Electrochemical Zero-Mode Waveguide Studies of Single Enzyme Reactions

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ABSTRACT

Because electron transfer reactions are fundamental to life processes, such as respiration, vision, and energy catabolism, it is critically important to understand the relationship between functional states of individual redox enzymes and the macroscopically observed phenotype, which results from averaging over all copies of the same enzyme. To address this problem, we have developed a new technology, based on a bifunctional nanoelectrochemical-nanophotonic architecture - the electrochemical zero mode waveguide (E-ZMW) - that can couple biological electron transfer reactions to luminescence, making it possible to observe single electron transfer events in redox enzymes. Here we describe E-ZMW architectures capable of supporting potential-controlled redox reactions with single copies of the oxidoreductase enzyme, glutathione reductase, GR, and extend these capabilities to electron transfer events where reactive oxygen species are synthesized within the ~ 100 zL volume of the nanopore.

INTRODUCTION

Despite their fundamental importance in life processes, direct electrochemical observation of single electron transfer events in biological systems is still quite challenging. The principal problem is that the generated currents are at, or below, the noise floor of electrical measurements that can be made at accessible gain-bandwidth product. Thus, prior approaches to measuring single molecules electrochemically have relied on amplifying the signal, for example by redox cycling. Alternatively, converting redox processes to photon emission events, *i.e.* luminescence, can circumvent the noise floor problem. Here, we utilize a new kind of bifunctional architecture - the electrochemical zero mode waveguide (E-ZMW) – that has both nanoelectrochemical and nanophotonic characteristics and which can therefore be used to couple biological electron transfer reactions to luminescence.

Although zero mode waveguides may be constructed as single nanopores, we utilize parallel arrays of nanopores, which exhibit several advantages, including: (1) the average occupancy of individual pores may be controlled by concentration, (2) the electrodes in each individual pore are connected to those in all the other pores, so they can all be controlled at the same potential, E_{appl}, and (3) the electrochemical behavior is integrated over the entire array. The defining characteristic of these architectures is that single molecule spectroscopic and electrochemical data can be acquired simultaneously. For example, flavins are fluorigenic molecules, because flavin adenine dinucleotide (FAD) cofactors are strongly fluorescent in the oxidized state, while they are non-emissive in the reduced state ($FADH_2$).[1]

MATERIALS AND METHODS

A. Fabrication of E-ZMW Devices

Nanopore arrays were fabricated *via* a combination of standard photolithography, layer-by-layer deposition, and focused ion beam (FIB) milling. These FIB-milled pores exhibit a conical frustum shape with typical top diameter, $d_{top} \sim$ 100-120 nm, and bottom diameter, $d_{bottom} \sim$ 60-70 nm. Immediately after milling the device, an ohmic background current was observed between the top and bottom electrodes in a dry environment. The presumed conducting layer was removed by immersing the device in dilute Au etchant solution for 1-3 min.

B. Electrochemical Measurements

Electrochemical measurements were performed with a CH Instruments electrochemical analyzer (Model 750E) using a Pt wire and Ag/AgCl (RE-5B, BASi) as auxiliary and reference electrodes, respectively. The nanopore-confined top- and bottom-ring electrodes were operated as separate working electrodes. All potentials are reported vs. Ag/AgCl reference at 300K. Both the reference electrode and auxiliary electrode were immersed in a 100 μL PDMS

reservoir in direct fluid contact with the E-ZMW device. Amperometric traces were acquired by fixing one electrode at an oxidizing potential and the other at a reducing potential.

