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Continuous Monitoring of Ammonia Removal Activity and Observation of Morphology of Microbial Complexes in a Microdevice▽

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Continuous monitoring of ammonia removal by microbial complexes and observation of their morphology were carried out using a microdevice. Consumption of NH_4^+ ions by active sludge could clearly be recorded over 48 h. Aggregation of the sludge could be observed in parallel by using confocal reflection microscopy.

Bacterial ammonia removal plays a critical role for material conversion, such as wastewater treatment and fermentation (5, 20). Active sludge used for the wastewater treatment removes ammonia mainly by nitrification, oxidizing ammonia (more properly, NH_4^+ ions) into nitrate (12). As with many other cases relevant to the monitoring of the metabolic activity of bacteria, high-throughput screening has been recently highlighted as one of the critical themes. For example, there may be a situation in which the effect of compounds including rare or expensive molecules is examined (18, 19). For high-throughput processing with samples and reagents of small volumes, the use of a microtiter plate is a solution. However, the analysis using this technique provides only fragmented data obtained by a series of one-shot measurements. On the other hand, changes in the concentration of reactants and products in a medium have been measured using mainly electrochemical probes installed in a fermentor. However, with the centimeter-scale instruments, it is unrealistic to examine the influence of compounds that are extremely expensive or difficult to obtain. As a first step toward this goal, we demonstrate the continuous monitoring of ammonia removal activity of active sludge based on a new approach.

Microfabrication techniques conventionally used for semiconductor chips have begun to be used for various other purposes, including biochemical analysis (2), chemical synthesis (13), and culture of animal and microbial cells (11, 14, 22, 23, 24), revolutionizing the methodologies in related fields. Promising advantages brought by miniaturization include reduction of sample and reagent volumes, high-throughput parallel processing, and reduction of production costs by batch fabrication, which usually accompanies miniaturization. On the other hand, electrochemical sensing in a microscopic volume has made drastic progress over the last 2 decades, and there exists a wealth of technical resources (17). Even for continuous monitoring of the status of microbes, detection based on the measurement of changes of impedance has been carried out (3, 8, 9). However, from the viewpoint of chemical sensing, the impedimetry provides only rough estimates that may be influenced by various factors. In situations in which an ion or a compound works as a good indicator, direct sensing of the target analyte is indeed preferable. To this end, we used an integrated ion-selective electrode to monitor changes in NH_4^+ concentration accompanying the ammonia removal. With an ion-selective electrode, excellent selectivity to a specific ion is achieved through the use of an ionophore that makes a complex with a target ion. The response of a cation-selective electrode to a target ion is usually larger than that to other cations by two or more orders of magnitude (1, 4, 15).

Our device was constructed by stacking substrates of glass, polydimethylsiloxane (PDMS), and polymethylmethacrylate (PMMA) (Fig. 1A). In the device, oxygen is supplied through the PDMS substrates. The oxygen concentration and diffusion coefficient in PDMS are 10 times and 1.5 times greater than those in culture medium, respectively (7). Compartments for microbial culture and an NH_4^+ ion-selective electrode were formed by replica molding (6). Electrode patterns were formed by thin-film microfabrication techniques. An NH_4^+ ion-selective membrane was formed in a through-hole (diameter, 1 mm) formed in the PMMA substrate. The membrane consisted of nonactin (3.5 weight percent [wt.%]; Fluka), potassium tetrakis (*p*-chlorophenyl)borate (0.35 wt.%; Fluka), polyvinyl chloride (32.30 wt.%; Fluka), dibutyl sebacate (32.95 wt.%; Wako), and tetrahydrofuran (62.30 wt.%; Fluka) (10). A 10 mM NH_4Cl solution was used as the electrolyte solution for the NH_4^+ ion-selective electrodes. Ag/AgCl electrodes were used as the internal and external electrodes for the NH_4^+ ion-selective electrode. An issue in conducting continuous monitoring has been the instability of the Ag/AgCl electrodes caused by the dissolution of AgCl. To solve the problem, AgCl was grown from three square pinholes (10 μm by 10 μm) formed in a polyimide protection layer into the silver layer (21). In addition, AgCl was grown continuously by applying a small constant current (10 nA) to the electrode by using a surrounding gold electrode (Fig. 1B) (21). The Ag/AgCl electrodes used in the device showed a 20-fold increase in lifespan compared with that of conventional structures, enabling reliable continuous

