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A Simple Voltage Controlled Enzymatic Nanoreactor Produced in the Tip of a Nanopipet

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ABSTRACT

We report the enzymatic cleavage of fluorescein diphosphate (FDP) by alkaline phosphatase (AP) confined in the tip of a nanopipet with a volume of approximately 100 attoliters. The amount of enzyme that reversibly adsorbs in the pipet tip was shown to be proportional to the enzyme concentration in the bath. Increasing the voltage applied to the pipet, up to 1.5 V, linearly controls the substrate turnover rate by increasing the flow of substrate out of the pipet. This work opens up possibilities of highly miniaturized and sensitive enzyme assays with high spatial resolution.

There are several advantages to performing chemical or biochemical reactions in confined, nanometer sized volumes. These include reduced mixing time, improved signal-to-noise ratio and minimized amounts of reagents. Enzymatic reactions are of particular interest as the basis for biosensors, DNA sequencing, or drug screening. To date two types of experiments have been performed. Ultra-sensitive fluorescence studies down to single enzyme level have been employed to investigate the heterogeneity of different enzymes. These experiments were performed in agarose gel with pore sizes of a few micrometers,[1] at a glass surface,[2] in nanometer sized zero-mode waveguides,[3] and in standard capillaries.[4,5] In these experiments, there was little or no control over the rate of delivery of the substrate to the enzyme. Enzymatic reactions have also been studied in larger volumes in microfluidic devices where the flow was used to control the substrate concentration delivered to enzymes in solution or immobilized on a surface.[6−8] To bridge the gap between these two types of experiments, we have studied an enzymatic reaction in the tip of a nanopipet, where the flow of substrate can be controlled by a voltage applied between two electrodes, one in the pipet and one in the bath.

We have previously studied the delivery of DNA,[9,10] proteins,[11,12] and small molecules[13] from a borosilicate nanopipet. Due to the tapered structure of the tip, there is a voltage drop in the tip region allowing the generation of a high electric field from modest applied voltages.[9,10] The electrodes are located away from the tip region, eliminating problems with electrolysis. The delivery from the nanopipet can be finely controlled by the applied voltage down to the single molecule level.[9,12] The only reaction reported in a nanopipet is between fluor-3 and calcium to produce a fluorescence near-field light source in the tip.[13,14] Here we describe studies on an enzyme reaction confined in a volume of about 100 attoliters (150 nm in diameter and ∼2 μm in length). The nanopipet is filled with the substrate fluorescein diphosphate (FDP) and inserted into a bath containing the enzyme alkaline phosphatase (AP) in a buffered aqueous solution (Figure 1). The substrate flow rate in the tip can be controlled simply by varying the voltage applied to the electrodes. Instead of immobilizing the enzyme in the pipet tip, as done for microchannels,[7] we found we can simply place the glass pipet in a solution containing the enzyme. The enzyme molecules then enter the pipet tip (by diffusion or with assistance by the voltage applied between both electrodes) and reversibly physisorb on the glass. The number of enzymes attached in the steady state scales linearly with the enzyme concentration in the bath, allowing us to straightforwardly vary the number of enzymes adsorbed to the tip. This seems to be a simple method to study enzymatic reactions in confined nanometer size volumes, creating a nanoreactor, with potential applications in miniaturized screening.

Enzyme activity was initially tested by adding 4 nM alkaline phosphatase to a solution of 1 μM FDP (Figure 2) and monitoring the fluorescence using a confocal microscope.[9] Before adding any enzyme, the FDP already shows a significant background fluorescence of 20 kHz when excited with 5 μW laser power. Further dilution shows that

