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Fluorescence imaging of the activity of glucose oxidase using a hydrogen-peroxide-sensitive europium probe

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Abstract

A method for optical imaging of the activity of glucose oxidase (GOx) using a fluorescent europium(III) tetracycline probe for hydrogen peroxide is presented. A decay time in the microsecond range and the large Stokes shift of 210 nm of the probe facilitate intensity-based, time-resolved, and decay-time-based imaging of glucose oxidase. Four methods for imaging the activity of GOx were compared, and rapid lifetime determination imaging was found to be the best in giving a linear range from 0.32 to 2.7 mUnit/mL. The detection limit is 0.32 mUnit/mL (1.7 ng mL^{-1}) which is similar to that of the time-resolved (gated) imaging using a microtiterplate reader. Fluorescent imaging of the activity of GOx is considered to be a useful tool for GOx-based immunoassays with potential for high-throughput screening, immobilization studies, and biosensor array technologies.

Keywords: Glucose oxidase; Enzyme imaging; Fluorescent probe; Time-resolved imaging; Enzyme activity assay

Glucose oxidase (GOx, EC 1.1.3.4)[1] is a flavoenzyme that catalyzes specifically the oxidation of glucose into gluconate and hydrogen peroxide Eq. (1) [1]. It is one of the most widely used enzymes due to its availability and stability. As a representative of oxidases, it has been studied in solution and in the immobilized state on solid surfaces [2] or encapsulated in microspheres [3] for applications in biosensors [4], industrial bioreactors [5], and bio-fuel cells [6]. Apo-glucose oxidase has also been utilized in studies for "nanowiring" [7] of the electron transfer. GOx has been applied not only to the electrochemical and optical detection of glucose [8] but also in GOx-labeled enzyme amplification analysis for immunoassays [9]. GOx-based enzyme immunoassays have been used for the detection and screening of, e.g., steroids, drugs, environmental pollutants, and special peptides [10]. Recently, GOx has been applied to microchip or microarray technologies for the development of protein chips for proteomics [11].

$$\text{glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconolactone} \xrightarrow{\text{hydrolysis}} \text{D-gluconate} + H_2O_2 + H^+ \quad (1)$$

Numerous methods for the determination of GOx have been developed. A set of assays have emerged based on the measurement of oxygen [12], pH [13], or H_2O_2 [14]. However, methods based on the measurement of H_2O_2 produced by GOx are of particular interest because H_2O_2 does not form a background but is produced only during enzymatic reaction. Since H_2O_2 cannot be easily visualized, it is usually converted into a colored or fluorescent product using peroxidase (POx).

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[1] *Abbreviations used:* GOx, glucose oxidase; EuTc, europium–tetracycline complex; EuTc-HP, europium–tetracycline–hydrogen peroxide; HP, hydrogen peroxide; Tc, tetracycline; FII, fluorescence intensity imaging; TRI, time-resolved imaging; PDR, phase delay rationing imaging; RLD, rapid lifetime determination imaging; POx, peroxidase; CCD, charge-coupled device; LED, light-emitting diode.

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In recent years, fluorescent imaging technologies have attracted substantial attention because of the multitude of information on both the spatial and the temporal characterization of the target analyte [15,16]. Confocal [17], multiphoton excitation [18], near-field [19], and decay-time-based imaging [20,21] technologies have been developed among others. Existing methods for imaging GOx are restricted mainly to scanning electrochemical microscopy [22,23] and scanning chemiluminescence imaging [24]. This is largely due to the fact that almost all fluorescence imaging methods for GOx need a second enzyme (POx) to visualize the H_2O_2 produced by the GOx-catalyzed reaction. There have been reports on H_2O_2-based imaging using dihydrorhodamine 123 [25], homovanillic acid [26], scopoletin [27], dichlorodihydrofluorescein diacetate [28] or others [29], all using POx along with a second (chromogenic or fluorogenic) substrate [30].