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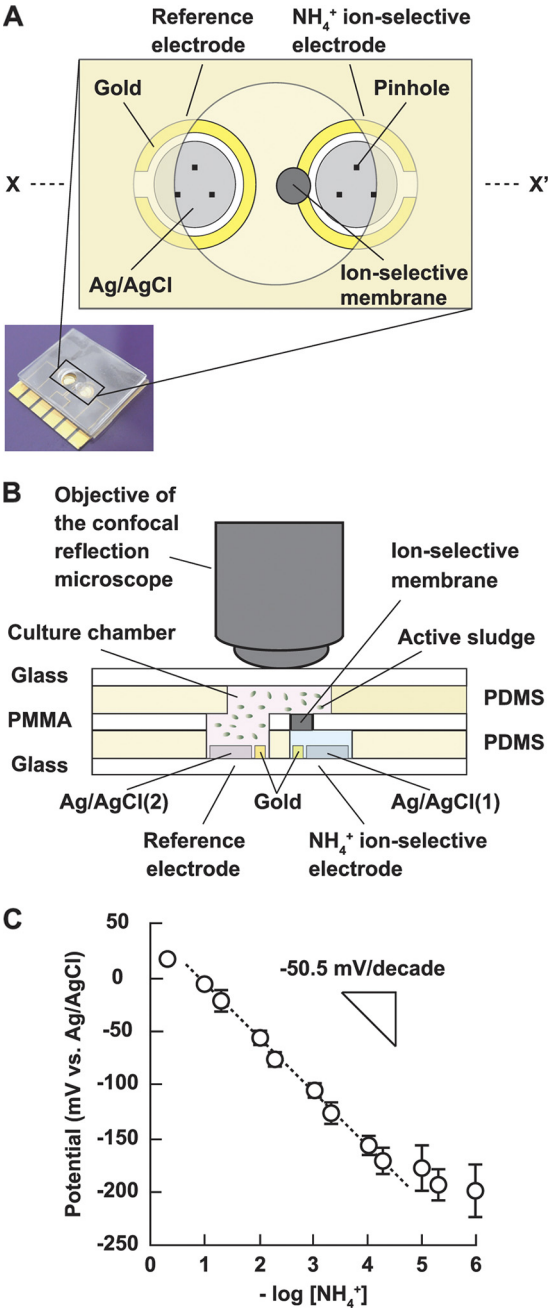

FIG. 1. Microdevice for continuous monitoring of microbial activity. (A) Top view of the device. The dimensions of the chip are 18 mm by 18 mm. (B) Cross-section along the line X-X' in panel A. (C) Dependence of the potential of the ion-selective electrode on NH_4^+ ion concentration. Potential of Ag/AgCl(1) with respect to Ag/AgCl(2) was measured. Five measurements were made, and averages and standard deviations are shown. Many of the error bars are behind the symbols.

monitoring over the period. The dependence of the potential of the ion-selective electrode on the concentration of the target ions is expressed with the Nernst equation. Actually, the calibration plot for the NH_4^+ ion-selective electrode was linear in a range of concentration between 5.0×10^{-5} M and 1.0×10^{-1} M, with a slope of −50.5 mV/decade (Fig. 1C). In addition to the excellent selectivity of the NH_4^+ ion-selective electrode, the concentration of NH_4^+ (50 mM) in our culture medium was 27 and 3.6 times higher than those of K^+ (1.81 mM) and Na^+ (13.7 mM) ions, respectively. Therefore, the interference by other ions is negligible.

The active sludge was sampled from a wastewater treatment plant (Yokosuka, Japan). The samples were precultured at 30°C for 3 days with shaking at 170 rpm. The sludge was centrifuged (3,000 × *g*, 5 min) and washed four times with distilled water. The sludge (0.1 g) was suspended in 700 μl artificial wastewater [6 mM $NaHCO_3$, 9 mM $(NH_4)_2SO_4$, 1.9 mM KH_2PO_4, 4.2 mM Na_2HPO_4, 0.4 mM CH_4N_2O, 17 μM $FeSO_4 \cdot 7H_2O$, 41 μM $MgSO_4 \cdot 7H_2O$, 1.5 μM $CaCl_2 \cdot 2H_2O$, 0.3 μM $ZnCl_2$, 0.2 μM $CuCl_2 \cdot 2H_2O$, 0.2 μM $MnCl_2 \cdot 4H_2O$, 0.4 μM $AlCl_3$, 0.1 μM $NiCl_2$]. NH_4Cl (50 mM) was added. Seventeen microliters of the solution was added in the reservoir of the device. The device was placed in an incubator whose temperature was maintained at 30°C. The morphology of the sludge was observed every 12 h using a Carl Zeiss PASCAL laser scanning microscope. For the confocal reflection microscopy (16), cells were illuminated by a 514-nm argon laser, and reflected light was collected through a 505- to 530-nm bandpass filter. The images were analyzed using Carl Zeiss LSM 5 PASCAL software.