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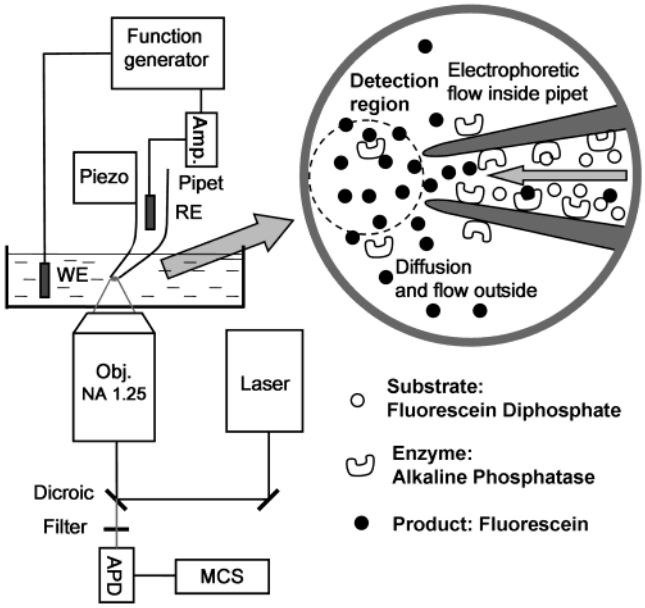

Figure 1. Schematic of the experimental setup and of the tip region of the nanoreactor (inset). The substrate is delivered by the pipet and the enzyme is added to the bath. The enzyme enters the pipet and physisorbs onto the pipet walls. Substrate is converted into fluorescent product by the enzyme in the tip, and fluorescence signal is detected just outside the pipet tip. The dashed circle outside of the tip shows the approximate detection region. WE: working electrode, RE: reference electrode, APD: avalanche photodiode, MCS: multichannel scalar card.

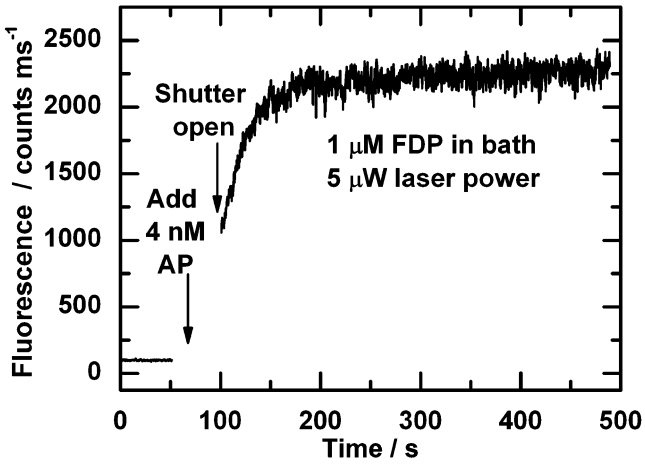

Figure 2. Fluorescence time trace with 1 μM FDP in solution. The shutter was closed after 50 s and 4 nM alkaline phosphatase was added. After mixing, the shutter was reopened and the fluorescence increase was monitored under the excitation of 5 μW laser at 488 nm.

this background fluorescence is due to highly fluorescent product, produced by the gradual autohydrolysis of FDP in an aqueous solution.[5] On the basis of the fluorescence intensity from the background, we conclude that about 1% of the FDP has already been converted to fluorescein before the enzyme was introduced. After adding 4 nM alkaline phosphatase into the solution, the fluorescence intensity increases with an initial slope of 29 kHz/s. This corresponds to a turnover rate of about 5 FDP molecules per enzyme per second.[15] For all subsequent experiments in the nanopipet we used FDP which has been purified. This reduced the background fluorescence to below 10 kHz for 1 μM FDP excited with 50 μW laser power.

Nanopipet experiments were performed by scanning the voltage applied to the electrode in the bath from -1.5 to 1.5 V in a triangular waveform. The pipet is filled with 1 μM purified FDP and the excitation laser is focused just outside the tip opening of the pipet. Note the FDP concentration is well below the Michaelis−Menten constant K_m measured to be 164 ± 36 μM in open volume (see Supporting Information Figure S1). When no enzyme is present, we measure a weak fluorescence signal, which is linear with the applied voltage, during the positive half of the voltage cycle (Figure 3A, lowest trace). The fluorescence signal depends on the average concentration of product molecules in the probe volume and is independent of the residence time of the molecules in the laser focus, as long as the molecules are not photobleached. We do not find any signs of photobleaching under our experimental conditions. The signal increases by a factor of 4 for 1.5 V applied voltage, which suggests a 4-fold concentration increase due to a small dielectrophoretic effect.[10]

