Enzyme Detection

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Detection of Single Enzyme Molecules inside Nanopores on the Basis of Chemiluminescence**

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Thus far, single-molecule detection has been implemented successfully by using fluorescence detection and in rare cases electrochemical cycling.[1–10] Amplification is possible in fluorescence excitation because the excited states of single molecules can be cycled up to a million times. In contrast, enzymes provide the benefit of chemical amplification, that is, the cycling of millions of substrate molecules to product molecules that are detected and quantified.[11] The product of certain enzyme reactions is light, which can be detected by using a range of sensitive optical devices. For example, a detection limit of 10^{-22}–10^{-19} mol of luciferase can be reached under special conditions.[12,13] These results show that chemiluminescence or bioluminescence detection can approach the single-molecule limit in sensitivity. Until now, individual enzyme molecules could not be monitored by chemiluminescence because they were in solution and able to diffuse freely. As a result, the light produced could not be confined to a small region. However, we believed it may be possible to detect chemiluminescence from a single enzyme molecule trapped in a nanopore. Such an approach could be important for studying gene expression or enzyme dynamics at the single-molecule level, and could maximize the detection power of the enzyme-linked immunosorbent assay. This study examined the single-molecule chemiluminescence detection of firefly luciferase randomly trapped in 50 nm nanopores.

Firefly luciferase is one of the most commonly used reporter genes, and the bioluminescent reaction catalyzed by luciferase is one of the most sensitive analytical tools for measuring gene expression.[14–16] The enzyme can detect trace amounts of adenosine triphosphate (ATP) by catalyzing ATP-dependent D-luciferin oxidation by oxygen to oxyluciferin, with the emission of light centered at 560 nm.[17–19] Nanopores were conveniently provided by a membrane filter made from a thin (ca. 6 μm) nanoporous polycarbonate film material (see Figure S1 in the Supporting Information) with precisely controlled cylindrical pores and a smooth flat surface (< 0.1 μm).[20] To trap the individual luciferase molecules into the nanopore chambers on the basis of an electrostatic interaction, we first bound the membrane to a quartz coverslip coated with poly-L-lysine (PLL). In this experiment, the spatial confinement of light will occur provided emission occurs inside the nanopores that contain the localized enzyme molecules. On the other hand, substrate molecules and ground-state product molecules (oxyluciferin and adenosine 5′-monophosphate) can diffuse freely in and out of the nanopores to minimize saturation of the reaction. This phenomenon is quite different from fluorescence detection, for which the product molecules need to be confined to enhance the efficiency of light collection.

Monitoring of the luciferase-derived chemiluminescence reaction was investigated by first loading luciferase (1 mg mL^{-1}, 2 μL) into the 50 nm pores in the polycarbonate membrane filter (13 mm diameter, 6 μm thickness) on a PLL-coated coverslip. The hydrophilic surface of the polycarbonate membrane filter helps it to stick firmly to the coverslip. Subsequently, the reagent solution (D-luciferin (1 mM) and ATP (1 mM) in 1X Hanks balanced salt solution (HBSS) was added to the membrane filter. Excess solution above and below the membrane was squeezed out. Bright-field optical and chemiluminescence images of the membrane before the addition of the reagent solution are shown in Figure 1 A,B (setup shown in Figure S2 of the Supporting Information). The bright patches indicate light that passed through the 50 nm pores (Figure 1 A). Chemiluminescence generated from the firefly luciferase molecules inside the 50 nm membrane pores was recorded by using an intensified

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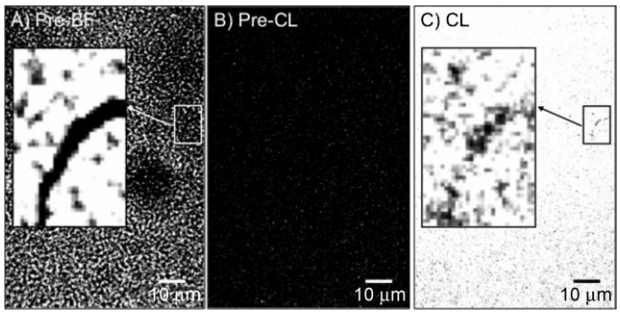

