Single-biomolecule observation with micro one-way valves for rapid buffer exchange

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This paper describes a method for the rapid exchange of buffer solution during single-molecule observation. We use a simple, transparent, all-plastic one-way valve integrated on a coverslip on a flow cell. The valve is formed using a membrane made of parylene covering a microhole. It opens when a buffer solution is introduced from the microhole (the flow pushes the cover membrane) and closes when suctioning the buffer solution (the membrane is pulled back and seals the microhole to prevent the diffusion of the solution). To check valve performance, we observed the response of a rotary biomotor, F_1-ATPase, for several buffer solutions in the fabricated chamber. © *2009 American Institute of Physics*. [DOI: 10.1063/1.3116102]

I. INTRODUCTION

The direct observation of single molecules, particularly biomolecular motors,[1,2] under several buffer conditions is important for the elucidation of molecular dynamics.[3,4] However, it is difficult to exchange buffer rapidly by conventional, manual buffer exchange methods owing to the drift of the observation stage and/or the diffusion of residual solution in the chamber. Here, we propose a method using Micro Electro Mechanical System (MEMS)-based one-way inlet and outlet microvalves placed at the tip of a tube connected to the coverslip of the chamber.

One-way valves are passive devices that open when applying forward flow and close when applying reverse flow. They are useful in microfluidic systems for chemical and biological applications. The one-way valve has been developed to be an ideal valve, having low forward pressure and small reverse leakage.[5,6]

Many MEMS one-way valves have been fabricated from various deformable materials including silicon, polymers, and metals.[7–11] Parylene is a particularly attractive material for the structure of such valves because of its transparency, deformability, and high biocompatibility. Thus several groups have used this polymer with silicon as the substrate.[11–15]

Here, we fabricate a parylene-based thin film valve on a transparent poly(methylmethacrylate) (PMMA) substrate; such a configuration is very compatible for conventional biochemical assay that uses a flow chamber with a glass slide. We also construct a chamber in which one-way valves are integrated near the observation field and connect the syringe pump to the valves through tubes. Using these valves, it is possible to define the exact starting point of chemical and physical reactions at the single-molecule level and to investigate the effect of several buffers on single molecules. Here, we observe a rotary biomotor, F_1-ATPase,[1] in the chamber and perform the following experiments. First, we detect the response of F_1-ATPase immediately after exposure to the inhibitory reagent NaN_3 and calculate the inactivation rate of the enzyme. Second, we exchange the reducing and oxidizing reagents alternately through the valves and observe the response of the rotor-stator cross-linked mutant of F_1-ATPase.

II. MATERIALS AND METHODS

A. Design of buffer exchange chamber

Schematic illustrations of the chamber are shown in Fig. 1. Parylene valves are placed inside the chamber at the inlet and outside the chamber at the outlet. The valves are integrated on the opposite surface of the PMMA substrate, which is used instead of a coverglass.

The valve can be moved by injecting a buffer. After buffer exchange, the valve becomes flat as a result of suction with reverse flow. The valve is set near the observation field so that the objective molecule can be exposed rapidly to the buffer injected.

B. Fabrication of microparylene caps

The fabrication process is shown in Fig. 2. First, 100-μm-diameter microholes were made on the PMMA substrate (5 mm thick/Mitsubishi Rayon) using a cutting machine (MM-100/Modia Systems, Japan). These holes serve as the fluid inlet/outlet for the valves. The substrate was preheated at 50 °C after rinsing it in ethanol. A dry photoresist (DRF SUNFORT [SPG-102]/Asahikasei) was deposited on each side of the substrate at 100 °C for 30 s. A thin sacrificial dry photoresist was patterned to cover the holes where the parylene valve was directly deposited. After developing the dry photoresist for 15 s in 1% Na_2CO_3, the PMMA substrate was washed with de-ionized water. Next, a 5-μm-thick parylene-N layer and an aluminum layer were deposited on both sides of the substrate. An S1818 photoresist layer was spun and patterned to serve as the etching mask of the parylene valve; the aluminum layer was patterned first, and then the parylene layer was etched using O_2 plasma. Finally,