Based on our previous reports on the europium–tetracycline (EuTc) fluorescent probe for determination of either H_2O_2 [31] or glucose either in solution [32] or using biosensor membranes [33,34] we present here a scheme for imaging GOx at neutral pH and without the need for a second enzyme. It also is shown that, in addition to intensity-based imaging of GOx, the long luminescence decay time of the probe enables time-resolved and lifetime-based imaging.

Materials and methods

Materials and reagents

All solutions were prepared in a 10 mmol L^{-1} 3-(*N*-morpholino)propanesulfonate (Mops) buffer of pH 6.9. Glucose oxidase (from *Aspergillus niger*; 185,000 Unit/g; used without further purification) was from Sigma–Aldrich (Steinheim, Germany) and tetracycline hydrochloride was from Serva (Heidelberg, Germany; www.serva.de). The activity of GOx is defined by the provider as follows: one unit will oxidize 1.0 μmol of β-D-glucose to D-gluconolactone and H_2O_2 per min at pH 5.1 and 35 °C. $EuCl_3$ hexahydrate was from Alfa Products (Danvers, MA, USA; www.alfa.com).

The EuTc stock solution was prepared by mixing 10 mL of a 6.3 mmol L^{-1} $EuCl_3$ solution with 10 mL of a 2.1 mmol L^{-1} tetracycline solution and diluting it to 100 mL with Mops buffer (the molar ratio of Eu^{3+} to Tc being 3:1). The glucose stock solution (0.277 mol L^{-1}) was stored overnight before use to warrant the equilibration of α and β anomers.

Instruments

Fluorescence measurements and time-resolved (gated) intensity detection were performed on a GENios+ microtiter plate reader (Tecan, Grödig, Salzburg, Austria; www.tecan.com). The excitation filter was set to 405 nm and the emission filter to 612 nm. All experiments were performed at a programmed temperature of 30.0 ± 0.1 °C. Either black Fluotrac200 microtiterplates or black plates with a flat transparent bottom (Greiner bio-one, Frickenhausen, Germany; www.greiner-lab.-com) were used.

Imaging device

The device used in this study has been previously reported [35,36] and was used with minor modifications. As shown schematically in Fig. 1, it is composed of a fast gateable CCD camera (SensiMod; from PCO, Kelheim, Germany; www.pco.de), a pulsable LED array with 96 UV light-emitting diodes (λ_{max} 405 nm, Roithner Laser Technik, Vienna, Austria; www.roithner-laser.com), a pulse generator (Scientific Instruments DG 535, Sunnyvale, CA, USA; www.srsys.com; not shown in Fig. 1), an optical emission filter (KV 550; Schott, Mainz, Germany; www.schott.com), and an optical excitation filter (BG 12; Schott), with a light-guiding adapter consisting of 96 optical fibers (diameter 3 mm) for matching the focus of the CCD camera. A computer was used for control and visualization of the experiments that were programmed in Interactive Data Language (IDL; from Research Systems, Boulder, CO, USA; www.rsinc.com).

The manipulation and calculation of the images, such as the rotation and cropping of the images, the subtraction of the dark image (blank, without illumination) from the fluorescent image, and the integration of the

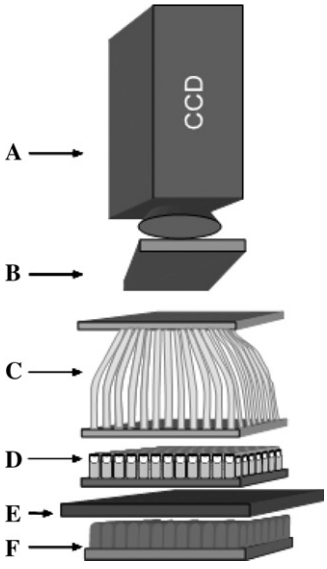

Fig. 1. Setup of the imaging system. (A) Fast gateable CCD camera; (B) optical emission filter (KV 550); (C) light-guiding adapter consisting of 96 optical fibers (diameter 3 mm); (D) 96-well microtiterplate (black with transparent bottom); (E) optical excitation filter (BG 12); (F) pulsable LED array with 96 UV light-emitting diodes (λ_{max} = 405 nm).