The ammonia removal activity of the sludge was monitored continuously using the NH_4^+ ion-selective electrode on the chip (Fig. 2A). With the medium seeded with the sludge, the NH_4^+ ion concentration decreased gradually, accompanying the growth of the microbes. The monitored changes were reproducible among different trials. Approximately 80% of the initially added NH_4^+ ions had been consumed by 48 h. On the other hand, no change was observed without the sludge. In addition, we compared the values obtained using the ion-selective electrode and a control method. One hundred millimolars of NH_4Cl was added to the culture medium with microbial complexes. The changes in the concentration were measured using the ion-selective electrode ($n = 2$ experiments) and a commercialized assay kit based on absorbance measurement ($n = 3$). The assay kit required at least 10 μl for a measurement. Therefore, we terminated the culture and extracted the entire sample from the device at each measurement time. In contrast, the concentration was monitored continuously using the ion-selective electrode. The concentrations at 0, 24, and 48 h of culture were 102, 42, and 28 mM with the ion-selective sensor and 98, 55, 28 mM with the assay kit.

The confocal microscopy images show that the sludge remained aggregated during the incubation period (Fig. 2A). To examine this point in more detail, live and dead cells were also visualized using a *Bac*Light LIVE/DEAD bacterial viability staining kit (Molecular Probes, Eugene, OR) after 48 h of culture. Here, membrane-permeant SYTO 9 labeled the nucleoid of live cells (green), whereas propidium iodide (PI) blocked by live cell membranes labeled the nucleoid of dead cells through their damaged membranes (red). SYTO 9 and PI were excited using an argon laser and a helium neon laser, respectively, and the fluorescence was detected through >560-nm and >505-nm long-pass filters, respectively. Mean signal intensities of PI and SYTO 9 in each optical slice were calculated as before. The result suggested that the sludge contained a large proportion of dead cells at both 0 and 48 h in the

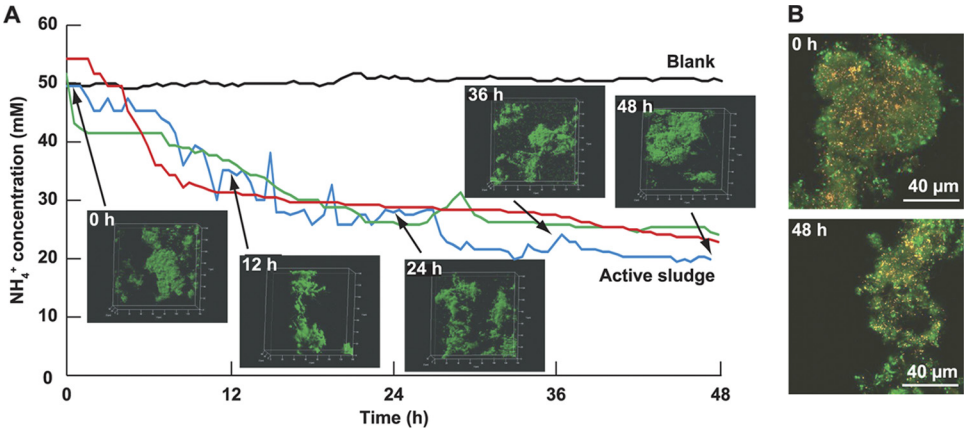

FIG. 2. Continuous monitoring of the ammonia removal activity and observation of the morphology of the active sludge. (A) Changes in the NH_4^+ ion concentration in the medium with or without the active sludge. The response curves in the medium with the active sludge were obtained by three independent experiments. The insets are representative images obtained using confocal reflection microscopy at the indicated times. Each projection shows fields of 140 by 140 by 30 μm^3 (depth × width × height). The thickness of each image stack was 0.8 μm. (B) LIVE/DEAD fluorescent staining at 0 and 48 h of culture. Scale bars indicate 40 μm.

device (Fig. 2B), which is reasonable considering the communication that influences the proliferation and death of the neighboring microbes. The ratio of the mean signal intensities (SYTO 9/PI) in an optical slice showed a slight increase during culture (1.24 ± 0.11 at 0 h, 1.70 ± 0.26 at 48 h [$n = 3$]), suggesting that the device can provide a suitable environment for maintaining the activity of the microbes.

In this study, ammonia removal activity was monitored with the medium at a volume of only 17 μl. This small volume will open up a new way of examining the influence of compounds that are expensive or difficult to obtain, such as signal molecules that play a crucial role in bacterial cell-cell communication (quorum sensing), on ammonia removal activity. Another advantage brought with the microfabrication technology is the capability to integrate the same electrode structure on a single chip. With the multiple electrodes and small volumes, more challenging applications will be considered, including the screening of signal molecules. The application of this device is not limited to the analysis of the NH_4^+ ions. The ion-selective membrane can be replaced with other ones for various other ions. We believe that this study will be an important step to establish an efficient high-throughput technique.

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