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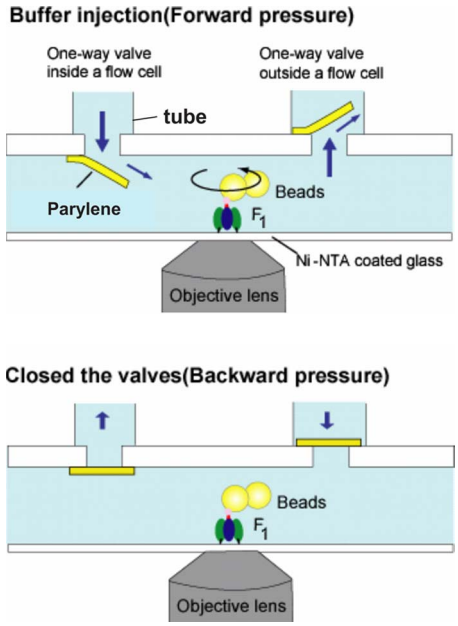

FIG. 1. (Color online) Schematic views of the buffer exchange chamber for single biomotor observation. Inlet and outlet micro-one-way valves are placed on the tip of a tube connected to the coverslip of the chamber. While the valves are opened with forward pressure, the exchange buffer is injected into the chamber (upper illustration). After replacing the buffer completely, the valves are closed with backward pressure (lower illustration).

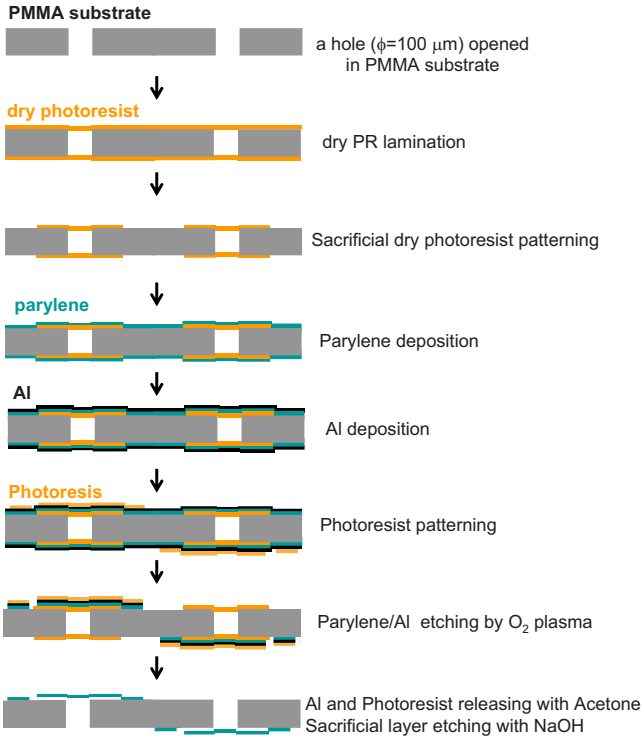

FIG. 2. (Color online) Fabrication process.

the aluminum and photoresist layers were chemically removed, and the sacrificial dry photoresist layer was released by immersing the substrate in 3% NaOH (at 50 °C) for more than 3 h.

C. Rotation assay of F_1-ATPase

F_1-ATPase from the thermophilic *Bacillus* strain PS3 was biotinylated at two cysteine residues on its γ-subunit (S107C/I210C) and His_{10} tagged on its β-subunits.[16] In this paper, we call this mutant F_1-ATPase for simplicity. For inhibition by NaN_3, this F_1-ATPase was used. For stator-rotor cross-link experiment, F_1-ATPase in which a cysteine residue was introduced at γR84 and βE391 was used.[17]

To visualize the rotation of the γ-subunit under a microscope, streptavidin-coated beads (440 nm in diameter) were attached to the γ-subunit. The flow chamber was composed of a Ni^{2+}-nitrilotriacetic acid (NTA)-coated coverglass (32 $\times$24 mm^2) and the valve-embedded PMMA substrate (10 $\times$15 mm^2) separated by two spacers 50 μm in thickness. First, we infused one-chamber volume ($\sim$3 μl) of 0.1 nM F_1 in buffer A (10 mM morpholinepropanesulfonic acid (MOPS)-KOH, *p*H 7.0/50 mM KCl/10 mg/ml BSA), waited for 2 min, and washed away unbound F_1-ATPase with 10 μl of buffer A. Then, we infused 6 μl of streptavidin-coated beads (0.56 μm, Bangs Laboratories, Carmel, IN), waited for 10 min, and washed away unbound beads with buffer A. Finally, we infused 6 μl of adenosine triphosphate (ATP) buffer (buffer A with 2 mM $MgCl_2$, 2 mM ATP, 1.25 mM creatine phosphate, and 0.1 mg/ml creatine kinase). We used the micro-one-way valves throughout the operation. For inhibition by NaN_3, the tube connected to the inlet of the valve was filled with the inhibition buffer (ATP buffer with 1 mM NaN_3) before the injection of F_1-ATPase and beads into the flow chamber. For the stator-rotor cross-link, the tube was filled with the reducing buffer [ATP buffer with 1 mM dithiothreitol (DTT)], and another tube was filled with the oxidizing buffer [ATP buffer with 250 μM 3-carboxy-4-nitrophenyl disulfide (DTNB)]. The infusing buffer volume was 6 μl and the flow rate of the syringe pump was 1 μl/s so that buffer exchange was completed within 6 s.

