pH Nanosensor

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Advisory Board

Fluorescent pH-Sensitive Nanoparticles in an Agarose Matrix for Imaging of Bacterial Growth and Metabolism**

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Bacteria distribute all over the biota and are present almost everywhere in our daily life. Some (such as *Lactobacillus* and *Steptomyces*) are beneficial to mankind. Others do more harm than good, especially in terms of food storage and health. The growth of bacteria causes enormous food spoil and—even worse—threatens food safety and the health of consumers.[1] More than 20% of food all over the world is being wasted because of spoilage.[2] This waste causes economic losses, is not acceptable in times of widespread starvation, and also induces health and environmental problems.[3] Thus, real-time monitoring of bacterial metabolism and growth is essential for food storage and also for monitoring food freshness.[4] Optical sensors possess the unique feature of enabling imaging of parameters such as hydrogen peroxide,[5] carbon dioxide,[6] cations,[7] temperature,[8] oxygen[9] and pH value.[10] To enable optimal spatial resolution in case of bacteria with their typical diameters between 2 and 10 μm, it is, however, mandatory to design nanosized sensors rather than using conventional imaging sensor layers or microsized sensor particles.[11]

Herein we present a rather simple, novel, and generally applicable method for making nanosensors of a size of typically 12 nm and show that they enable two-dimensional monitoring of physiological pH values. The pH nanosensors are composed of a core containing both hydrophilic and hydrophobic domains (see below) and a mainly hydrophilic outer coating. The sensor nanoparticles contain two fluorescent dyes. The first one is a lipophilic commercial fluorescein (H-110)[12] and acts as a green-emitting pH probe. The second is a pH-insensitive and highly photostable fluorinated porphyrin that acts as a red-emitting reference dye. Both dyes can be simultaneously photo-excited with a 405 nm light emitting diode (LED). The nanosensors were then immobilized in a conventional layer of agarose as a highly biocompatible sensing layer for fluorescence imaging. The agarose contains nutrients as used for the growth of *Escherichia coli* (*E. coli*). Figure 1 shows a schematic representation of the pH-sensitive nanoparticles in an agarose film in a Petri dish.

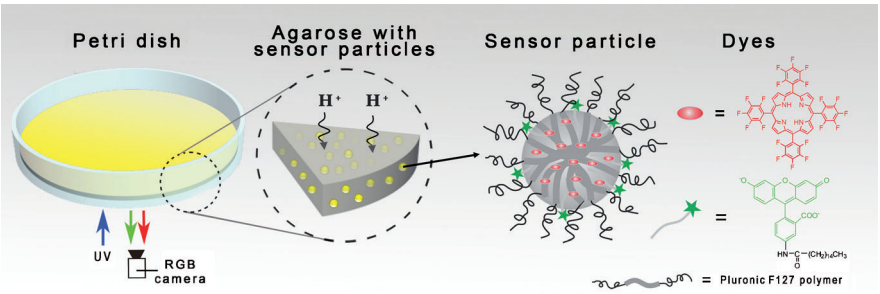

Figure 1. Scheme for preparing a pH-sensitive agarose Petri dish.

The pH nanosensors were prepared, in a one-pot reaction, from the biocompatible and commercially available copolymer Pluronic F-127 as the template. Similar materials have been used for drug delivery[13] and electrochemiluminescence,[14] but not for sensing purpose to date. This copolymer contains relatively hydrophobic poly(ethylene oxide) domains in the center of its chains but hydrophilic poly(ethylene glycol) (PEG) fragments at its ends. At a certain concentration, the polymer forms stable micelles in aqueous solutions. The core of the resulting micelles is relatively hydrophobic and can retain hydrophobic dyes, in this case the lipophilic pH indicator (green stars with a hydrophobic tail in Figure 1), but also the red-emitting reference dye 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (TFPP; shown as red ellipsoids). The hydrophilic outer PEG domains of the micelles make them stable in aqueous solution.

To obtain sensor nanoparticles, the Pluronic copolymer and the hydrophobic dyes are dispersed in 0.85 M hydrochloric acid. The addition of tetraethoxysilane to the system then induces the formation of silica nanoparticles wherein the dyes are firmly retained. We presume, on the basis of the excellent response to changes in pH value, that the hydrophilic pH-sensitive headgroup of the fluorescein probe and the PEG chains are exposed outside the nanoparticle, as shown in Figure 1.

Transmission electron microscopy (TEM) reveals a uniform size of the nanosensors, with an average diameter of

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12 nm, and good monodispersity without any tendency towards aggregation (Supporting Information, Figure S1). They are stable in aqueous solutions (for at least 6 months) if kept at 4 °C in the dark, in buffer solutions, and even in cell culture media. Cytotoxicity tests using normal rat kidney (NRK) cells showed them not to be toxic (Supporting Information, Figure S2). This property can again be attributed to the beneficial effect of the PEG groups, located on the surface of the nanosensors, which are known to reduce cytotoxicity, improve stability in aqueous solutions, and prevent penetration of cell membranes (Supporting Information, Figure S3).[15] These features also warrant that the nanosensors do not interfere in the growth or metabolism of bacteria. In contrast to most other pH-sensitive nanosensors,[16] the nanosensors reported herein cannot be taken up by cells through endocytosis, and thus will neither cause adverse effects on metabolism nor induce apoptosis.