Figure 1. A) Bright-field and B) chemiluminescence images before the enzyme reaction. C) Chemiluminescence image of the enzyme reaction after the addition of the reagent solution. Exposure time of the bright-field image: 1 s. The boxed regions show magnified areas in (A) and (C) at the same position on the membrane.

charge-coupled device (ICCD) with 5 seconds exposure (Figure 1C). Because of the high pore density, individual pores were not resolved. The dark pixels within the region in the box in Figure 1A depict imperfections in the membrane. The fact that chemiluminescence was decreased in the same areas in Figure 1C confirms that the chemiluminescence emission came from the nanopores and not from the liquid layers above or below the membrane. There were also no major shifts in the positions of the nanopores. The efficient entry of individual luciferase molecules into the nanopore chambers was possible because these molecules are anionic at physiological pH values[16,21] and were readily trapped by the positively charged PLL-coated coverslip behind the nanopores. The chemiluminescence intensity detected at 22.4 °C by the ICCD camera was clearly different before (Figure 1B) and after (Figure 1C) the addition of the reagent solution.

We monitored the kinetics of the luciferase in the 50 nm pores on the coverslip by imaging the membrane pores (Figure 2). An ICCD image was taken every 5 seconds to record the chemiluminescence intensity per 1×2 pixel area at 13 different locations (corresponding to the different lines on the graph in Figure 2) on the same nanopore membrane. The average chemiluminescence intensity for the 13 locations is shown as a black solid line in Figure 2. Given the $100\times$ objective and the pore density, one pixel (i.e., 0.25 μm) is enough to define one pore. However, the pore may move slightly because the microscope may vibrate. Therefore, we carefully chose 1×2 pixel areas to allow for shifting of the nanopore positions and integrated over the pixels. The extra pixels were not problematic, since they should be "dark".

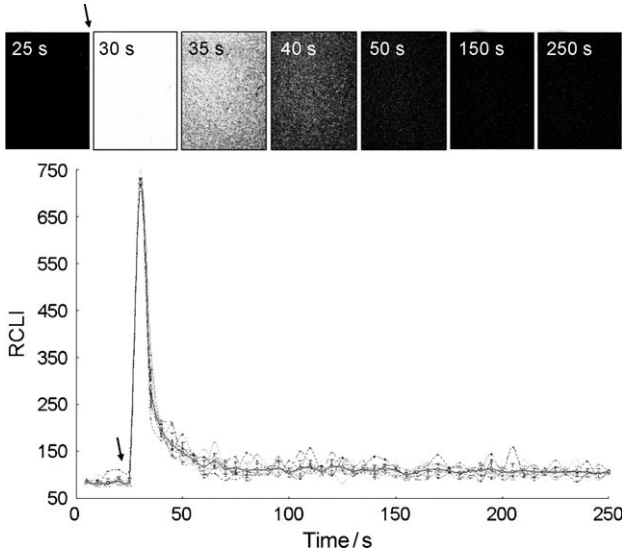

Figure 2. Top: representative chemiluminescence images of the luciferase enzyme reaction in 50 nm pores; bottom: typical time-course curves for the firefly luciferase enzyme reaction in 50 nm pores. The reagent solution (1 mM, 10 μL) was added to the membrane with 50 nm pores already filled with luciferase (1 mg mL^{-1}, 2 μL). Exposure time: 5 s. RCLI = relative chemiluminescence intensity.

Although the individual chemiluminescence intensities in the selected regions showed some fluctuations, all curves exhibited the same trend. As shown in the representative chemiluminescence images at each time point in Figure 2, there was a rapid increase in the chemiluminescence at 30 seconds after the addition of the reagent solution at $t = 25$ s (arrow). The intensity quickly decreased within 1–2 frames after the maximum intensity point. However, the intensity at the final frame (at 250 s) was still noticeably higher (20%) than the intensity at the initial point. These data showed a similar pattern to the enzyme-reaction time course previously observed in an O-ring chamber (i.d. 3/16 in.),[12] and were also consistent with the known kinetics of luciferase that result from inhibition of the enzyme by the reaction products oxyluciferin and dehydroluciferyl adenylate.[22–24] Thus, confinement in the nanopores did not affect the enzyme activity.

