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Potassium Sensitive Optical Nanosensors Containing Voltage Sensitive Dyes

Xiaojiang Xie[§a], Agustín Gutiérrez[a], Valentin Trofimov[b], Istvan Szilagyi[a], Thierry Soldati[b], and Eric Bakker*[a]

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Abstract: Ionophore-based ion-selective optical nanosensors have been explored for a number of years. Voltage sensitive dyes (VSDs) have been introduced into this type of sensors only very recently, forming a new class of analytical tools. Here, K^+-sensitive nanospheres incorporating a lipophilic VSD were successfully fabricated and characterized. The nanosensors were readily delivered into the social amoeba *Dictyostelium discoideum* in a non-invasive manner, forming a promising new platform for intracellular ion quantification and imaging.

Keywords: Nanosphere · Optical sensor · Potential · Valinomycin · Voltage sensitive dye

1. Introduction

Established nanoscale ionophore-based optical ion-selective sensors contain several major components that include a chromoionophore (a hydrophobic pH indicator), a lipophilic ion exchanger, an ionophore (ion receptor), a matrix material and a surfactant to form and stabilize the structure.[1–3]

Similar to so-called bulk optodes, a classical cation nanosensor functions on the basis of ion exchange between hydrogen ions and the cationic analyte, and for an anion-responsive sensor, on the coextraction of hydrogen ions and the anionic analyte.[4–6] In this type of sensors, the chromoionophore indicates the level of the hydrogen ions in the organic sensing material and thereby indirectly quantifies the analyte in the aqueous sample.[7–10]

While abundant research work has been dedicated to the fundamental understanding and application of the chromoionophore-containing ion-selective sensors, their pH cross-response always remained a key drawback. Recently, it was demonstrated that one can operate nanoscale sensor suspensions in a so-called exhaustive detection mode that can overcome the pH cross-response in a specific pH window.[11,12] However, an exhaustive detection mode will always cause a dramatic perturbation of the sample and is, unfortunately, not a universal sensing scheme that could be applied in biological imaging.

The root cause of the pH cross-response is in the chromoionophore itself because it is a receptor for hydrogen ions, making H^+ inevitably the reference ion. Eliminating the reference ion H^+ should potentially help to overcome the pH dependence of this type of sensor. Recently, we have demonstrated that voltage-sensitive dyes (VSDs) can be applied to ionophore-based ion-selective nanospheres and result in sensor responses that are indeed pH independent.[13]

VSDs have been useful optical indicators for membrane potential measurements in cells and organelles that are too small for electrodes.[14] VSDs are generally categorized by their response to the change in local electric field, and one distinguishes fast-response and slow-response probes.[15] Most slow-response VSDs exhibit polarity-dependent optical characteristics. The absorption and/or emission spectra in polar and nonpolar solvents can be very different, making these dyes solvatochromic. As the electric field changes, the slow-response VSDs will redistribute between the aqueous and the organic phases to adapt to the membrane potential change. Because of the repartition kinetics, the response times are often relatively slow, typically hundreds of milliseconds. The fast-response VSDs behave mainly on the basis of electrochromism (the Stark effect) and reorientation, regardless of any redistribution, and thus are capable of achieving response times of several microseconds. However, the relative signal change in absorbance or fluorescence is much smaller, rendering them not sufficiently sensitive to ion imaging applications. This work focuses therefore on redistributing solvatochromic dyes to design ion imaging reagents.

2. Method and Materials

2.1 *Materials*

Pluronic® F-127 (F127), bis(2-ethylhexyl) sebacate (DOS), tetrahydrofuran (THF), methanol, potassium tetrakis-[3,5-bis(trifluoromethyl)phenyl]borate (KTFPB), 3,3'-dioctyldecyloxacarbocyanine perchlorate (VSD) and reference dye Lumogen Red were obtained from Sigma-Aldrich. Cell culture media (HL5-C medium including glucose supplemented with vitamins and micro-elements, and LoFlo medium) were obtained from ForMedium. All solutions were prepared by dissolving appropriate salts into deionized water (Mili-Q). All salts used were analytical grade or better.

