Materials Science and Engineering C 33 (2013) 4809–4815

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

In vivo optical detection of pH in microscopic tissue samples of *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form 26 June 2013
Accepted 30 July 2013
Available online 7 August 2013

Keywords:
Fluorescence
HPTS
pH
Tissue
Arabidopsis thaliana

ABSTRACT

Minimally invasive *in vivo* measurement of pH in microscopic biological samples of μm or μl size, e.g. plant cells, tissues and saps, may help to explain complex biological processes. Consequently, techniques to achieve such measurements are a focus of interest for botanists. This paper describes a technique for the *in vivo* measurement of pH in the range pH 5.0 to pH 7.8 in microscopic plant tissue samples of *Arabidopsis thaliana* based on a ratiometric fluorescence method using low-loss robust tapered fiber probes. For this purpose tapered fiber probes were prepared and coated with a detection layer containing ion-paired fluorescent pH-transducer 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (c-HPTS). A fluorescence ratiometric approach was employed based on excitation at 415 nm and 450 nm and on the comparison of the fluorescence response at 515 nm. The suitability of tapered fiber probes for local detection of pH between 5.0 and 7.8 was demonstrated. A pH sensitivity of 0.15 pH units was achieved within the pH ranges 5.0–5.9 and 7.1–7.8, and this was improved to 0.04 pH units within the pH range 5.9–7.1. Spatial resolution of the probes was better than 20 μm and a time response within 15–20 s was achieved. Despite the minute dimensions of the tapered fiber probes the setup developed was relatively robust and compact in construction and performed reliably. It has been successfully employed for the *in vivo* local determination of pH of mechanically resistant plant tissues of *A. thaliana* of microscopic scale. The detection of momentary pH gradients across the intact plant seems to be a good tool for the determination of changes in pH in response to experimental treatments affecting for example enzyme activities, availability of mineral nutrients, hormonal control of plant development and plant responses to environmental cues.

1. Introduction

New discoveries in biology [1] or medicine often rely on the development of novel detection approaches and measurement techniques among which the development of chemical or biochemical sensors may play a crucial role. Complex biological processes like morphogenesis, plant defense mechanisms, plant stress tolerance and signaling processes inside living organisms and cells, are among topics of major interest to experimental botanists [2]. Optical sensors are of particular importance in investigations in such fields since they are capable of providing (bio)chemical information together with imaging of the investigated object. Miniaturization of such sensors can provide locally specific information on the (bio) chemical composition of plant tissues, cells and even organelles. In turn, this has enabled precise quantification of gradients in (bio) chemicals of interest and allowed monitoring of changes in their spatial and dynamic patterns.

For *in vivo* (bio)chemical mapping, probes based on optical fibers offer an excellent tool which can be scanned inside samples at a spatial resolution of 1 μm or less, providing comprehensive time and spatially resolved information. For this purpose optical fibers are shaped by flame or laser brushing or by Focused Ion Beam to form a small cone at the tip [3–6]. When a suitable transducer or bioreceptor is immobilized onto a fiber tip, and excited by light guided through the fiber, a fluorescence response of the analyte of interest can be collected microscopically and registered by photomultiplier. The performance of this technique (known as near-field scanning optical microscopy (NSOM) [3,4,7–11]) has been systematically advanced, e.g. by application of nanoparticles for the enhancement of the fluorescence response [12].

NSOM can serve as an alternative to conventional fluorescence (confocal) microscopy [13–15] based on observation of fluorescence of markers, transducers or PEBBLEs [16,17], more or less homogeneously spread within bio-samples. Apart from the detection of pH, methods based on NSOM can be used to monitor oxygen, glucose [3,7–9], nitric oxide [18], glutamate [19],and carcinogens like benzo(alpha)pyrene tetrol and benz(alpha)pyrene [4,11] among others (see [5] for comprehensive survey). Macroscopic bio-samples of larger than millimeter size can be locally characterized by conventional electrochemical pH-meters with reduced electrode size [20] or by commercially available optical sensors [21,22] with 100–150 μm diameter

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http://dx.doi.org/10.1016/j.msec.2013.07.045

optical fibers. Local detection of samples of μm or μl dimensions requires microscopic resolution, i.e. resolution between intracellular and macroscopic scales. Samples of such sizes, e.g. of tissues or droplets, are frequently explored in (experimental) botany and medicine. For their characterization sufficiently robust, compact and minimally invasive methods are required. Detection of pH with fine spatial recognition based on decay time characterization using fiber probes tapered to 20–100 μm has already been demonstrated [23,24].

This paper deals with an approach to *in vivo* measurement of pH of microscopic and mechanically resistant plant tissues of *Arabidopsis thaliana*. Unlike the results already presented [23,24,30] the proposed approach is based on the self-referenced ratiometric optical response of originally complexed 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (c-HPTS) transducer applied onto tapered fibers with a tip diameter of 20 μm or less. This unique approach allowed us to determine pH between 5.0 and 7.8 with a spatial resolution comparable to the size of an individual cell.

2. Experimental

2.1. Fabrication of fiber tapers

In order to reach micrometer-scale spatial resolution, the diameter of the optical fibers was reduced from the conventional 125 μm by a tapering process.

Graded-index multimode fiber of own production was used for all experiments. The parameters of the fiber were similar to the commercially available ones, i.e. cladding/core diameter 125/50 μm, parabolic refractive index profile, numerical aperture 0.2 and optical loss at 1300 nm was around 1–2 dB/km.

Approx. 2 m long parts of the fiber were locally heated by indirect flame heating and pulled. The shape and properties of the prepared tapers depended on the pulling speed, tension of fiber and temperature of heating. The aim was to prepare short and nearly adiabatic tapers of minimum optical losses. The reproducibility of the tapering process depended on precise control of the process parameters and the antivibration arrangement of the fabrication setup.