The fluorescence intensity increases when 0.8−4 nM alkaline phosphatase is added to the bath, while the triangular shape of the signal is preserved (Figure 3A). Delivery of the negatively charged substrate takes place during the positive half-cycle. The nonfluorescent substrate is then converted to fluorescent product, which is detected just outside the pipet tip as it flows out of the pipet. The product leaves the detection volume and is rapidly diluted in the bulk solution. Therefore there is no build up of fluorescent product in the detection region, unlike in the bulk experiment (Figure 2). The fluorescent signal is linear with applied voltage in the range from 0.5 to 1.5 V (Figure 3C). When less substrate is delivered out of the pipet, during the back scan, the fluorescent signal decreases accordingly. Furthermore, the intensity measured at a given voltage increases linearly with the enzyme concentration (Figure 3D). This process is reversible, and the intensity reduces again when diluted back down to lower enzyme concentration.

To locate where the enzymatic reaction occurs, and to determine if enzyme enters the pipet, an image has been taken while the pipet is scanned through the laser focus (Figure 4) with an applied voltage of 0.5 V. There is high fluorescence just inside the pipet tip extending over about 2.0 μm. In a further experiment, we used goat anti-rabbit F(ab′)$_2$-conjugated alkaline phosphatase bound to Alexa 647-labeled rabbit IgG antibody so the enzyme can be detected independently from the product. Illuminating the pipet tip showed a strong fluorescent signal from the Alexa 647 fluorophore that underwent rapid photobleaching (data not shown). We therefore conclude that the enzyme−antibody complex is immobilized at the pipet tip.

We used fluorescence correlation spectroscopy to determine the flow dynamics. Autocorrelation curves were measured just outside the tip and, for comparison, in a 470 nM solution of fluorescein. When the laser was focused just outside the pipet tip with 200 mV applied voltage, the