2.2 *Nanosensor Preparation*

The K^+-selective nanospheres were prepared by dissolving 0.3 mg of KTFPB, 0.01 mg of VSD, 0.005 mg of Lumogen red, 8 mg of DOS, 4.5 mg of F127, and 1.2 mg of valinomycin in 3.0 mL of methanol to form a homogeneous solution. 0.2 mL of this solution was pipetted and injected into 5 mL of deionized water (or cell culture) on a vortex with a spinning speed of 1000 rpm. The resulting clear mixture was blown with compressed air on the surface for 30 min to remove methanol, giving a clear particle suspension.

*Correspondence: Prof. Dr. E. Bakker[a]
E-mail: Eric.Bakker@unige.ch
[a]Department of Inorganic and Analytical Chemistry
[b]Department of Biochemistry
University of Geneva
Quai Ernest-Ansermet 30, CH-1211 Geneva

2.3 *Instrumentation and Measurement*

The size of the nanospheres was measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Inc.) instrument. Fluorescence responses of the nanospheres were measured with a fluorescence spectrometer (Fluorolog3, Horiba Jobin Yvon) using disposable poly(methyl methacrylate) cuvettes with path length of 1 cm as sample container. The excitation wavelength was 480 nm. The desired analyte concentration in the nanosphere suspension was achieved by addition of calculated volumes of stock solutions or amounts of solid.

For transmission electron microscopic (TEM) imaging of the nanospheres, the suspension was dispersed onto a Formvar/Carbon film-coated TEM grid, counterstained with uranyl acetate, dried in the air and visualized using a FEI Tecnai™ G2 Sphera transmission electron microscope.

For confocal imaging, cells of the AX2(Ka) strain of *Dictyostelium discoideum* expressing the ABD-GFP protein (consisting of an actin binding domain fused to a green fluorescent protein) were grown in HL5-C.[16] Cells were detached, centrifuged twice and resuspended in LoFlo, then plated on glass-bottom cell culture dishes. To these, 2 mL of either LoFlo or the potassium nanospheres suspended in LoFlo were added. The particle-containing medium was replaced with LoFlo after approximately 20 minutes to remove uningested extracellular particles. Image acquisition was carried out with a laser confocal microscope (Zeiss LSM780).

3. Results and Discussion

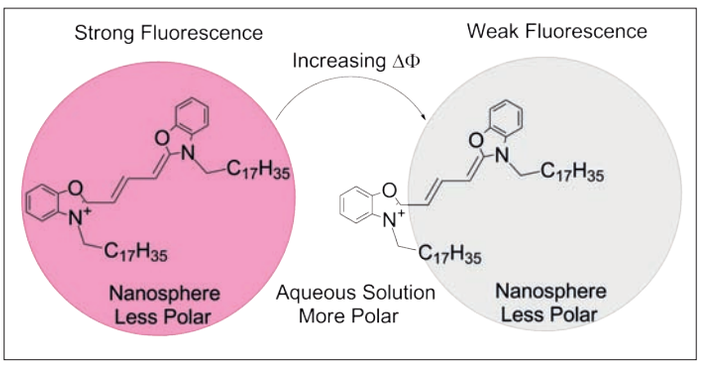
3.1 *Sensor Mechanism*

For an established K^+-selective ion optode containing a neutral chromoionophore, ion exchanger (TFPB), and K^+ ionophore, the response mechanism is based on the partition equilibrium between H^+ and K^+ at the aqueous-organic interface. Here, the VSDs doped ion sensors function on a similar but somewhat different basis. As shown in Scheme 1, the positively charged VSD can essentially exist in two different micro-environments. The aqueous phase is more polar than the nanospheric organic phase. In a simplified manner, one could postulate that the role of H^+ is now replaced by the positively charged VSDs. The VSD used here is more lipophilic compared with the one used in our previous report, 3,3'-dibutylthiacarbocyanine iodide. Nevertheless, the core structure is similar, giving lower emission intensity in the polar aqueous environment than in the organic nanoparticle core.

