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Facile Preparation Method of a Disposable Capillary Biosensor Using an Ion-selective Optode Membrane and a Dissolvable Enzyme Membrane and Its Application to Urea Sensing

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A facile preparation method of a disposable capillary biosensor immobilizing a plasticized poly(vinyl chloride) (PVC) membrane ion-selective optode containing a lipophilic pH indicator dye and an ionophore, and a dissolvable poly-(ethylene glycol) (PEG) membrane containing an enzyme is developed. The method involves the sequential introduction of membrane cocktail solution followed by air introduction. Repeating this process allowed facile preparation of a capillary biosensor comprised of two layer structure, which can be used for single-step biosensing just by sipping the sample with capillary action. Here a urea biosensor was prepared as a proof-of-concept.

Microfluidics, an almost two-decade old technology, has recently steered debate in the analytical science community on its future potential.[1] However, it is generally agreed that microfluidics is an enabling technology that may function as an integral part of a certain total analytical system. Therefore, in order to develop a practical microanalytical system, developments of both integration method and integral parts are indispensable subjects.

On the other hand, the combination of membrane science and microfluidics has yielded various miniaturized integral parts like micropumps, microfilters, microdialysis, microsensors, and microreactors.[2] Among them, ion-selective optode membranes have also been applied in a microfluidic format.[3] This allows a simple, selective, and direct measurement of ions present in a miniscule volume of sample solution. Ion-selective optode membranes are well-established in analytical devices.[4–6] The basic principle is based on the ion-pair extraction equilibrium between an organic membrane phase containing lipophilic pH indicator dye together with an ionophore and an aqueous phase containing the primary ion.[7–10] However, some clinically relevant analytes require indirect measurements in which an enzyme may initially be used to react with a certain organic analyte yielding an ion followed by optode membrane detection. Macroscale systems have already demonstrated this concept by covalent immobilization or photocrosslinking of enzyme on the surface of an optode membrane.[11,12] These immobilization methods allow close proximity of the enzyme to the sensing membrane that may result in shorter analysis time. Nonetheless, covalent enzyme immobilization requires meticulous steps with long reaction time and may also affect the native structure of the protein. This may lessen the activity of the enzyme. It is, therefore, necessary to develop a simple and straightforward method which can preserve the biological integrity of the enzyme while having a local optode membrane inside the microchannel.

Previously, our group has proposed capillary assembled microchip (CAs-CHIP) as a general integration method of various chemical functions on a single microfluidic device by simply embedding functionalized square glass capillaries into poly(dimethylsiloxane) (PDMS) microchannels which are the same size as the outer dimensions of square capillaries.[13] Recently, we also presented a simple fluid delivery method allowing the distribution of one microliter sample solution into eight different sensor capillaries embedded in a CAs-CHIP.[14] In this case, one microliter of sample solution flows as a long sample plug in the main PDMS microchannel of a CAs-CHIP embedding eight different sensor capillaries. When the solution comes in contact with the edge of each sensing glass square capillaries, it spontaneously and simultaneously is distributed into each independent sensor capillary by capillary action. Then multiple and simultaneous detection of eight different chemical species including enzyme activities and electrolytes was achieved by direct reactions with the analytes and the reagents immobilized inside the capillaries.

In order to expand this method for multi-analyte sensing including clinically relevant organic analytes, development of a capillary biosensor enabling single-step analysis, which involves an enzyme reaction and subsequent selective detection of enzymatic reaction product, is necessary.

Here, we proposed a simple preparation method of a disposable capillary biosensor just by simply introducing membrane cocktail solution and air alternately to form two-layer structure of a hydrophobic ion-selective optode membrane and a hydrophilic and dissolvable enzyme release membrane inside a square glass capillary. In this case, sample introduction by capillary action allows spontaneous release of enzyme by dissolving the membrane then enzymatic reaction takes place in the bulk solution. The enzymatic reaction product can be extracted into ion-selective optode membrane exposed to the solution phase by dissolving the outer hydrophilic membrane, in which this extraction facilitates the release of protons of the lipophilic pH indicator dye in optode membrane phase. Therefore, enzymatic reaction and subsequent selective analysis of enzymatic reaction product can be possible (Figure 1). Furthermore, although the optode membrane prepared by plasticized PVC is fairly hydrophobic, the dissolvable enzyme release membrane is basically composed of a hydrophilic polymer, PEG, offering a hydrophilic inner capillary surface. This made the capillary biosensor compatible with the capillary action sample introduction described above. In addition, enzyme is entrapped into biocompatible PEG membrane so that the preservation of the biological integrity is expected. The ultimate intention of this research is to combine this capillary with the other developed capillary biosensors on a single microfluidic device, CAs-CHIP, to simultaneously detect different chemical species in a certain biological sample. This kind of technology is expected to be a promising tool in the field of