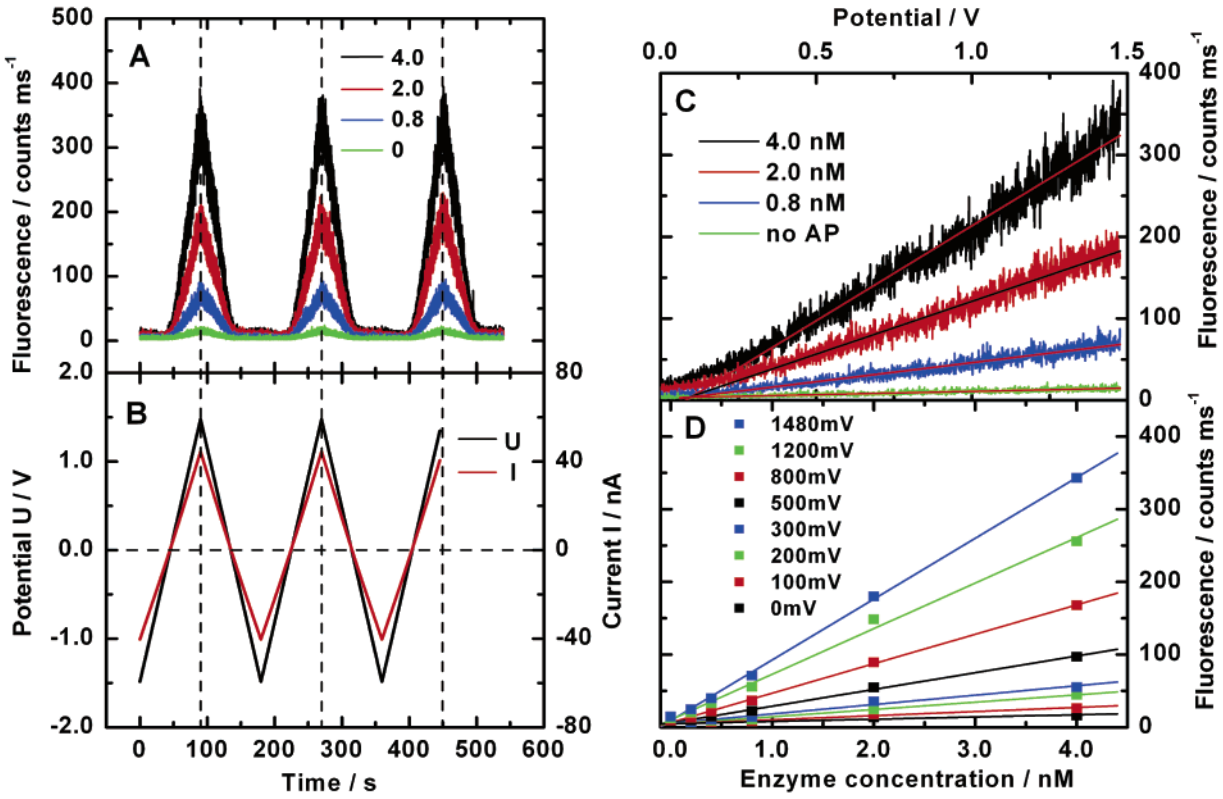

Figure 3. (A) Fluorescence vs time traces during triangular voltage scans (−1.5 to 1.5 V) with 1 μM FDP inside the pipet and increasing concentrations (0, 0.8, 2.0, and 4.0 nM) of AP in the bath, at 50 μW laser power. (B) Simultaneously recorded voltage and current. (C) Fluorescence as function of applied voltage at different enzyme concentrations; data taken from A and linear fits. (D) Fluorescence as function of enzyme concentration at different voltages; data taken from A and linear fits.

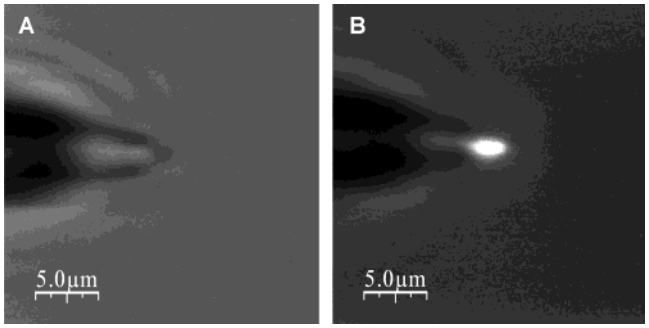

Figure 4. Optical images of the pipet obtained by scanning the pipet through the detection volume emulating global illumination. Under white light illumination (A) and combined white light and laser illumination (B). The fluorescent area is located inside the pipet, the image shows the cross section of the slightly bent vertically mounted pipet with the scanning plane, 500 mV applied voltage.

autocorrelation curve could only be fitted to a combined diffusion and flow model:[16]

$$G(t) = G_0 \frac{1}{1 + t/t_d} \sqrt{\frac{1}{1 + (r_0/z_0)^2 t/t_d}} \exp\left(-(t/t_f)^2 \frac{1}{1 + t/t_d}\right)$$

where t_d and t_f are diffusive and flow half-times, r_0 is the beam radius, measured as 280 nm in these experiments, z_0 is 1.4 μm, and G_0 the correlation amplitude. The diffusive and flow half-times were found to be 83 μs and 81 μs,

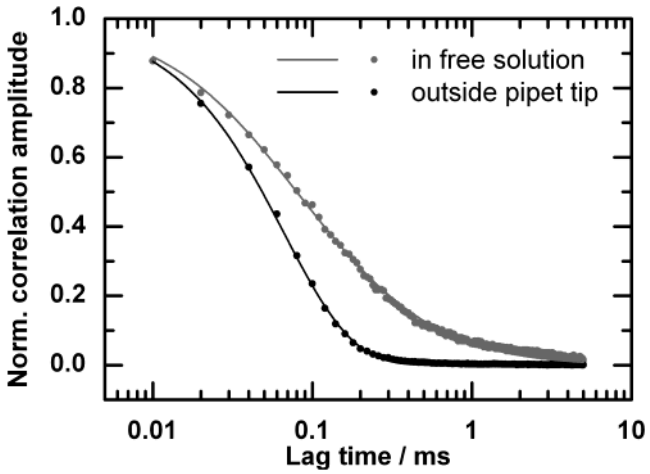

Figure 5. Normalized autocorrelation curves measured in free solution (with 470 nM fluorescein) and just outside the pipet tip (1 μM FDP in pipet, +0.2 V, 4 nM enzyme in bath). The data were fitted with a 3D diffusion model and a 3D combined diffusion and flow model, respectively.

respectively (Figure 5). In the free solution, the three-dimensional diffusion model[16]

$$G(t) = G_0 \frac{1}{1 + t/t_d} \sqrt{\frac{1}{1 + (r_0/z_0)^2 t/t_d}}$$

fits the data very well with $t_d = 83$ μs. These measurements

show that there is not only diffusion of molecules away from the tip but also an equally fast contribution of directed flow out of the pipet.

