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Ultrabright planar optodes for luminescence life-time based microscopic imaging of O_2 dynamics in biofilms

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ABSTRACT

New transparent optodes for life-time based microscopic imaging of O_2 were developed by spin-coating a μm-thin layer of a highly luminescent cyclometalated iridium(III) coumarin complex in polystyrene onto glass cover slips. Compared to similar thin-film O_2 optodes based on a ruthenium(II) polypyridyl complex or a platinum(II) porphyrin, the new planar sensors have i) higher brightness allowing for much shorter exposure times and thus higher time resolution, ii) more homogeneous and smaller pixel to pixel variation over the sensor area resulting in less noisy O_2 images, and iii) a lower temperature dependency simplifying calibration procedures. We used the new optodes for microscopic imaging of the spatio-temporal O_2 dynamics at the base of heterotrophic biofilms in combination with confocal imaging of bacterial biomass and biofilm structure. This allowed us to directly link biomass distribution to O_2 distribution under both steady state and non-steady state conditions. We demonstrate that the O_2 dynamics in biofilms is governed by a complex interaction between biomass distribution, mass transfer and flow that cannot be directly inferred from structural information on biomass distribution alone.

1. Introduction

Molecular oxygen (O_2) is a key molecule for important biogeochemical and metabolic processes (Fenchel and Finlay, 2008; Glud, 2008). It is produced by oxygenic phototrophs (cyanobacteria, algae and plants) and is the preferred terminal electron acceptor in biological breakdown of carbohydrates since it generates the highest energy yield compared to other electron acceptors. Higher O_2 levels are critical e.g. for anaerobic microorganisms and processes, and for aerobic organisms due to formation of reactive oxygen species that can damage cellular processes. The O_2 distribution in biological systems can thus have strong effects on biogeochemical conversion rates and growth yields affecting the morphology of tissues and cell clusters. Local variations in O_2 respiration or production rates coupled with mass transfer limitations, e.g. due to the presence of diffusive boundary layers, can lead to steep spatial gradients of O_2 that respond dynamically to environmental parameters such as light or flow (Fenchel and Finlay, 2008). Oxygen concentration can vary strongly at μm to mm scale and precise quantification of the O_2 distribution and dynamics is a prerequisite for understanding the performance and regulation of many metabolic conversion rates, biotechnological and biomedical processes.

Oxygen dynamics can be monitored at high spatio-temporal resolution with electrochemical or fiber-optic O_2 microsensors (Klimant et al., 1995, 1997; Kühl, 2005; Revsbech, 2005). However, the spatial coverage is limited due to the one-dimensional nature of such measurements, and it is difficult to describe the spatial heterogeneity of systems with microsensor techniques, especially under non steady state conditions. With the development of planar optodes, a new tool for mapping the spatial distribution of O_2 became available (Glud et al., 1996; Kühl and Polerecky, 2008). Planar optodes use luminescent O_2 indicators immobilized in a polymeric matrix, which is permeable to O_2 and can be fixed on foils or glass surfaces. The measuring principle is based on the dynamic collisional quenching of the indicator luminescence by O_2 (DeGraff and Demas, 2005). Using sensitive gated CCD camera systems, O_2 dependent levels of luminescence and its exponential decay characteristics can be imaged, ultimately resulting in a description of the two dimensional distribution of O_2 (Holst et al., 1998; Liebsch et al., 2000; Oguri et al., 2006).

Hitherto planar O_2 optodes have mostly been based on the use of either ruthenium(II) polypyridyl complexes or metallo-porphyrines as O_2 indicators (Amao, 2003; Wolfbeiss, 2005). These indicators exhibit moderate luminescence brightness with luminescence life-times in the μs–ms range. Recently, novel optical sensor materials were developed, based on the use of cyclometalated iridium(III) coumarin complexes as O_2 indicators with an exceptionally bright luminescence (Borisov and Klimant, 2007; DeRosa et al., 2004). These

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new O_2-sensitive dyes are suitable for application in ultrathin transparent sensor layers, enabling very short response times and spatial resolution at the single cell level. A thin layer and short response time are required e.g. when fast changes in O_2 concentrations under non steady state conditions are monitored.

