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A Cell-Penetrating Ratiometric Nanoprobe for Intracellular Chloride

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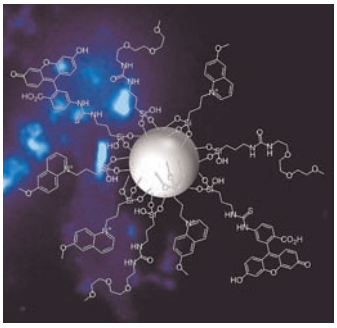
ABSTRACT



NanoChlor, a nanoparticle-based fluorescent probe for chloride that is both ratiometric and capable of spontaneously penetrating neuronal cells at submillimolar concentrations, was designed and studied. NanoChlor is built on silica nanoparticles grafted with 6-methoxyquinolinium as the chloride-sensitive component and fluorescein as the reference dye. A Stern–Volmer constant of 50 M^{-1} was measured in Ringer's buffer at pH 7.2, and the response to chemically induced chloride currents was recorded in real time in hippocampal cells.

Over the past few years, the design of fluorescent probes[1] has been extended from molecules to nanoparticles. By providing multivalent scaffolds directing the assembly of cooperating subunits, nanoparticles made it possible to venture beyond single-molecule sensing schemes and opened new opportunities for the determination of analytes in the complex environment of a living system.[2] Intracellular chloride, one of the most elusive targets, is a prime example. As the most abundant inorganic anion in living organisms, its transport across both the plasma membrane[3] and intracellular membranes[4] is involved in several key physiological processes, including cell volume regulation, organellar acidification, and neurotransmission. Dysfunctional chloride transport is linked to a number of diseases, such as cystic fibrosis,[5] *myotonia congenita*,[6] and nephrolithiasis.[7] The availability of fast and reliable methods to measure intracellular chloride levels is therefore of primary importance.[8]

Commercial fluorescent probes for the determination of intracellular chloride belong to the quinolinium or similar classes of fluorophores, which are quenched by halides through a collisional mechanism.[9] Several derivatives were developed over the years to improve water solubility and

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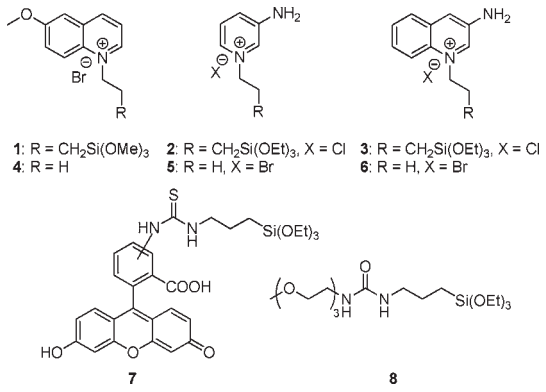
Scheme 1. Structures of Candidate Fluorophores and Reference Compounds



sensitivity to chloride.[10] However, they still suffer from poor cell permeability (which requires potentially disruptive loading techniques or high dye concentrations)[11] or a single-wavelength (on−off) response.[12] Ratiometric measurements, in which a double wavelength emission reading provides an internal reference, are immune from artifacts arising from environmental effects. So far, only genetically encoded ratiometric probes[13] have overcome both limitations but require transfection procedures and suffer from other limitations, such as cell-dependent efficacy and side toxicity.

In this context, nanoparticles have emerged as an attractive, more versatile, approach. The association of sensitive components and reference dyes on the same particle provides a straightforward way to convert intensity-based probes into their more accurate ratiometric counterparts.[14] However, only a few examples of nanoparticle-based intracellular probes for chloride are known,[15] one of which is ratiometric,[15b] but they all require special techniques for loading, such as the use of gene guns or transfection agents. We reasoned that the surface functionalization of silica nanoparticles with quinolinium- and pyridinium-based dyes should result in a positively charged surface, which is known to promote their cellular uptake.[16] Indeed, we here describe the first chloride probe that is both ratiometric and capable of spontaneously entering cells at a submillimolar concentration. The NanoChlor sensor is obtained by assembling a chloride-sensitive quinolinium fluorophore, a reference dye, and a solubilizing moiety on the surface of 20-nm silica nanoparticles.

