 mM (sodium-saturated form) of NaCl. The excitation light (514 nm from mode-locked argon ion laser) was focused on a small volume sample (about 0.1 ml). First, the intensity decay of each sample was measured at low excitation intensity without observable photo-bleaching. Then, after the samples were exposed to various excitation intensities for 30 min, the frequency-domain intensity decays were measured in the time interval indicated in Fig. 5 from A to B. The Na^+ free form mean lifetime increased from 1.13 to 1.74 ns and that for the Na^+ -bound form slightly decreased from 2.36 to 2.17 ns. The steady-state intensity of the Na^+ -bound form decreased about 2.5-fold compared to the initial value, whereas that of free form increased about 1.2-fold (Fig. 5).

Combined intensity and lifetime data indicate that the photochemical reactions under intense illumination create fluorescent photoproducts or different relative contributions to total fluorescence between existing multicomponent fluorescence of Sodium Green. The comparison of photobleaching of Sodium Green in buffer with photobleaching of fluorescein in glycerol (dotted line in Fig. 5, top) indicates that Sodium Green can be regarded as one of the more photostable fluorescein-based probes. To facilitate comparison of these changes, the phase angles in Fig. 5 are plotted using the same scale as in Fig. 4.

CONCLUSIONS

We have characterized Sodium Green as a potential lifetime-based probe for intracellular free sodium measurements and imaging. Many difficulties encountered with intensity measurements can be circumvented by the use of lifetimes. The major advantage of lifetime-based imaging is that it does not require wavelength ratiometric probes for quantitative analyte measurements. Fluorescence lifetime imaging allows image contrast to be created based on the lifetime of the probe at each point of the image. Since the mean lifetime of Sodium Green is relatively short, less than 2.5 ns, it is expected that more appropriate techniques for sodium imaging would be frequency-domain methods previously described (30–33). The phase and modulation of Sodium Green are very sensitive to Na^+ , so one now has the ability to create $[\text{Na}^+]$ images of cells in ways similar to those reported for $[\text{Ca}^{2+}]$ images of cells (30).

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FIG. 1.
Absorption spectra of free (solid line) and Na⁺-bound (dashed line) forms of Sodium Green.

FIG. 2. Emission spectra of Sodium Green at various NaCl concentrations. Inset is the Na^+ -dependent intensity at the emission maximum (530 nm).

FIG. 3. Frequency-domain intensity decays (top, phase angle; bottom, modulation) of Sodium Green at various sodium concentrations.

FIG. 4. Na^+ - and K^+ -dependent phase angles (top) and modulations (bottom) for Sodium Green at the modulation frequency of 151.8 MHz for various solution compositions. The solution contained only NaCl (A), only KCl (B), and combined salts $[\text{NaCl}] + [\text{KCl}] = 135 \text{ mM}$ (C).

FIG. 5. Effects of intense illumination on intensities (top) and phase angles (bottom) of free and Na⁺-bound forms of Sodium Green. Photobleaching of fluorescein in glycerol is included (top, dotted line). A and B indicate the time interval for frequency-domain data acquisition for photobleached samples. Arrows with numbers indicate the excitation intensity (in mW) applied to samples.

SCHEME I.

Chemical structure of Sodium Green.

TABLE 1
Global Intensity Decay Analysis of Sodium Green in the Presence of Various Concentrations of Na⁺

NaCl (mM)	a_1	a_2	a_3	f_1	f_2	f_3	$\bar{\tau}$ (ns)
0	0.444	0.503	0.053	0.106	0.536	0.357	1.13
0.5	0.331	0.563	0.105	0.059	0.432	0.509	1.47
1	0.195	0.630	0.175	0.025	0.354	0.620	1.71
2	0.286	0.525	0.189	0.037	0.294	0.669	1.81
4	0.149	0.518	0.333	0.013	0.195	0.792	2.08
8	0.092	0.449	0.459	0.004	0.136	0.860	2.23
15	0.092	0.424	0.483	0.006	0.122	0.872	2.26
48	0.098	0.308	0.594	0.006	0.075	0.919	2.36
144	0.058	0.284	0.658	0.003	0.064	0.933	2.39

Note. Global decay times: $\tau_1 = 0.09 \pm 0.02$ ns, $\tau_2 = 0.40 \pm 0.03$ ns, $\tau_3 = 2.53 \pm 0.05$ ns, $\chi^2_R = 2.3$.

TABLE 2
Global Intensity Decay Analysis of Sodium Green in the Presence of Various Concentration of K⁺

KCl (mM)	a_1	a_2	a_3	a_4	f_1	f_2	f_3	f_4	$\bar{\tau}$ (ns)
0	0.355	0.547	0.059	0.038	0.081	0.535	0.103	0.280	1.09
7	0.327	0.560	0.070	0.040	0.072	0.526	0.118	0.283	1.11
17	0.324	0.512	0.106	0.057	0.063	0.426	0.158	0.353	1.29
27	0.447	0.287	0.200	0.065	0.085	0.233	0.291	0.391	1.42
57	0.413	0.305	0.216	0.066	0.075	0.238	0.301	0.385	1.41
171	0.345	0.199	0.381	0.074	0.053	0.131	0.449	0.366	1.42
507	0.258	0.071	0.589	0.081	0.034	0.040	0.589	0.338	1.40

Note. Global decay times: $\tau_1 = \{0.09\}$ ns (This decay time was held fixed during the analysis), $\tau_2 = 0.39 \pm 0.03$ ns, $\tau_3 = 0.69 \pm 0.08$ ns, $\tau_4 = 2.87 \pm 0.33$ ns; $\chi^2_R = 2.9$.