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Ultra-Small, Highly Stable, and Sensitive Dual Nanosensors for Imaging Intracellular Oxygen and pH in Cytosol

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Supporting Information

ABSTRACT: We report on the first dual nanosensors for imaging of pH values and oxygen partial pressure in cells. The sensors have a unique nanostructure in that a soft core structure is rigidized with a silane reagent, while poly(ethylene glycol) chains form an outer shell. Lipophilic oxygen-sensitive probes and reference dyes are encapsulated inside the hydrophobic core, while a pH-sensitive probe is covalently attached to the poly(ethylene glycol) end-group on the shell. The core/shell structure renders the nanosensors well dispersed and highly stable in various kinds of aqueous media. Their average size is 12 nm, and they respond to both pH and oxygen in the physiological range. They do not pass cell membranes, but can be internalized into the cellular cytosol by electroporation, upon which they enable sensing and imaging of pH values and oxygen with high spatial resolution. The nanosensor strategy shown here is expected to be applicable to the development of various other kinds of multiple nanosensors for in vivo studies.

Optical chemical sensors, compared to respective electrodes, possess the unique feature of enabling multiple sensing.[1] Examples include dual sensors for oxygen and pH,[2−6] oxygen and temperature,[7−10] carbon dioxide and oxygen,[11,12] pH and temperature,[13−17] and others.[18,19] A planar triple sensor for pH, oxygen and temperature,[20] and a quadruple sensor layer for simultaneously measuring oxygen, pH, carbon dioxide and temperature also have been reported.[21] However, these sensors are mostly constructed in the form of planar thin films, but nanosized sensors are needed for intracellular studies. Yin et al.[17] recently have reported on an intracellular pH and temperature dual nanosensor based on a fluorophore-labeled thermo-responsive polymer. However, both the pH and temperature responsive signals were reported by the same luminescent moiety which induces serious cross-talk so that it is difficult to attribute the cause for the signal changes observed.

The design of multiple nanosensors for intracellular applications is challenging in view of the material requirements when aiming for selective sensing of individual parameters. Gas sensors, for example, need hydrophobic materials with good gas permeability, while sensors for pH, ions or hydrophilic analytes (such as glucose) require hydrophilic materials. It is difficult to integrate all these features in a dual nanosensor for pH and oxygen which, however, is very desirable in view of the significance of these two parameters in cell metabolism. In addition, the selection of proper indicators for multiple sensing is critical. They are expected to possess distinguishable spectra or luminescence lifetimes and, ideally, to be excitable at the same wavelength and the same light source. Most importantly, the two probes must not undergo fluorescence resonance energy transfer even if located in close proximity.

We report here on an optical nanosensor for simultaneous measuring (and imaging) of pH and oxygen values in cells. The measurement of pH and oxygen can provide abundant information for understanding of cellular growth, metabolism, signaling and the fundamental processes of physiology, but also in cell-based high-throughput screening.[22,23] Deviations of pH and oxygen also are associated with the growth of cancer cells.[24−27] A dual nanosensor will enable the measurement of pH and oxygen at exactly the same site. This is highly advantageous over methods based on the use of two kinds of individual sensors.[23,28−30]

Figure 1 shows a schematic of the method for preparation which relies on a one-pot approach.[31,32] Its core is made from a commercially available and highly biocompatible polymer referred to as Pluronic F-127. This is a nonionic, surfactant triblock copolymer composed of a central hydrophobic chain of poly(propylene oxide) flanked by two hydrophilic chains of

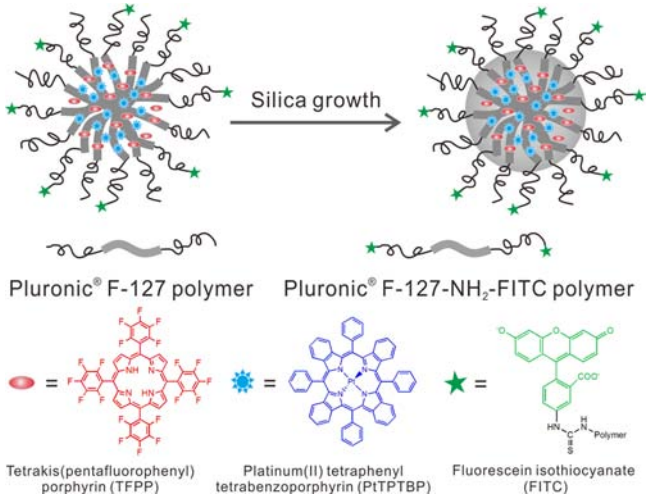

Figure 1. Schematic of the preparation of the dual nanosensor for oxygen and pH, and chemical structures of the probes used.