III. RESULTS AND DISCUSSION

A. Performance of micro-one-way valve

The experimental setup is shown in Fig. 3(a). The inlet and outlet valves are connected to syringe pumps through the tubes. A Scanning Electron Microscope (SEM) image of the fabricated one-way valve is shown in Fig. 3(b). The image shows that the central region of the valve has dents along the microhole edge; we think that this configuration might improve the sealing performance because the diameter of the dent is almost the same as that of the hole.

The valve opens very smoothly when applying forward pressure and becomes flat when applying backward pressure [Fig. 3(c)]. During the open-and-close motion, the hinge of the valves did not break with the water flow and remained fixed to the PMMA substrate. The performance of the one-way valves was measured with a pressure sensor (AP-12S, Keyence) and a precise mass flowmeter (ASL1430, Sensirion), as shown in Fig. 4. A microhole without a valve was also tested as a negative control. As a result, in the forward flow direction, the initial release pressure of the valve is very low (less than 0.2 kPa); this pressure might be needed for the initial movement of the valve detaching from the PMMA

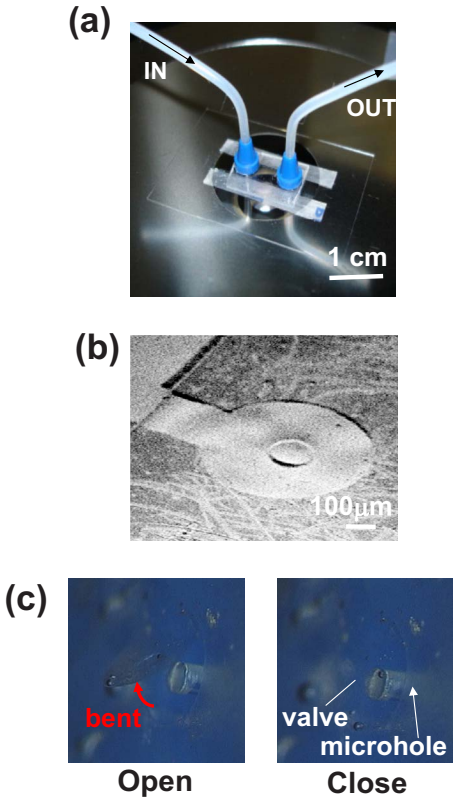

FIG. 3. (Color online) (a) The buffer exchange chamber on an observation stage of the microscope. (b) SEM image of the valve. (c) Photos of the parylene micro-one-way valve. The valve is moved upside when opened (left) and becomes flat when closed (right).

substrate. We observed no plastic deformation on the valve up to 6 kPa of pressure. In the reverse flow direction, we found no observable leakage up to −8 kPa.

B. Observation of F_1-ATPase in the fabricated chamber

To check valve performance, we observed F_1-ATPase, in which the γ-subunit rotates in the $\alpha_3\beta_3$ ring during ATP hydrolysis, in the chamber.[18] F_1-ATPase is also part of the ATP synthase binding with its partner motor, F_0, in cells.[1] There are many inactivation manners for this enzyme.[19–22]

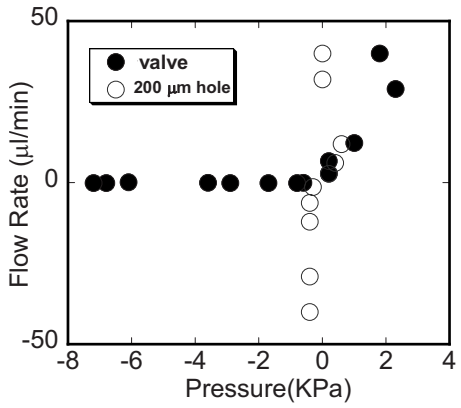

FIG. 4. Flow characteristics with (blue circles) and without (white circles) the parylene valve.