Here we present new thin layer optodes based on the new iridium (III) coumarin complexes immobilized in a 1 µm thick layer on a microscope cover slip. We compare the O_2 measuring characteristics of the new optodes with other thin planar optodes based on ruthenium(II) polypyridyl and platinum(II) porphyrin complexes (Kühl et al., 2007) and apply the new optodes for combined microscopic imaging of biomass, O_2 dynamics and spatial gradients in heterotrophic microbial biofilms under different flow regimes.

2. Materials and methods

2.1. Optode preparation

Three different types of luminescent O_2 indicators were used in this study: i) ruthenium(II)-tris-4,7-diphenyl-1,10 phenantroline (Ru-DPP), ii) platinum(II)-meso-tetra(pentafluorophenyl)porphyrin (Pt-TFPP), and iii) iridium(III) acetylacetonato-bis(3-(benzothiazol-2-yl)-7-(diethylamino)-coumarin) (IrC). Ru-DPP and IrC were synthesized as described elsewhere (Klimant and Wolfbeiss, 1995; Borisov and Klimant, 2007). Pt-TFPP was obtained commercially (Frontier Scientific Inc., USA). All luminescent dyes were immobilized in the same polystyrene (PS) matrix (ST316310/1 LS223989 JV, Goodfellow Ltd., Cambridge, UK) to guarantee similar gas diffusion characteristics within the optode. Dye concentrations (mg indicator per g PS) were 18.75 mg Ru-DPP/g, 25 mg Pt-TFPP/g, and 15 mg IrC/g. Thin-film optodes were fabricated by spin coating ~1 µm thick indicator layers onto 20×50 mm silanized microscope coverslips (Kühl et al., 2007).

2.2. Optode calibration setup

Optodes were calibrated at 20 °C in freshwater with defined O_2 concentrations. The optodes were placed in a holder on the window of a small water filled tank. The temperature of the tank was controlled by a cryostat (Julabo F25 HD, Germany). Oxygen levels in the calibration chamber were varied by flushing the water with defined gas mixtures of N_2 and O_2 at a flow rate of 0.6 l min^{-1}. Mixtures were generated with a PC-controlled programmable gas mixing system using electronic mass flow controllers (Sensorsense, Netherlands). The O_2 concentration in the water was increased step wise, at time intervals including at least 2 min of steady state at each O_2 concentration. Additionally, O_2 levels in the water were monitored with a fiber-optic O_2 minisensor system (Fibox 3, Presense GmbH, Germany). The temperature dependence of the planar sensor luminescence was measured at two different O_2 concentrations in steps of 5 °C over a range of 5–30 °C.

2.3. Life-time imaging system and image calibration

Luminescence life-time and intensity of the optodes was imaged with a modular luminescence life-time imaging system (Holst et al., 1998) consisting of i) a fast gate-able 12 bit SVGA CCD (1280×1024 pixel) camera (SENSICAM-SENSIMOD, PCO AG, Germany) equipped with a macro lens (Xenoplan XNP 1.4/17, Schneider-Kreutznach, Germany) and a 590 nm (30 nm bandwidth) bandpass filter, ii) a custom built trigger box driving two high power blue LED's (1 W Luxeon Star, 470 nm, Lumileds) for the excitation of the Ru-DPP and IrC optodes, or two UV power LED's (405 nm 1 W, Roithner Lasertechnik GmbH, Austria) for excitation of the Pt-TFFP optode, and iii) a custom-built PC-controlled pulse-delay generator. Image acquisition and hardware control were done with a custom made software program (Holst and Grunwald, 2001).