C. Fluorescence Measurements

A custom-built confocal microscope was used to collect all fluorescence data. 458 nm excitation radiation from a continuous wave laser was passed through a spatial filter and a quarter wave plate to produce TEM_{00} circularly polarized light. A dichroic filter reflected the collimated beam into the back aperture of a 40x, 1.30 NA oil immersion objective to produce a diffraction-limited spot with 40 μW power at the focal plane. Fluorescence emission was collected by the same objective (epi-fluorescence) and passed through three emission filters before being focused through a 30 μm diameter confocal pinhole before detection by a single-photon avalanche photodiode). A hardware correlator was used to collect single molecule fluorescence data. The fluorescence optical path was augmented with a trans-illumination path to assist in locating the ZMW pores. To access the trans-illumination path, collimated radiation from a 632.8 nm laser was used to illuminate the back (upper) surface of the ZMW array. A mirror was selectively rotated into the detection path to allow monitoring of the transmitted radiation by a video-rate CCD camera. Once located, the pore array was indexed to the motion of a piezoelectric stage to reliably center the *x*-*y* focal position of the objective on the ZMW pore of interest.

RESULTS AND DISCUSSION

A schematic cross-sectional diagram of a single E-ZMW nanopore is given in **Figure 1**. These structures consist of an array of nanopores on an SiO_2 or glass substrate. The geometry is chosen such that electromagnetic field does not propagate through the pore, *i.e.* zero-mode waveguide behavior,[2] when it is irradiated from the glass half-space. The trapped radiation can interact with molecules contained in the ~100 zL (1 zL = 10^{-21} L) volume bounded by the radiation field and enclosed within the nanopore. The Au layer functions simultaneously as both an optical cladding (OC) layer to confine the radiation and as one of two working electrodes (WE) to drive faradaic electrochemistry.

In order to direct the single GR enzyme to the bottom electrode, we begin with a Au ring WE/OC decorated with immobilized cystamine. A linker, PQQ, is added, which presents 3 separate carboxylic acid moieties for carbodiimide coupling. We then identify the pores that contain a single GR molecule and proceed to measure the on-time, $P(\tau_{on})$, and off-time, $P(\tau_{off})$, distributions which are then used to characterize single molecule kinetics through the exponential waiting time distribution, $P(t) = P_0 e^{-kt}$.[3] Importantly, these distributions can be measured over many different single molecules and over multiple cycles of the same molecule, enables us to characterize the static and dynamic heterogeneity, respectively, as a function of E_{appl} at the single molecule site at the bottom OC/WE.[4]

To extend this idea, we developed the capability to generate enzyme substrates, such as reactive oxygen species (ROS), *in situ* and deliver them to an immobilized enzyme in the same nanopore by using the top (Pt) annulus as a generator electrode. The oxidative potential of the solution is used to set the GSH/GSSG ratio in the glutathione redox buffer system. After characterizing the florescence dynamics of E-ZMW surface-immobilized GR, both in buffer and in the presence of GSSG, we obtain the detailed time records of GR fluorescence emission to look for evidence of single enzyme turnover.

Typical data are shown in **Figure 2**. In the top trace, the enzyme GR (which is homodimer containing 2 FAD cofactors) is in the oxidized state, and a number of emission-related spikes are observed. In contrast, when the Au electrode is poised at a reducing potential, E_B = -0.6 V *vs.* Ag/AgCl, the emission events decrease significantly, as expected. Furthermore, the top electrode can also be controlled at a potential sufficient to convert dissolved O_2 to H_2O_2, the result being a shift in the GSH/GSSG ratio, which is also reflected in the GR emission dynamics.

CONCLUSIONS

The confined environment of the E-ZMW makes it possible to achieve *in situ* control over reaction conditions and delivery of reactants, thus enabling the study of single electron transfer events in the enzyme GR. To accomplish this, we elaborated the basic E-ZMW architecture to produce a dual-electrode nanopore structure with the capacity to synthesize and deliver substrate molecules *in situ* and on-demand and to exploit this capability to characterize single ROS-enzyme reactions *in situ*.