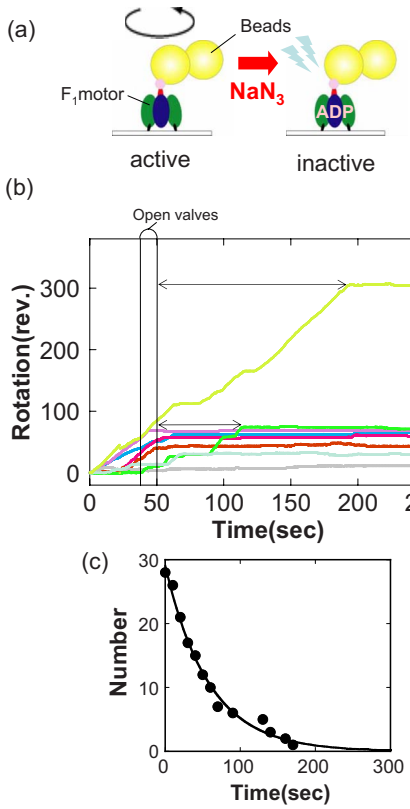

FIG. 5. (Color online) NaN_3 inhibition of F_1-ATPase. (a) Scheme of NaN_3 inhibition of F_1-ATPase. NaN_3 is considered to stabilize ADP inhibition state, in which the F_1-ATPase fails to dissociate ADP from the catalytic site (Ref. 8). (b) Time course of NaN_3 inhibition in the fabricated chamber. Here, we observed F_1-ATPase at 2 mM ATP. After 45 s, valves are opened and NaN_3 containing buffers are introduced into the chamber. We close the valves at 51 s and continue recording. Arrows indicate duration of the actively rotating F_1-ATPases after closing the valves. (c) Duration of the active F_1-ATPase in NaN_3 containing ATP buffer. Solid line shows a single-exponential function with k $(=1/\tau)$ (rate of conversion from the rotation to the pause) $=0.017$ s^{-1}.

There is as yet no report on the visualization of the response of F_1-ATPase immediately after injecting inhibitory materials. NaN_3 is the most commonly used inhibitor for the crystallization of F_1-ATPase and is known to inhibit ATPase activity by stabilizing adenosine diphosphate (ADP) inhibition [Fig. 5(a)].[22,23] However, how NaN_3 affects rotation remains unclear. Thus, we monitored the behavior of F_1-ATPase after treatment with NaN_3 in the fabricated chamber. Actively rotating F_1-ATPase molecules were observed in the fabricated chamber at 2 mM ATP [we monitored eight molecules in Fig. 5(b)]. We introduced the inhibition buffer containing NaN_3 through the micro-one-way valve [at 45 s in Fig. 5(b)], and then closed the valves [at 51 s in Fig. 5(b)] and continued recording. F_1-ATPase occasionally falls into pause caused by ADP inhibition during ATP hydrolysis.[16] F_1-ATPase trapped in this inhibition state can be easily recovered, and the rotation is restarted at high ATP concentrations. Therefore, the brief pauses observed in the inhibition and ATP buffers may be attributed to the ADP inhibition as is, not to NaN_3-induced inhibition. The rotation of F_1-ATPase was almost completely prevented several tens of seconds after the valves were closed. The rotation rate was not distinguishable from that observed in 2 mM ATP after the