The localization of the VSDs is governed by the phase boundary potential difference ($\Delta\Phi$), which can be related to the analyte activity (K^+ in this case) in the sample according to Eqn. (1), where R is the gas constant, T is the absolute temperature, F is Faraday's constant, $a_{K^+}(org)$ and $a_{K^+}(aq)$ are the activity of uncomplexed K^+ in the nanosphere and in the sample, and $\Delta\Phi^{0,aq\rightarrow org}_{K^+}$ is the standard ion transfer potential for K^+.

$$\Delta\Phi = \Delta\Phi^{0,aq\rightarrow org}_{K^+} + \frac{RT}{F}\ln\frac{a_{K^+}(aq)}{a_{K^+}(org)} \quad (1)$$

Note that the same $\Delta\Phi$ should also apply to VSDs, therefore, as $\Delta\Phi$ changes, so does the distribution of the VSD. Fig. 1 shows the emission spectra of the K^+ selective nanosphere suspension with various KCl levels in the sample, covering the normal intracellular K^+ levels. Owing to the excellent selectivity of valinomycin to K^+, the nanospheres gave no response to other common cations such as Na^+, Mg^{2+}, Li^+ and Ca^{2+} in this concentration window. The excitation was chosen at 480 nm, which is close to the 488 nm laser used for fluorescence microscopy. An emission peak around 595 nm that originated from the reference dye Lumogen red was also observed. $\Delta\Phi$ increases as the K^+ concentration in the sample increases, forcing the VSDs to accumulate in the more polar aqueous region, and resulting in a decrease in the emission intensity of the VSDs. Compared with previously reported nanospheres with more hydrophilic voltage sensitive dye, the relative intensity change was smaller, *i.e.* the detection limit for this system was higher. This is likely caused by the higher lipophilicity of the VSD used in this work, which results in a significantly different standard ion transfer potential for the VSD.



Scheme 1. Representative illustration for the VSD distribution in the K^+ nanosphere according to the phase boundary potential difference ($\Delta\Phi$)

3.2 *Choice of Materials*

Since the fluorescence of the VSD depends on the polarity of the microenvironment, the nanosphere materials choice becomes very important. Here, the nanospheres were composed of a hydrophobic nonpolar compound DOS and an amphiphilic block copolymer F127. The nanospheres exhibited a long shelf-life of

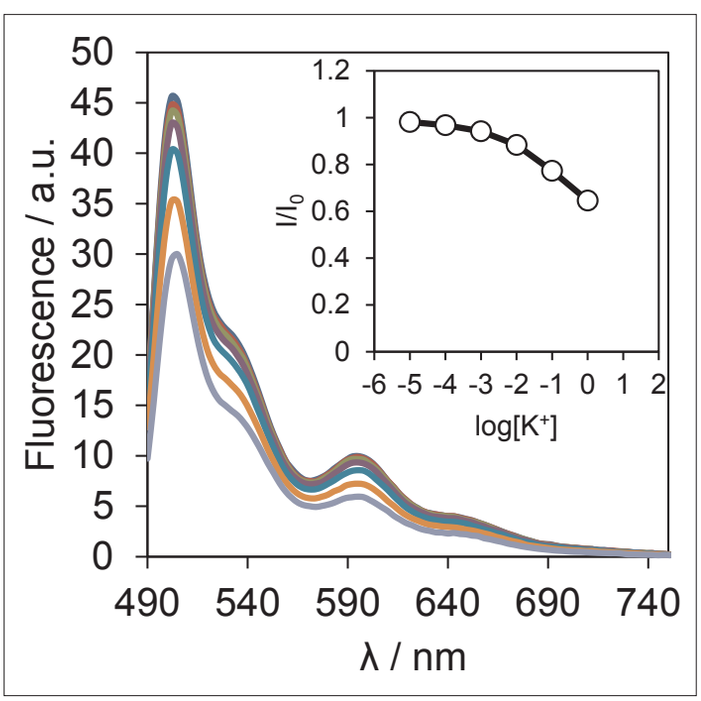


Fig. 1. Fluorescence spectra for the K^+ nanosphere with different KCl background. Excitation: 480 nm. Inset: Calibration using the normalized emission intensity at 510 nm. I_0 is the intensity without addition of KCl.

at least several months with a narrow size distribution in solution (polydispersity index, ca. 0.1) and small average diameter (<100 nm typically).

The same matrix material (DOS+F127) has been used for conventional nano-optodes and for ion releasing/up-taking nanospheres.[2,17] Fig. 2 shows a TEM image of the K^+-selective nanospheres. Note that aggregation during the sample preparation stage of the TEM experiment may influence the apparent size distribution.