Silica-graftable quinolinium[17a] and pyridiunium[17b] derivatives are easily synthesized in one step by alkylation of pyridine and quinoline starting materials with 3-(halopropyl)trialkoxysilanes (Figure 1a). This allowed us to test a small library of silica nanoparticles functionalized with halide sensitive fluorophores **1**−**3** (Scheme 1, where **4**−**6** are the corresponding models lacking the trialkoxysilyl moiety). Dyes **1**−**3** were grafted to 20-nm commercial silica nanoparticles (Ludox AS-30) by condensation with surface silanols (Figure 1a) in a 1:1:1 mixture of ethanol, water, and acetic acid at 80 °C. Nanoparticles **NP1**, **NP2**, and **NP3** were recovered as a water solution by ultrafiltration and diluted with an equal volume of DMSO to prevent precipitation. The nanoparticles appear as 20-nm-diameter spheres by transmission electron microscopy (TEM). Size measurements were confirmed by dynamic light scattering (see Supporting Information (SI)).

The absorption and emission maxima of the reference fluorophores increase in the order **5** < **4** < **6** (Table 1). All three compounds conveniently display a large Stokes shift (70 to 100 nm), which helps minimize the background excitation signal in fluorescence microscopy. While the spectral properties of **NP1** and **NP2** do not deviate significantly from those of reference dyes **4** and **5**, comparison of the UV−vis spectrum of **NP3** with that of reference dye **6** shows that the absorption band of **6** becomes broadened

Table 1. Photophysical Characterization of Model Nanoparticles and Reference Compounds[a]

entry	λ_{abs}/ nm	ε_{max}/ M^{-1} cm^{-1}	λ_{em}/ nm	K_{SV}/ M^{-1}	f_a/ %
NP1	318, 342	[b]	444	92 ± 2	87 ± 1
NP2	325	[b]	408	30 ± 1	86 ± 1
NP3	375	[b]	472	[c]	[c]
4	318, 342	5700, 4120	442	155 ± 2	100[d]
5	325	3430	408	33 ± 1	100[d]
6	395	3160	472	[c]	[c]

[a] A solution of nanoparticles (18 μM of dye) in Milli-Q water was titrated with NaCl (0 to 200 mM) at 25 °C. The fluorescence intensity at λ_{em} was corrected for dilution and fitted to a modified Stern−Volmer equation (see SI). The results are given as the mean ± s.d. of two titrations. [b] The extinction coefficients on nanoparticles were not measured. [c] **6** and **NP3** are not quenched by chloride. [d] The data were fitted to the classical Stern Volmer equation, which does not include this parameter.

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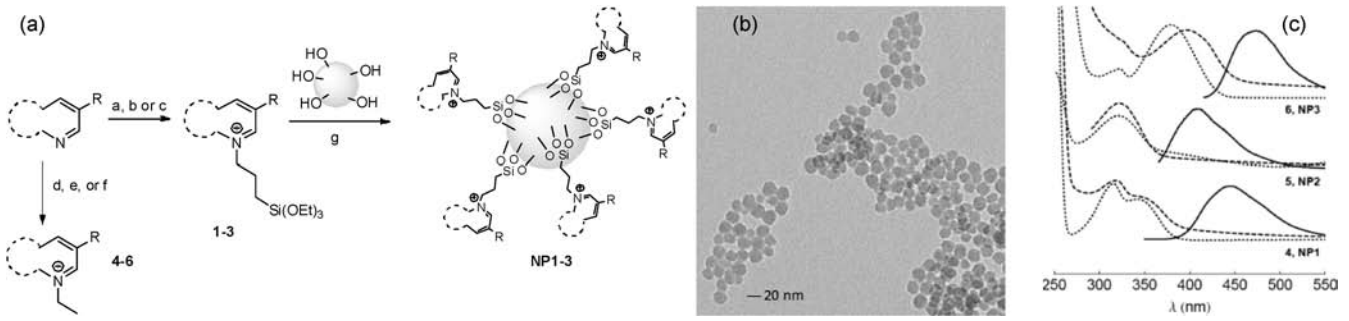

Figure 1. (a) Synthesis of model nanoparticles and reference compounds. (b) TEM image of **NP1**. (c) Absorption (dashed lines) and emission (solid lines) spectra of model nanoparticles. The absorption spectra of the reference compounds (dotted lines) are shown for comparison. (Step a) (3-Bromopropyl)trimethoxysilane, neat, 95 °C, 24 h, 86%. (Step b) (3-Chloropropyl)triethoxysilane, EtOH, 90 °C, 3 h, 79%. (Step c) (3-Chloropropyl)triethoxysilane, EtOH, 90 °C, 6 h, 68%. (Step d) Ethyl bromide, neat, 90 °C, 3 h, 83%. (Step e) Ethyl bromide, CH_3CN, 80 °C, 2 h, 89%. (Step f) Ethyl bromide, EtOAc, 70 °C, 16 h, 78%. (Step g) Ludox AS30, EtOH/H_2O/AcOH, 80 °C, 16 h.

and red-shifted (from 375 to 395 nm) upon immobilization on silica.