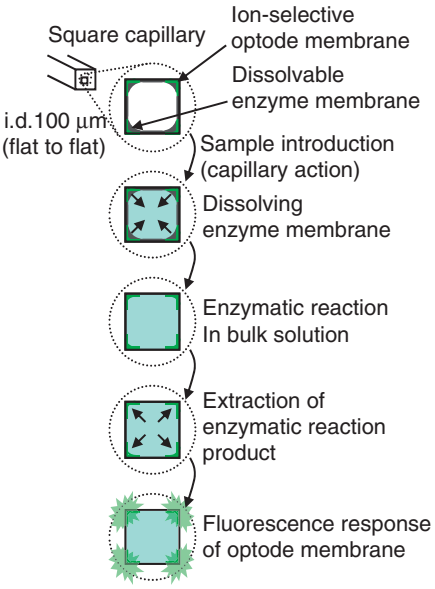

Figure 1. Single-step sensing scheme of capillary biosensor composed of two-layer structure using ion-selective optode membrane and dissolvable enzyme membrane.

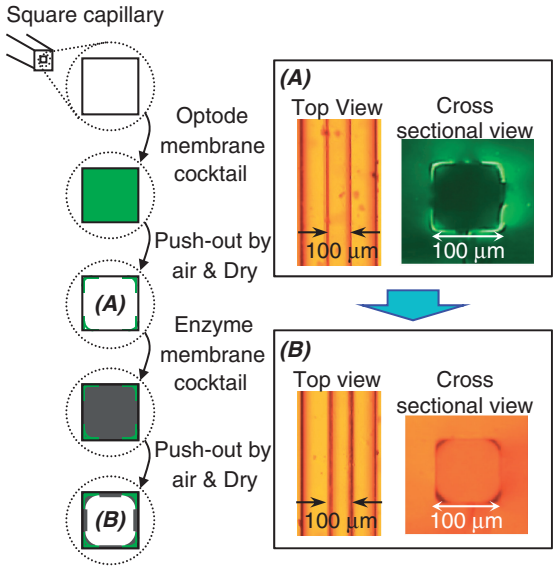

Figure 2. Simple procedure for capillary biosensor preparation and photographs for each immobilization step. (A) Optode membrane on a glass surface (cross-sectional view shows a fluorescent image for ease of recognizing optode membrane). (B) Enzyme membrane on an optode membrane.

life sciences. Here a urea biosensor was prepared as a proof-of-concept.

Figure 2 shows the preparation procedure of capillary biosensor composed of ion-selective optode membrane and dissolvable enzyme membrane. First, a membrane cocktail solution containing poly(vinyl chloride-*co*-vinyl acetate-*co*-vinyl alcohol) (30 mg), 2-nitrophenyl-*n*-octyl ether (60 mg), ammonium ionophore I (nonactin) (2.8 mg), chromoionophore XI (fluorescein octadecylester) (2.2 mg), and tetrahydrofuran (600 mg) was introduced into a clean square capillary (20 cm length, 100 µm square inner dimension) followed by air infusion to push out the cocktail. Then, remaining membrane cocktail at the four corners of the square capillary was dried at 70 °C for 1.5 h to complete ammonium-selective optode membrane preparation. Next, a mixture of PEG 1000 (1000 mg), urease (10 mg), and HEPES–tetramethylammonium hydroxide buffer (pH 7, 1 mL) was introduced and pushed out by air infusion. The capillary was vacuum dried for 1.5 h to complete the biosensor preparation. As described above, the method proposed here allowed very simple and easy preparation of capillary biosensor by simply repeating the membrane cocktail and air infusions.

Figure 2A shows a photograph of the optode membrane composed of plasticized PVC. Since the membrane contained a fluorescent lipophilic pH indicator dye (chromoionophore XI), cross section is shown as a fluorescent image. As can be seen, four corners of square capillary were successfully modified with an ammonium ion-selective optode membrane. Estimated thickness was ca. 3–5 µm from the fluorescent image.