Prepared tapered fiber probes without a supplementary protective thin layer were beveled by micro-grinding using a home-made micro-beveler with lapping foils of 2 μm roughness (3M) rotating at 7200 rpm. This process allowed us to control the size and beveling (sharpness) of tips of tapered fiber probes interacting with analyzed objects.

2.2. Preparation of detection layers and their characterization

Detection layers were prepared by immobilization of a pH-transducer into a sol–gel matrix. Firstly, the fluorescent pH-transducer 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) was modified. The HPTS (>97%, Aldrich) was reacted with hexadecyltrimethylammonium bromide (CTAB, >99%, Sigma) [25] forming complexed c-HPTS. All employed chemicals were used without any purification. The sample of 500 mg of HPTS was dissolved in 50 mL of deionized water under heating at 50 °C. The sample of 695 mg of CTAB was dissolved in 100 mL of deionized water under heating at 50 °C. Both solutions were mixed together. The molar ratio of HPTS to CTAB was equal to 1:2. A white-yellow precipitate of ion paired c-HPTS was formed immediately after the mixing of the solutions. The precipitate of the ion paired c-HPTS was filtered and dried in the oven at 70 °C for 24 h.

The sol used for the immobilization of prepared pH-transducer was prepared by mixing two sols based on triethoxy(ethyl)silane (ETES, 97% Fluka) and (3-glycidyloxypropyl)trimethoxysilane (GLYMO, 98% Aldrich) organosilicates [25]. The first sol was prepared by dissolving 22 mL of GLYMO in 36.7 mL of ethanol (EtOH, ACS reagent, ≥99.5% absolute, Sigma-Aldrich). A mixture of 5.5 mL 1-methylimidazole with 7.2 mL of deionized water was added drop-wise into the formed sol. The second sol was prepared by dissolving 21.5 mL of ETES in a mixture of 37 mL of EtOH and 7 mL of 0.1 M hydrochloric acid (Fluka). Both sols were mixed together keeping the molar ratio GLYMO:ETES equal to 1:1. Finally, 5 mg of prepared c-HPTS was carefully mixed with 1 mL of freshly prepared sol. The resulting sol was aged for 72 h at ambient temperature and then employed for the deposition of detection layers on prepared fiber probes.

The aged sol containing the c-HPTS transducer was deposited onto tapered fiber probe by dip coating. The tips of the probes were briefly (10 s maximally) immersed into a mixture of 40% HF, 65% HNO_3 and water in 1:1:1 volume ratio immediately before the layer deposition in order to clean their surface. The probes were then put into a micromanipulator holder (Narishige) mounted on an inverted microscope (NIB-100, Novel Optics). A 10 μl drop of the aged sol was put onto a glass slide, positioned in the inverted microscope and after approx. 20–30 s delay the tip of the probe was carefully put into a contact with the drop of sol using the micro-manipulator and immediately withdrawn. Due to an increased viscosity of the sol, caused by solvent evaporation during the 20–30 s delay, a small droplet of sol was formed at the tip of the probe. Coated tapered fiber probes were dried and sintered in one step at 140 °C for 4 h and characterized by optical microscope.

Since it is difficult to characterize layers deposited onto small tapered fiber probes, planar Pyrex® substrates (OpticC s.a.) were simultaneously coated using the same procedure and characterized by fluorescence spectrometry using the Fluorolog-3 (Horiba-Jobin Yvon) fluorimeter equipped with a photomultiplier. Coated substrates were sealed inside a PTFE cell and brought into contact with a set of Britton-Robinson colorless buffer solutions of different pH but of the same ionic strength (0.15 mol · L^{-1}). pH values of the buffer solutions were determined by a conventional pH-meter (Jenway 3305) calibrated by standard pH buffer solutions of pH 4.01 and pH 7.01 (Carl Roth GmbH). Excitation spectra ranging from 390 nm to 490 nm at the emission wavelength 515 nm were recorded at various pH values.

2.3. Detection setup and calibration

Tapered fiber probes coated with detection layer were involved in the detection setup depicted in Fig. 1. A high-power light-emitting diode (LED) (OEM, 350 mA, 3.2–3.6 V, 1.3 W max. input power) emitting at 450 nm and a laser diode (TopGaN, TGL415-50PMG) emitting at 415 ± 5 nm were used as excitation sources. Beams of excitation light from both sources were combined using a Y-optical coupler 125/50 μm of coupling ratio 50:50. Switching between the sources was carried out by manual switching of the LED and LD power sources. A second Y-coupler 125/50 μm was placed between the tapered fiber probe and the source and detection parts. A coupling ratio of 99:1 (detection 99%:excitation 1%) was chosen to provide effective collection of weak fluorescence responses and to reduce the intensity of excitation sources.

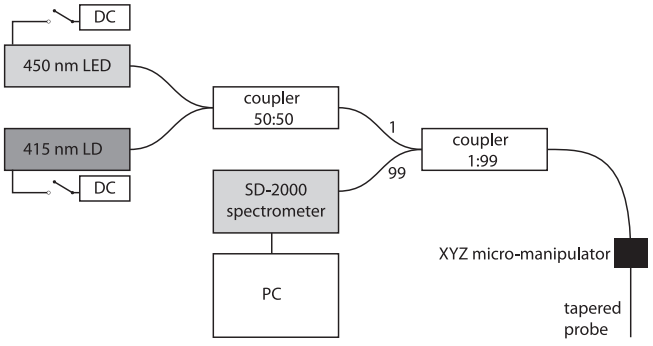

Fig. 1. Scheme of the experimental setup.