To maximize the signal, we measured the chemiluminescence intensities generated from the firefly luciferase molecules at 30 °C at concentrations ranging from 8 nM to 8 mM with a 5 minute exposure (Table 1 and Figure 3). The average intensities rose with an increase in the concentration of luciferase but quickly reached saturation. Above an 8 μM concentration of luciferase there was no dramatic increase in intensity (Figure 3A). However, a linear dependence is evident at the low concentration range (Figure 3B) that extended to 800 nM, corresponding to a maximum number of molecules per pore of six.

Table 1: Standard firefly luciferase calibration data (see Figure 3).

Concentration of luciferase [μM]	Theoretical number of molecules[a] (per pore)	CLI (mean ± SD)[b]
0.008	0.06	219 ± 46 (n = 13)
0.08	1 (0.57)	229 ± 30 (n = 11)
0.2	1.41	303 ± 51 (n = 13)
0.8	6 (5.65)	403 ± 51 (n = 11)
8	57 (56.5)	448 ± 33 (n = 15)
80	565	471 ± 55 (n = 11)
800	5650	490 ± 60 (n = 11)
8000	56500	555 ± 47 (n = 21)

[a] Predicted number of luciferase molecules within a height of 6 μm in a 50 nm nanopore area. [b] Average chemiluminescence intensity originating from the luciferase enzyme reaction inside each pore (1×2 pixels) minus the background signal; SD = standard deviation.

The number of enzyme molecules trapped in the nanopores was limited by the size (i.e., volume) of the nanopores. Therefore, we converted the concentrations into the expected number of luciferase molecules per 1.178×10^{-17} L volume ($3.14\times(25\text{ nm})^2\times6$ μm pores; Table 1, second column). The observed chemiluminescence intensity (CLI) in the third column of Table 1 is the averaged integrated intensity at each location from the chosen areas of 1×2 pixels minus the background signal originating from dark counts, stray light, and luminescence from the buffer solutions and from the coverslips in the nonpore areas. The size of one pixel in the image, corrected for the magnification of $100\times$, is 0.25×0.25 μm^2. The average number of 50 nm pores located in

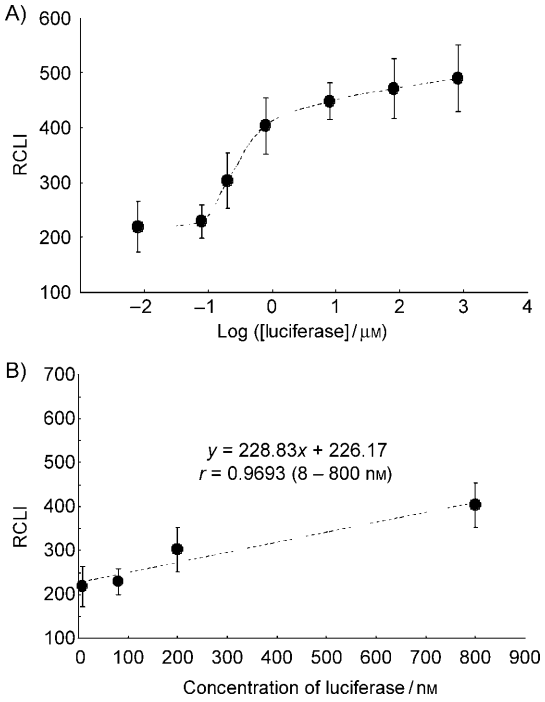

Figure 3. Dependence of the intensity per area of 1×2 pixel local regions on A) the logarithmic concentration of firefly luciferase in the range of 8 nM–8 mM and B) the (linear) concentration range of 8 nM–800 nM. Sample: solution of luciferase (2 μL) at various concentrations; exposure time: 5 min; temperature: 30 °C; other reaction conditions were the same as for Figure 2.