Bacteria are routinely cultivated in Petri dishes on agarose gel containing nutrients. The growth of bacteria produces protons and this induces local acidification. We have integrated our pH nanosensors into a standard agarose/nutrient matrix (see Figure 1) to see whether pH values can be monitored over time in a Petri dish, which was autoclaved before use. Because of their ultra-small size, excellent dispersity, and stability in aqueous solutions, they can uniformly distribute in the agarose gel without aggregation. This is particularly beneficial in case of pH imaging with high spatial resolution, for example when making use of super-resolution methods, such as STED,[17] PALM,[18] or STORM.[19]

Figure 2 shows the pH response of the agarose sensor film in a Petri dish. The green luminescence emitted by the pH probe is very sensitive to pH value whilst the red luminescence of TFPP is sensitive to neither pH nor oxygen at various concentrations (Figures S4 and S5 in the Supporting Information). TFPP was chosen because fluorinated porphyrins are resistant to oxidation by reactive species.[20] On encapsulation of the TFPP in the hydrophobic core of the particles, they are well protected from interferences by potential quenchers.[21] Thus, its red emission can act as a (constant) reference signal for ratiometric readout. In fact, the color tint of the red and green emission undergoes a distinct change from red to green when pH values are increased stepwise from 5.5 to 9.0. This situation will enable (semiquantitative) colorimetric read-out.[22]

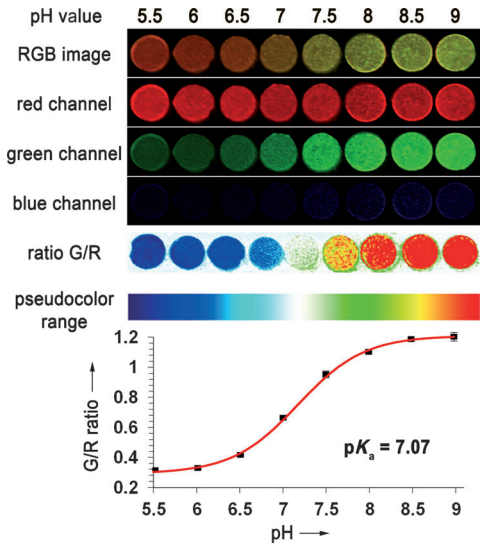

Figure 2. Optical response of the pH-sensitive agarose Petri dish, see text for details.

The emission spectra of the two dyes in the nanosensors spectrally match the red-green-blue (RGB) channels of commercially available digital cameras (Supporting Information, Figure S6 and S7). This property enables read-out of pH values, not only by spectral ratiometric, but also photographic techniques[23] using digital cameras or even the integrated cameras of mobile phones, as previously shown.[24] Respective experiments, shown in Figure 2, demonstrate that the intensity of the red channel—as expected—does not vary with pH value. The green luminescence, in contrast, strongly increases with rising pH value as indicated by the increase in the brightness of the green channel of the RGB memory. The ratio of the intensities between the green and red reference channel (G/R) is related to pH values as shown in the sigmoidal plot in Figure 2, bottom. This calibration plot and its small error bars (relative standard deviation, $n = 3$) demonstrate that the pH-sensitive agarose has a very good pH response and excellent pH resolution. The pK_a of this system is 7.07, which is quite suitable for precise pH measurement in the physiological pH range.

Heat sterilization (120 °C, 30 min) of the Petri dishes containing integrated sensor layers prior to their use in bacterial cultivation causes only a slight decrease in the luminescence intensities of the two dyes. This is an important feature in terms of practicability. We are aware that ratiometric read-out of two dyes is compromised if differential photobleaching does occur. Figure S8 in the Supporting Information shows that both the reference dye and the indicator partially photobleach over time, but that the G/R ratio remains virtually constant for at least 120 min of continuous illumination with a 405 nm LED array. The rate of photobleaching is approximately 1% per hour. This warrants long-term accuracy, not the least because continuous illumination is not needed. In fact, photographic pictures can be taken in more than 1 min intervals but acquired in less than 0.2 s.

The nanoparticles have a good dispersibility in water and can be evenly distributed in the agarose layer. This matrix contains a large fraction of water so that protons can easily diffuse in and out. Furthermore, the pH-sensitive headgroup of the probe H-110 is located on the nanoparticle surface, which is beneficial with respect to rapid pH equilibration with the environment. Single nanoparticles respond to changing pH values within seconds. When using the comparatively thick sensor layers (approximately 4 mm), diffusion is much slower and the response time is around 20 min (pH change from 8.5 to 5.5; see Supporting Information, Figure S9) which is indeed not the most rapid response compared to other pH sensors,[10a] but is sufficient to visualize bacterial growth. Leakage tests (Supporting Information, Figure S10) showed that neither the dye nor the nanoparticles leach out of the agarose matrix on exposure to water over 48 h.