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poly(ethylene glycol) (PEG). Its molecular weight is ~12 500 Da. Such polymers also are referred to as poloxamers.[33] This PEG-terminated copolymer and its fluorescein conjugate (referred to as F-127-NH_2-FITC) were mixed with the hydrophobic luminescent probe for oxygen and the (inert) references dye in 0.85 N hydrochloride solution, upon which micelles are formed because of hydrophobic interaction.[34] To prevent self-quenching of the closely packed fluorophores on the surface of the nanoparticles, we have used unlabeled polymer to spatially separate them. Both the oxygen probe platinum(II) *meso*-tetraphenyltetrabenzoporphyrin (PtTPTBP) and the (inert) reference dye 5,10,15,20-tetrakis-(pentafluorophenyl) porphyrin (TFPP) are evenly distributed in the hydrophobic center of the micelle. The hydrophilic PEG chains containing the pH-sensitive probe fluorescein, on the other side, are directed outward as shown in Figure 1. Following the growth of a silica layer under acidic conditions, ultra-small nanosensors are obtained. The suspension was dialyzed for 7 days to remove unreacted chemicals, and then filtered through a 0.1-μm filter to remove large aggregates.

Transmission electron microscopy shows the dual nanosensors have uniform size with an average diameter of 12 nm, and are monodispersed without any aggregation (Figure S1). They are stable in aqueous solutions for at least 6 months without forming aggregates if kept at 4 °C in the dark, and also in cell culture media. Cytotoxicity tests using the AlamarBlue assay (Figure S2) showed that the nanosensors are not toxic to normal rat kidney (NRK) cells at various concentrations. This is attributed to the presence of PEG groups on the surface, which is known to reduce cytotoxicity, improve stability in biological systems, and to render them membrane-impermeable (Figure S3).[35] The dyes suffer from some photobleaching under strong laser excitation (408-nm laser was used to excite TFPP and PtTPTBP, and a 488-nm laser for fluorescein) but this plays no major role because ratiometric readout is applied (Figure S4).

Figure 2 shows the response of the nanosensors to pH and oxygen. The green luminescence emitted by the fluorescein fluorophore is very sensitive to pH and has a fast response to changes of pH. This is attributed to the specific structure of the nanosensors, where the pH sensitive probe is located on the terminating PEG group of the polymer, and thus is exposed outward and easily accessible for protons. The calibration plot and small error bars given in Figure 2B reveal a large signal change that occurs between pH 5 and 8. Response is governed by a pK_a value of 6.4. This makes the sensors suitable for measurement of physiological pH values. The red luminescence of TFPP is not sensitive to either pH or oxygen. Rather, it acts as a reference signal for ratiometric readout. A distinct color change from red to green occurs if the pH is increased from 3.0 to 9.0 (Figure 2B, inset picture). This also enables pH values to be read out not only via ratiometric spectroscopy but also by photographic (RGB) techniques.[36]

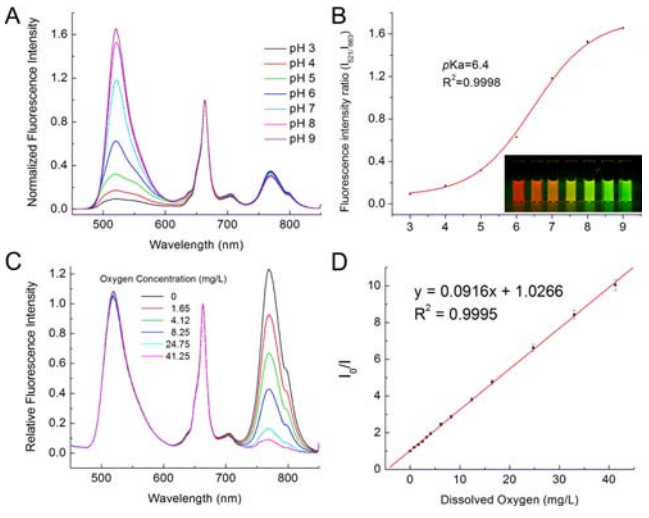

Figure 2. (A) pH-dependent spectra; (B) respective calibration plot of the dual nanosensors; (C) oxygen-dependent spectra; (D) Stern–Volmer plot of the dual nanosensors at different concentration of dissolved oxygen.

The near-infrared (NIR) emission (with a maximum at 769 nm) of the probe PtTPTBP is strongly quenched by oxygen. The ratio of the luminescence intensities in oxygen-free and in oxygen-saturated solution is as high as 10.6. This makes PtTPTBP a most useful probe for sensing and imaging of oxygen concentration in cells. There is a linear relationship between the dissolved oxygen concentration and the intensity ratio I_0/I (where I_0 is the luminescence intensity of PtTPTBP in oxygen-free solution, and I the one in solutions of various pO_2). Linear response is also due to the fact that all probes are located in an identical microenvironment. All signal changes are fully reversible as shown in Figure S5.