Nanoparticles **NP1**−**3** were titrated in duplicate with chloride and the potentially interfering anions bromide and iodide. Their quenching profiles were fitted to a modified Stern−Volmer equation (see SI) that accounts for an inaccessible population of fluorophores. Both **NP1** and **NP2** respond to chloride, bromide, and iodide. In both cases, less than 15% of the grafted dye molecules is inaccessible to the analyte. **NP3**, on the other hand, is only sensitive to bromide and iodide. As reported for similar dyes, bromide and iodide quench **NP1**−**3** more effectively than chloride, but they are not expected to interfere at the trace levels found in mammalian cells.[18] Together, **NP1**−**3** form an interesting library of halide sensitive dyes featuring large Stokes shifts, nonoverlapped emission bands, and a wide range of sensitivities, which could be of use in the development of sensor arrays. Comparison with the reference compounds reveals that, while the performance trend remains the same, the Stern−Volmer constants are systematically lower for nanoparticles. This behavior can be attributed to a number of causes: the lower diffusion coefficient of nanoparticles, the limited collision angle due to immobilization, and the electric double layer on the charged surface. However, chloride Stern−Volmer constants for **NP1**−**2** are still in the suitable range for intracellular measurements.

In the end, compound **1** emerged from this screening as the most sensitive chloride fluorophore for use in a nanoparticle-based sensor. In light of this result, we prepared the fully functional ratiometric system NanoChlor (Figure 2) by cografting dye **1**, the chloride-insensitive fluorescein derivative **7**,[19] and the short poly(ethylene glycol) derivative **8** on the surface of silica nanoparticles. Compound **8** plays the double role of improving water solubility and preventing nonspecific interactions with biomolecules.

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(19) The emission of fluorescein in NanoChlor was found to be pH independent in a wide pH range (Supporting Information).

The absorption spectrum of NanoChlor in Ringer's buffer at pH 7.2 clearly shows the absorption bands of 6-methoxyquinolinium **1** at 318 and 342 nm and of fluorescein **7** at 502 nm (Figure 3a). The emission spectrum recorded upon excitation at 318 nm shows two bands of comparable intensity, one at 442 nm from **1** and one at 510 nm from **7**. One distinct advantage of building ratiometric sensors on nanoparticles is the possibility of tuning the loading ratio of two fluorophores in order to obtain emission spectra of comparable intensity. In this case, we estimated a loading ratio of 10:1 (**1** vs **7**) by fitting

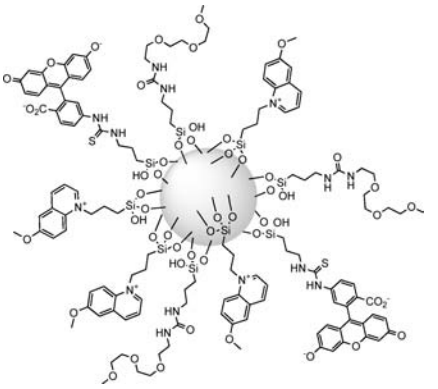

Figure 2. Structure of the NanoChlor assembly.

the absorption spectrum of NanoChlor to the sum of the absorption spectra of the individual fluorophores and a simulated Rayleigh scattering baseline.[20] Comparison with the ratio of emission intensities gives a quantum yield ratio (Φ_1/Φ_7) of 0.16.

The performance of NanoChlor as a fluorescent probe was evaluated by titrating the nanoparticles with chloride in a chloride-free Ringer buffer at pH 7.2. A typical titration is shown in Figure 3a. The Stern−Volmer constant for **1** grafted on NanoChlor is $49 \pm 2\ M^{-1}$, comparable to