ACKNOWLEDGMENT

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FIGURES

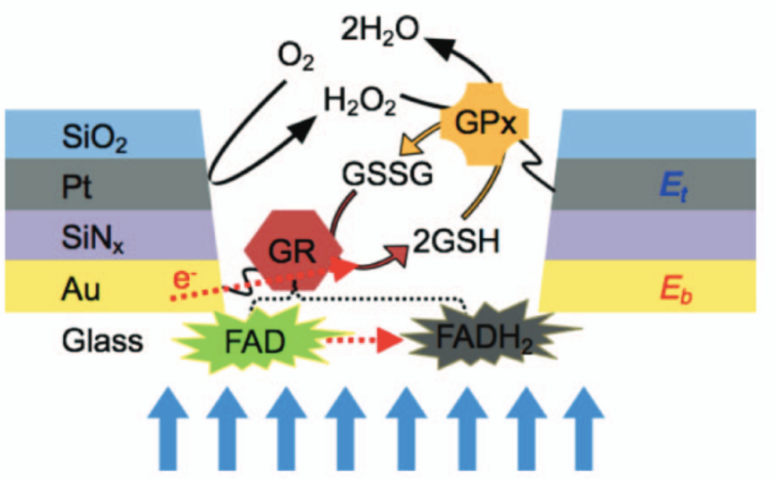

Figure 1. Schematic diagram of the single enzyme E-ZMW experiment. The ZMW confines the optical radiation to the portion of the structure enclosed by the bottom Au working electrode. A single GR enzyme molecule is immobilized on the bottom electrode. The top Pt working electrode is poised at a potential competent to generate ROS, *e.g.* H_2O_2. Single GR enzyme turnover is indicated by the observation of an emission discontinuity, characteristic of FAD $\leftrightarrow$ $FADH_2$.

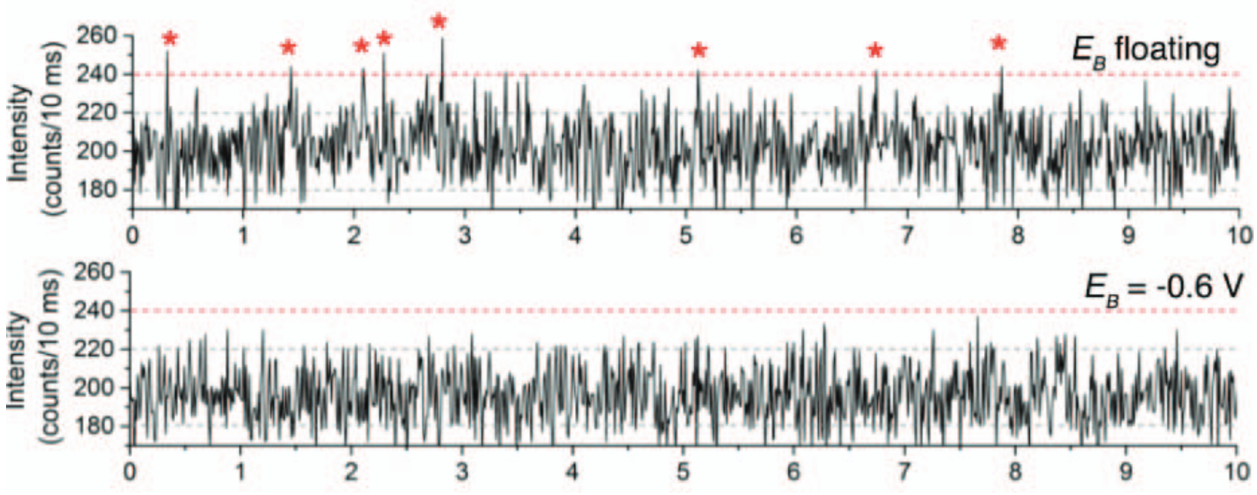

Figure 2. Single enzyme luminescence time traces. (*Top*) E_B (= potential of the bottom Au OC/WE) floats. Asterisks (*) denote events exceeding the 99% confidence level for photon emission. (*Bottom*) E_B = -0.6 V, a potential at which GR is expected to be pinned in the reduced, non-emissive state, $FADH_2$.