Life-time imaging with the system was done by acquiring luminescence intensity images (using a binning of 2), within two different time windows, w1 and w2, after the eclipse of an excitation light pulse. The luminescence life-time (τ) was calculated as (Gerritsen et al., 1997):

$$\tau = \frac{\Delta t}{\ln(I_{w1}/I_{w2})} \quad (1)$$

where Δt is the time delay between the start time of recording of the two time windows, and I_{w1} and I_{w2} are the corresponding luminescence images.

Since the three O_2 indicator dyes have different light absorption and life-time characteristics, we optimized the measuring protocols for each dye. In this study, we used excitation light pulses of 5, 5 and 7 µs for Ru-DPP, IrC and the Pt-TFPP optodes, respectively. Two luminescence intensity images (I_{w1} and I_{w2}) were acquired over 3 µs. Image one (I_{w1}) was acquired at 0.1 µs while the second image (I_{w2}) was acquired at 3.1 (Ru-DPP), 4.1 (IrC) or 7.1 µs (Pt-TFPP) after the excitation light pulse. These measurements were repeated and image pixel values integrated on the CCD chip over an exposure time period up to 400 ms to improve the signal to noise ratio. The first intensity window image is acquired by accumulation of Iw1 pulses over the integration time, and then the same is done for the second intensity window image. In this study we used exposure times of 100, 300 and 400 ms for IrC, Ru-DPP and Pt-TFPP optodes, respectively. Different exposure times were necessary to achieve comparable pixel intensity values on the camera, i.e., to obtain minimum grey values of >400 after subtraction of a dark image.

Image analysis was done in the custom made software *Look@-MOLLIdata* (Polerecky, http://www.mpi-bremen.de/Binaries/Binary4997/Polerecky-EADS-report-2005.pdf) and the freeware *ImageJ* (http://rsbweb.nih.gov/ij/). We analysed randomly selected regions of interest (ROI's) ($n = 6–10$) in the acquired images, each containing ~5000 pixels; regions at the very sensor edge or in the unevenly illuminated periphery of the field of view were avoided. From the ROI's we derived the average luminescence intensity (pixel value) of image 1 and image 2, as well as the average life-time and its standard deviation. Stern–Volmer plots of the data were fitted with a modified

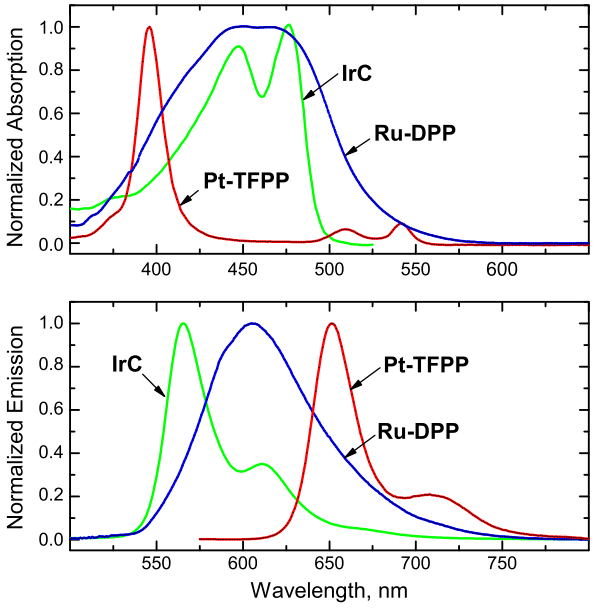

Fig. 1. Absorption and luminescence emission spectra of optical O_2 indicators immobilized in polystyrene: ruthenium(II)-tris-4,7-diphenyl-1,10 phenantroline (Ru-DPP), platinum(II)-meso-tetra(pentafluorophenyl)porphyrin (Pt-TFPP), and iridium(III) acetylacetonato-bis(3-(benzothiazol-2-yl)-7-(diethylamino)-coumarin) (IrC).