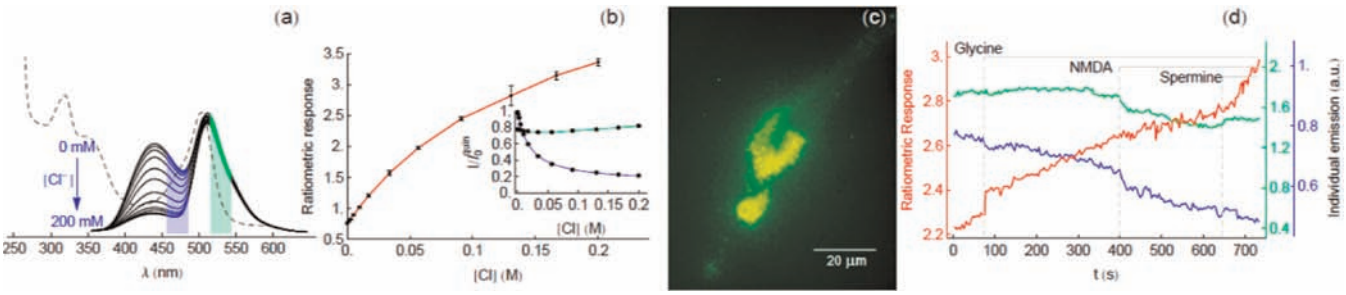

Figure 3. (a) Absorption (dashed line) and emission (solid lines) spectra of the nanoprobe in the presence of an increasing concentration of [Cl^-]. (b) Calibration curve calculated as the ratio of the integrated emission in the intervals 455−485 nm and 520−550 nm, corresponding to the emission channels used in the epifluorescence experiments. Inset: Emission intensity at 442 (blue) and 535 nm (green) as a function of [Cl^-]. (c) Fluorescence microscopy of hippocampal cells incubated with NanoChlor. (d) Time course of the intensity of the quinolinium (blue), fluorescein (green), and ratiometic (red) channels in a typical cell.

that of **NP1** ($52 \pm 1\ M^{-1}$) in the same conditions. The lower sensitivity with respect to the experiments performed in water (Table 1) is due to the effect of the higher ionic strength. The emission of **7** is unaffected (within 10%) by the presence of chloride. This behavior identifies a clear ratiometric response. The ratiometric calibration curve (Figure 3b) is linear up to 40 mM [Cl^-], after which it bends downward due to the unquenched population of buried **1** fluorophores.

Preliminary *in vitro* experiments were carried out on CHO cells in order to investigate the uptake ability of NanoChlor. Nanoparticles were incubated with cells for increasing times at 37 °C, and their uptake was measured by epifluorescence microscopy experiments (see SI). Remarkably, NanoChlor can be loaded effectively at 100 μM (with respect to units of **1**), a concentration which is 2 orders of magnitude lower than those typically required by commercial chloride indicators.[21] The uptake reaches a maximum after 8−16 h, and leaking only starts after 20 h. The nanoparticles are localized in the cytoplasm without entering the nucleus. No cellular toxicity was observed.

The ability of the nanoprobe to monitor variations of chloride levels inside live cells was then tested on primary cultures of hippocampal cells harvested from embryonic rats (Figure 3c and d). These cells are widely used to test several physiological properties of neurons and undergo strong variations of their intracellular chloride levels upon receptor activation with external stimuli (see SI). The cells were incubated with NanoChlor at [**1**] = 100 μM at 37 °C for 8 h. After that time, different chemical stimuli (glycine, *N*-methyl-D-aspartic acid (NMDA), and spermine; see SI) that are known to induce chloride influx in neuronal cells were applied while recording the time course of the fluorescence response with an epifluorescence microscope. Inspection of Figure 3d shows that all stimuli appear to affect the emission of the chloride sensitive quinolinium component of NanoChlor. However, ratiometric analysis (Figure 3d, red) reveals that only glycine and spermine do induce true alterations (respectively fast and slow) of intracellular chloride levels, while the effects observed on individual channels upon NMDA addition arise from artifacts that cancel out in the ratiometric signal. Control experiments confirm that no variation of the ratiometric response is observed, over the same experimental times, when chemical stimuli are not applied (see SI).

In conclusion, we developed a nanosensor for intracellular chloride by cografting 6-methoxyquinolinium and fluorescein on 20-nm silica nanoparticles. Unlike commercially available probes, NanoChlor displays ratiometric response and requires submillimolar concentrations. With respect to the previously reported nanoparticle-based ratiometric sensors,[15] it spontaneously penetrates living cells. Finally in contrast with genetically encoded probes, it appears to be nontoxic and potentially more reproducible. More experiments will be needed to confirm its scope and explore the cellular uptake mechanisms, intracellular localization, and the effects of intracellular pH on the ratiometric response.

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Supporting Information Available. Experimental details, synthesis and characterization of dyes and nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.