To demonstrate microbiological practicability, we have grown *E. coli* on the pH-sensitive agarose in a Petri dish and monitored their growth and metabolism by acquiring an RGB pictures once per hour under 405 nm illumination. As shown in Figure 3, the pH-sensitive Petri dish initially displays

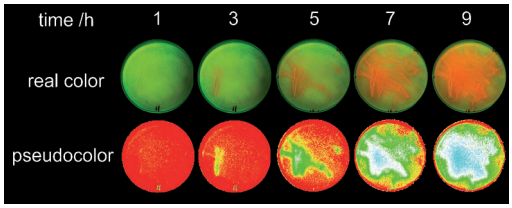

Figure 3. Photographing the time course of bacteria growth and metabolism in a 9.0 cm Petri dish (see text for details).

a homogeneous green fluorescence, indicating a starting pH value of 7.8. After 2 h of growth and metabolism, it can be seen that significant acidification of the agarose layer has occurred. The red region shown in the real color picture (Figure 3) is a grown colony. The protons produced by the bacteria during growth have caused a substantial change in the local pH value as the intensity of the green luminescence is strongly reduced. The red luminescence of TFPP is becoming the predominant color and can be associated with the region where the colony grows best.

The change of pH value was further visualized by the RGB photographic readout. Pseudo-color pictures are shown in Figure 3 (bottom). They underpin the fact that metabolism of *E. coli* lowers local pH values. After 9 h, the pH value of the region containing a large concentration of bacteria has dropped from 7.8 to 6.5. A 12 h time course (in 1 h intervals) and the respective pH change during the bacterial growth can be seen in Video V1 in the Supporting Information.

The novel pH-sensitive nanoparticles described herein have distinct features including non-toxicity, smallest size reported to date, and ease of incorporation into a biocompatible matrix so to obtain a film that enables imaging of pH value during bacterial growth and metabolism. Compared to the widely used sensor plates and layers containing absorptiometric pH indicators (which enable bacterial growth to be monitored visually) we note that a) our sensors do not undergo leakage of the (water-soluble) pH indicators; b) our nanoparticles are not internalized; and that c) the usual difficulties that sometimes prevent precise photometric or reflectometric determination of pH values (due to intrinsic absorptions and scattering of light by bacteria) are overcome by making use of fluorescent nanoparticles as this approach enables pictures to be acquired from on top or through the bottom wall of a Petri dish, and in a self-referenced mode. The method is expected to be superior to the use of sensor plates in a microtiter plate format which does not enable imaging and lacks nanoscale resolution.[26] Other features include a constant ratio of both dyes at any site (which is useful in terms of precision), and the lack of aggregation of dyes and nanoparticles in the agarose layer. In quite recent work we have shown that this material also allows two fluorescent probes to be incorporated. This development has led to nanoparticles capable of simultaneously sensing pH value and oxygen in cellular cytosol.[27]

We presume that the method may also be adapted to contactless real-time sensing of other parameters, for example, oxygen or carbon dioxide. We envision that the method will even enable different species of bacteria to be distinguished from their different metabolisms. Other future applications may be in the detection of bacterial stress[25] (such as in high-throughput screening), the freshness of food (by integrating it, along with a bar-code, into packed food), and in medical microbiology.

Experimental Section

Preparation of pH sensitive nanoparticles: Typically, of Pluronic F127 (200 mg; Sigma–Aldrich, www.sigmaaldrich.de) were mixed with a dichloromethane solution of 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (TFPP; 0.8 mL of a 2.0 mM solution from Sigma–Aldrich; www.sigma-aldrich.com) and a solution of the pH probe 5-hexadecanoyl aminofluorescein in acetone (H-110; 1.0 mL of a 2 mM solution from Invitrogen; www.invitrogen.com). The mixture was brought to dryness with a flow of nitrogen at room temperature. Then hydrochloric acid (0.85 M, 3.12 mL) was added to the residue. A stable and optically transparent solution was formed while rigorously stirring it. This was followed by dropwise addition of tetraethoxysilane (360 μL), and the solution was continuously stirred for 105 min. Next, dimethoxydimethylsilane (30 μL) was added to terminate the growth of the nanoparticles. The mixture was stirred for 24 h at room temperature. Finally, the solution was dialyzed (cut-off molecular weight 14000; from Carl-Roth; www.carlroth.com) against doubly distilled water for 2 days to remove residual chemicals. The suspension was then filtered through a 0.1 μm Whatman filter paper to remove large aggregates.

Preparation of the pH sensitive agarose Petri dish: Typically, agarose (400 mg) and EC broth (850 mg; from Sigma–Aldrich) were dissolved in distilled water (20 mL) at 75 °C. Once the mixture had become transparent, a solution of nanoparticles (2.0 mL , as described above) was added to the mixture. Stirring for 2 min yielded a homogeneous mixture which was poured into a Petri dish to form an agarose layer on cooling to room temperature. The resulting pH-sensitive agarose layer was steam sterilized at 120 °C at 1.1 bar for 30 min by the usual procedure.[28]

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