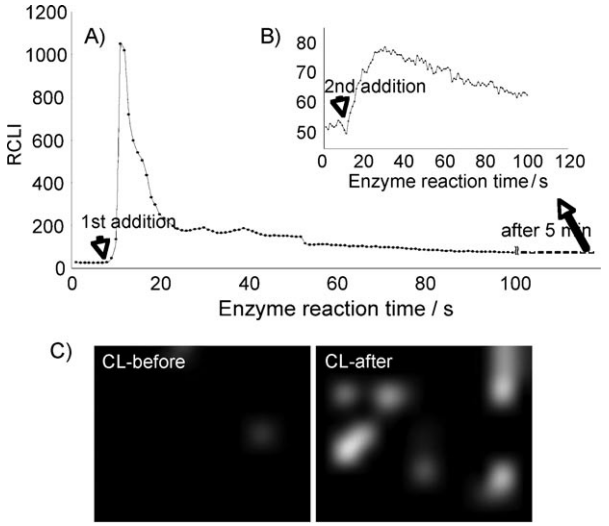

Figure 4. Monitoring of the recycling of luciferase. A) Time course for the first addition of reagents to the membrane already filled with luciferase (1 mg mL^{-1}). B) Time course for the second addition of reagents. C) Chemiluminescence images of selected 11×9 pixels immediately before (left) and after (right) the second addition. Exposure time: 1 s; other reaction conditions were the same as for Figure 2.

one pixel was approximately two according to the SEM image of the membrane (see Figure S1 in the Supporting Information). Since we used the corrected CLI at the corresponding locations (Figure 1B,C), there was no need to normalize the CLI.

As shown in Figure 3 and Table 1, single luciferase molecules in the 50 nm pores could be detected. The total number of luciferase molecules in the starting volume of 2 μL was not important because most of the liquid did not go into the nanopore chambers. The remaining molecules were either squeezed out or stayed on top of the pores and did not produce distinct spots because their emission was not confined. The second lowest luciferase concentration studied was 80 nM in the loading solution. Hence, the number of molecules per nanopore chamber (1.178×10^{-17} L) was approximately one. The experimental CLI count of each luciferase molecule was 229.2 ($n=11$). According to previous calculations,[12] the total intensity count from a single enzyme molecule should be approximately 217.7 on average. The experimental CLI thus corresponds to 105.3 ± 13.9 % of the theoretical CLI. The CLI for the bright regions at 8 nM was not very different from that at 80 nM (Table 1), which is consistent with the fact that the molecule number cannot be smaller than 1. At 0.2 μM, each pore should contain either one or two luciferase molecules, according to Poisson statistics. Thus, the average value should be about 1.5, which is almost identical to the experimental CLI of 1.41.

To confirm the long-term activity of luciferase, chemiluminescence was monitored after a second addition of the substrates to the membrane pores (Figure 4). Although the CLI intensity was not high, the second reaction was readily recognizable (Figure 4B). This result confirms that the catalytic species was not consumed during the reaction; therefore, the signal can be integrated over prolonged reaction times.[11] The reasons for the much lower CLI for the second reaction are: 1) inhibition by the reaction products,[22–24] 2) the depletion of substrate molecules inside the pores, and 3) the loss of trapped enzyme molecules during washing and manipulation, as the enzyme was not immobilized in the pores. The last effect is evident in Figure 4C, in which individual bright spots are visible, as opposed to the spatially featureless emission in Figure 1C. The drastically diminished molecule numbers led to a pore occupancy of much less than unity, so single molecules were isolated. The fractional decrease in CLI is much slower in Figure 4B than in Figure 4A, which is consistent with a slower consumption of the substrates by the decreased number of enzymes.

By accumulation over a prolonged enzyme reaction time to maximize the signal, we detected single luciferase molecules. If our results are compared with the BioTek report on the detection of as few as 60 protein molecules,[13] we observed a 60-fold increase in sensitivity. Our approach is different from single-enzyme fluorescence studies in that product confinement to increase the light-collection efficiency is not needed. Instead, the emission is spatially confined as a result of trapping (but not immobilization) of the enzyme molecules, whereas the substrates and products can still diffuse freely. In principle, the spatial resolution is thus improved. Although the signal is weaker in this method, there is no photobleaching. Our approach is therefore complementary to fluorescence detection and extends the

realm of single-enzyme studies and assays to the plethora of chemiluminescence reactions in biological chemistry.

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