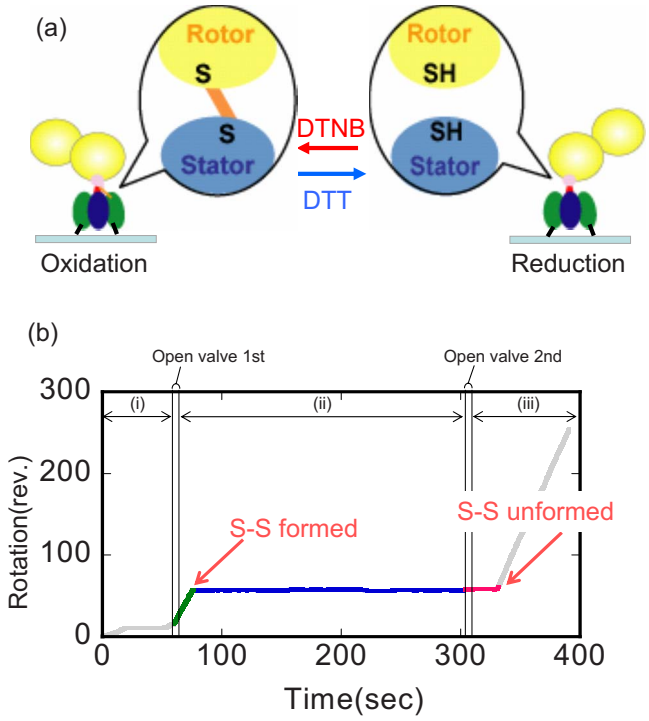

FIG. 6. (Color online) Stator-rotor cross-linking experiment under fabricated chamber. (a) Scheme of disulfide bond formation between rotor (γR84C) and stator (βE391C). The disulfide bond is reduced by DTT and cross-linked by oxidation with DTNB. (b) Time course of rotation of mutant F_1-ATPase. F_1-ATPase rotates at 200 nM ATP under DTT-containing buffer [(i) phase] and then DTNB-containing buffer is introduced into the chamber (open valve first time). Rotation was paused after cross-linking by disulfide bond formation between the rotor and the stator [(ii) phase] and then DTT-containing buffer was introduced (open valve second time).

valves were closed. However, once F_1-ATPase lapsed into a long pause, it did not restart rotation during our observation (>10 min). NaN_3 inhibition was assumed to correspond to the long pause, so we analyzed the duration of rotation of each molecule before the long pause from the time the valves were closed [arrows in Fig. 5(b)]. The histogram was plotted as a time-dependent decay and the data are well fitted with a single exponential of 0.017 s^{-1} (=rate of inhibition) [Fig. 5(c)]. This lifetime coincides well with the NaN_3 inhibition rate estimated from the bulk-phase measurement (0.019 s^{-1}). NaN_3 stabilized the ADP inhibition of F_1-ATPase within 53 s, on average, after the infusion. By using the buffer exchange chamber, it was possible to directly analyze the duration from the starting point of reaction for single molecules.

In another buffer exchange experiment, we changed the oxidizing and reducing buffers alternately through the valves and observed the response of mutant F_1-ATPase in which cysteine residues were introduced into the rotor and stator. These residues formed a disulfide bond that cross-linked the rotor and stator under oxidizing condition at a high efficiency (~90%) in bulk measurement.[17] The disulfide bond in this region is reduced by a DTT-containing buffer (reduction) and reformed by oxidation with a DTNB-containing buffer (oxidation) [Fig. 6(a)]. If the fabricated valves work properly, it is expected that F_1-ATPase will switch its state, rotating actively in the reducing buffer and stopping its rotation in the oxidizing buffer.

First, the rotation of F_1-ATPase was observed under reducing condition [Fig. 6(b), (i) phase]. Then, the oxidizing buffer containing DTNB was injected into the chamber by opening the valves [Fig. 6(b); open valve first phase]. After the valve was closed, F_1-ATPase stopped its rotation by forming a disulfide bond between the stator and the rotor within several seconds [Fig. 6(b), (ii) phase]. When the buffer was exchanged with the reducing buffer [Fig. 6(b); open valve second phase], the cross-linked F_1-ATPase resumed its rotation [Fig. 6(b), (iii) phase].

There was no drift of the chamber or damage to the molecules during the buffer exchange. This valve will be useful for various experiments, such as the determination of the effects of several buffers on single molecules.

IV. CONCLUSION

In this paper, we fabricated a buffer exchange chamber for observing a biomotor using a simple parylene-based one-way valve. The fabrication process of the one-way valve is technically simple, and the test results show that this valve has a low reverse flow leakage and a low membrane-induced flow resistance. Using the buffer exchange chamber, we analyzed the inhibition of F_1-ATPase by NaN_3 reagent and found that NaN_3 inhibition is characterized by the state in which rotation stops for a long time in an irreversible manner. In another experiment, reducing and oxidizing buffers are automatically exchanged through the valve for cross-linked mutant F_1-ATPase. Since we injected a buffer through the one-way valve using a syringe pump, it was possible to observe the same molecules continuously. We think that our method will be useful for reducing buffer injection time, which will be helpful in analyzing the nature of proteins more precisely.

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