Our data can be explained using the following minimal model. Substrate flows out of the pipet during the positive cycle and the flow rate is proportional to the applied voltage. At some point in the cycle, enzyme enters the pipet tip by diffusion or with assistance by voltage and forms an equilibrium physisorbed layer. The amount of enzyme absorbed is proportional to the enzyme concentration in the bath. The adsorption process is in a steady state when we cycle the voltage, but the enzyme desorbs over period of minutes when the enzyme concentration in the bath is diluted. The physisorbed enzyme cleaves the substrate that flows out of the tip and the product is detected by fluorescence just outside the pipet. The linearity of the fluorescence signal with applied voltage shows that the flow of substrate is proportional to the voltage and that the enzyme is not saturated.

Since we have calibrated the fluorescence signal (see Supporting Information) and know the flow rate out of the pipet, then, assuming all the product flows through the probe volume, we have a direct measurement of the number of substrate molecules turned over per second at a given applied voltage. We have also shown the turnover rate is linear with applied voltage and enzyme concentration in the bath. What we do not know is the turnover rate for the enzyme at a given applied voltage and the number of enzymes in the tip. However, if we use the data obtained at zero applied voltage, then we can use the turnover rate measured in bulk solution for an order of magnitude estimation of the number of enzymes adsorbed in the pipet.

When the applied voltage is zero and the enzyme concentration is 4 nM in the bath, we measured a fluorescence intensity of 16 counts ms^{-1} under 50 μW laser power, which corresponds to 0.3 molecules in the confocal volume. With no applied voltage there is no flow, and the diffusion time was measured to be 80 μs. Therefore, in every second, 3750 molecules pass through the detection region. The turnover rate of the enzyme in solution, at 1 μM substrate, is 5 molecules s^{-1}, so we can estimate that there are 800 enzyme molecules in the pipet. However, the enzyme turnover rate is reported to decrease by a factor of 6^7 on absorption on a glass wall. If so, the number of enzymes absorbed in the pipet tip could be estimated at between 240 and 4800 molecules over the 0.2−4 nM enzyme concentration range used in these experiments. If we assume one enzyme occupies 10×10 nm^2 space on the surface, the area covered by all the enzymes is ~ 0.5 μm^2. For a conical pipet with tip radius $r = 75$ nm and 3° cone angle, this area will extend about 1 μm into the tip. This is consistent with the image of the pipet tip where the length of the fluorescence region was about 2 μm.

In conclusion, we have shown that it is possible to carry out enzymatic reactions in the tip of a nanopipet using the applied voltage to linearly control the substrate flow. No surface attachment is required as the functional enzyme physisorbs onto the pipet tip in proportion to its concentration in solution. Further improvements in sensitivity should also be possible by increasing the substrate concentration in the pipet. The approach developed in this work opens up analytical possibilities of highly miniaturized and sensitive enzymatic assays. The pipet can be scanned over living cells[17] or nanostructures[18] using ion conductance distance control allowing these measurements to be performed with high spatial precision. In particular, alkaline phosphatase is commonly used as tag on secondary antibodies so this method could be applied to sensitively detect and locate these tagged antibodies by controlled voltage driven delivery of substrate locally from the nanopipet. This would avoid the photobleaching, which limits the observation times when the proteins are fluorophore tagged. Therefore, our method would allow prolonged measurements. This could be potentially further extended to fusion proteins of a target protein with alkaline phosphatase for protein mapping on the surface of living cells, providing a new contrast mechanism with high molecular specificity.[19]

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Supporting Information Available: Experimental details and figure of Michaelis-Menten kinetics (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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