Single nanosensors for oxygen and pH have been described and were used for intracellular studies after having been internalized via endocytosis.[23] The dual nanosensors reported here do, however, not pass the cell membrane which was to be expected in view of the PEG shell. Thus, they are likely to be useful for *extracellular* research, for example to study interstitial fluids or serum in presence of erythrocytes. Other conceivable applications include sensing in microfluidic devices and femtoliter microarrays.[37]

To make the nanosensors useful for intracellular (cytosolic) studies, they have to be forced to pass the cell membrane. This is highly desirable in view of the oxygen gradient across cellular membranes,[38] and because the pH values in lysosomes are different from those in the cytoplasm.[39] The knowledge of cytosolic pH is important in terms of cell signaling and other cellular events.[40] Two techniques are widely used for delivering cell-membrane-impermeable nanoparticles into cells. Among these, microinjection is more complicated, time-consuming, and limited to injecting only a single cell at one shot. Electroporation (where pores are generated by applying short electrical pulses) is more simple and convenient, and enables nanoparticles to be delivered smoothly into the cytosol of many cells at the same time.[41] However, this technique requires nanoparticles to possess (a) adequate size because of the rather small pores generated, and (b) good dispersity and stability in cell culture media. Most formerly described nanosensors[23,42] either have too large size, or form aggregates. Thus, they are not amenable to electroporation.

The dual nanosensors described here were successfully delivered via electroporation, and the results of respective microscopic studies are shown in Figure 3. The green luminescence of the fluorescein, the red luminescence of TFPP, and the NIR emission of PtTPTBP were recorded and are shown in Figure 3A–C. Unfortunately, our confocal laser scanning microscope is only equipped with a 650-nm longpass filter, which made it impossible to distinguish the red luminescence of the reference dye (TFPP) from the NIR

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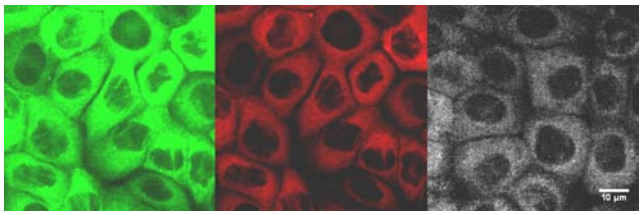

Figure 3. Confocal laser scanning microscopy images of the nanosensors internalized into normal rat kidney cells via electroporation. (A) The green luminescence of immobilized FITC on the surface of the dual nanosensors as seen with a 520-nm bandpass filter; (B) red luminescence of both TFPP and PtTPTBP as seen with a 650-nm longpass filter; (C) NIR luminescence of nanoparticles dyed with PtTPTBP only after delivery via electroporation.

emission of the oxygen probe (PtTPTBP). We therefore have prepared nanoparticles that contained the oxygen indicator only. Figure 3C shows that these nanoparticles are readily delivered into the cytosol by electroporation, and that the dual sensors are uniformly distributed in the cytosol without entering into the nucleus. One can also see that the dyes are associated with the nanoparticles and that no leaching can be detected. It shall be mentioned that the luminescence of both the reference dye (TFPP) and the oxygen probe (PtTPTBP) are not easily detected by photomultipliers with their poor sensitivity in the near-IR. To enhance brightness, high concentration of nanoparticles heavily loaded with dyes were used, which caused the Earle's Balanced Salts solution to become more viscous. This compromises the diffusion of the nanosensors into cells during electroporation.

In summary, we report on the first dual nanosensors for sensing pH and oxygen on a cellular level. They have a unique nanostructure in that a soft core structure is rigidized with a silane reagent, while poly(ethylene glycol) chains form an outer shell. The fluorescent indicator probes are firmly retained in the core or covalently immobilized in the outer PEG shell. The dual nanosensors have an ultra-small size and display excellent sensitivity. They are stable in various aqueous solutions without forming aggregates. The nanosensors are not membrane-permeable in either direction (outside → in; inside → out) but can be delivered into the cytosol via electroporation in a controlled manner and in a highly reproducible fashion. This new kind of nanomaterial enables, for the first time, confocal imaging of these two important parameters with very high resolution, even possible with nanometer resolution using nanoscopy.[43−45] The method, in addition, has a wide scope with respect to other dual (if not triple) optical nanosensors, not the least because it is based on the use of easily accessible materials. This enables such nanosensors to be prepared also by those not skilled in the synthesis of nanomaterials. The unique structure of the nanoparticles provides three different sites for further modification: (1) the hydrophilic PEG outer coating may be modified with hydrophilic probes for sensing ions and hydrophilic substances such as glucose; (2) probes can also be attached to the silica shell via sol−gel techniques; (3) the lipophilic core provides a host to encapsulate hydrophobic probes for sensing gases, such as oxygen as shown in this work, or carbon dioxide.

■ ASSOCIATED CONTENT

Supporting Information

The preparation of the nanosensors, transmission electron microscopic image, photostability, endocytosis test, and cytotoxicity test. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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