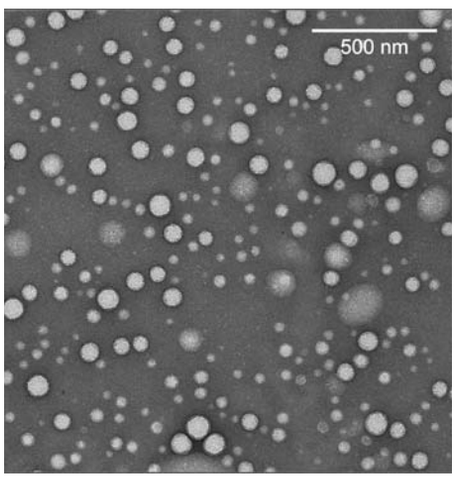


Fig. 2. TEM image for the K^+ sensitive nanospheres.

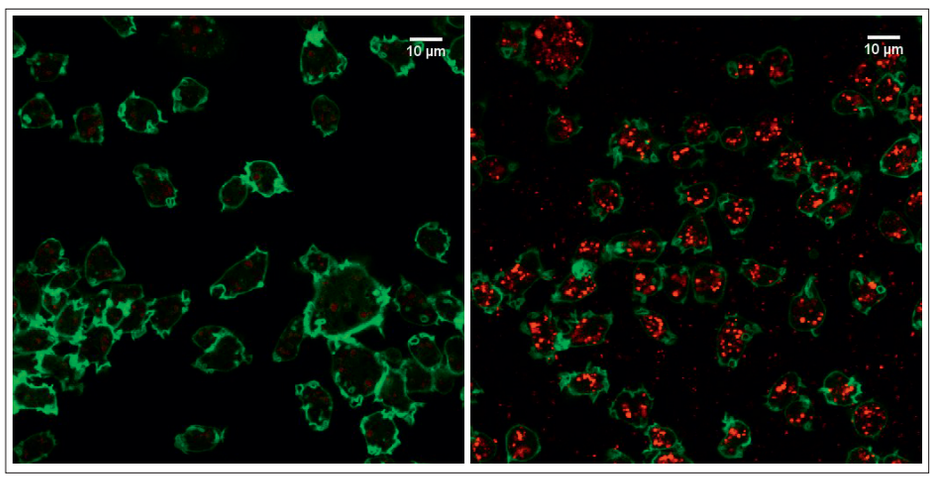


Fig. 3. Confocal fluorescence images of *D. discoideum* without addition (left) and with addition (right) of K^+ sensitive nanospheres into the cell culture medium. The green fluorescence comes from the green fluorescence proteins (GFP) localised at the actin cytoskeleton, while the red color is due to the emission of the reference dye Lumogen red within the nanospheres.

The use of DOS and F127 as matrix ensured a relatively large polarity difference between the core and the surrounding aqueous phase. The polarity of the nanosphere core could potentially be further decreased if a less polar surfactant than F127 is used.

3.3 *Nanosphere Uptake by Dictyostelium discoideum*

It has been demonstrated previously that nanoscale ion sensors can be applied to intracellular imaging with improved characteristics.[18,19] Nanospheres composed of F127 and DOS are newly introduced research tools that have not yet been applied in intracellular analysis.

As a first attempt, the VSDs doped nanosensors were applied to the social amoeba *Dictyostelium discoideum* in order to quantify the ion concentration changes during the membrane trafficking of phagocytosis and to understand its relevance to host–pathogen interactions. *D. discoideum* is a professional phagocyte and very similar to mammalian phagocytes of the innate immune system in morphology and behavior.[20] The nanospheres were added to the cell culture medium containing *D. discoideum* and incubated for *ca.* 20 min before monitoring by fluorescence confocal microscopy. As shown in Fig. 3, before addition of the nanospheres, only slight red autofluorescence was observed. After addition of the nanospheres to the cell culture medium, the nanospheres are readily taken up and concentrated in the endosomes by the cells, indicating the feasibility of applying such nanosensor to these phagocytes. The exact mechanism of the cellular uptake and a more quantitative analysis of the ion concentration, K^+ in this case, are currently being studied in our groups.

4. Conclusion

VSDs have been recently applied in ion-selective optical nanosensors, making the sensors pH-independent. Here, a lipophilic VSD was successfully used to prepare K^+-sensitive nanospheres, resulting in a sensor response range covering the intracellular K^+ level. The nanospheres can be readily delivered into *D. discoideum*, making them promising nanoscale ion imaging tools for intracellular measurements.

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