Figure 2B shows the dissolvable membrane containing an enzyme formed on the optode membrane. Since the PEG membrane cocktail is viscous solution, thicker membrane of approximately 10 µm of maximum membrane thickness was formed. This membrane thickness can be varied by choosing the amount of PEG in membrane cocktail. In Figure 2B, hydrophilic PEG membrane was homogeneously formed along the longitudinal direction of the capillary. This homogeneity is rather important for mass production of capillary biosensors by simply cutting the long capillary into small pieces. Relative standard deviation (RSD) calculated by the fluorescence intensities obtained for capillary pieces prepared from the same capillary was within 5% ($n = 4$); however, RSD values for capillary to capillary deviation was larger than 10%. This deviation is mainly attributed to the change of viscosities for each membrane cocktail solution occurred by evaporation of the solvent during the immobilization procedure. Change in viscosity leads to the change in membrane thickness. Concerning the optode membrane, since the degree of protonation of the dye is independent of the thickness, analytical values can be simply corrected by using this parameter. Concerning the dissolvable membrane, change in membrane thickness leads to the change in enzyme concentration, which may reflect to the change in response time. In our experiments, response times were not significantly changed; however, tolerable concentration range of enzyme should be defined for practical analysis.

To verify the performance of an ammonium-sensing capillary, different concentrations of ammonium chloride in HEPES buffer was introduced into the capillary shown in Figure 2A. Figure 3 shows a typical response. In this case, dissolvable enzyme membrane was not immobilized. Since the optode membrane was immobilized at the four corners of the square capillary, two clear lines are seen in the fluorescence images shown in Figure 3A. Response time was approximately 5 min and it corresponded well with our previously obtained values.[15] In Figure 3B, average fluorescence intensity of two lines was plotted against ammonium ion concentrations, and it was found that the response range was from 10^{-5} to 10^{-2} M. It clearly illustrated that as the ammonium ion was extracted with the ammonium ionophore I present in the membrane, a corresponding proton from fluorescein moiety in chromoiono-

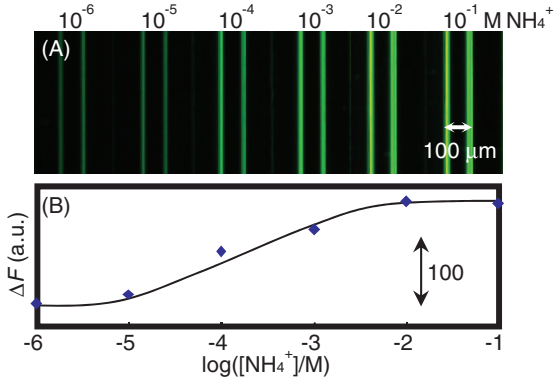

Figure 3. Typical responses of capillary sensors immobilizing optode membranes for ammonium ions. (A) Fluorescence images of capillaries responded for each concentrations of ammonium ions. (B) Response curve for ammonium ions.

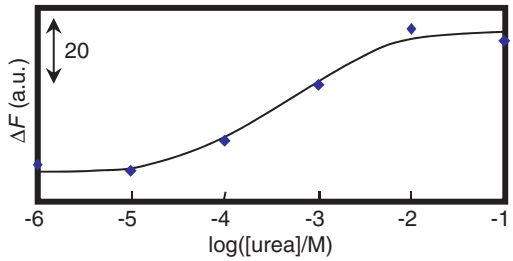

Figure 4. Typical response curve of capillary biosensor immobilizing optode membrane for ammonium ion and dissolvable PEG membrane containing urease for urea detection at the response time of 30 min.

phore XI released into the aqueous phase resulting a strong green fluorescence.

The measurement of urea undergoes two chemical processes with an initial enzyme reaction then followed by the ion sensing with the optode membrane. Figure 4 shows a typical response curve for urea obtained by the capillary biosensor immobilizing both ammonium ion-selective optode membrane and dissolvable PEG membrane containing urease at the response time of 30 min. Since the time required for dissolving PEG membrane was estimated to be 50–60 s[16] and that for ammonium response of optode membrane was ca. 5 min as described above, this slow response can be attributed to the slow enzyme kinetics. However, this response can be improved by simply increasing the concentration of enzyme in PEG membrane. The registered response range obtained here was between 10^{-5}–10^{-2} M urea, which fully covered the clinically relevant concentration range for urea analysis (10^{-4}–10^{-3} M).

This demonstrated the single-step analysis of urea with two-step chemical processes involving an enzymatic reaction and subsequent detection of product was quite successful.

In conclusion, a facile method of capillary biosensor preparation was developed. This novel preparation method can be applied to other single-step bioanalysis schemes for any two-step chemical processes involving the detection of an ionic product formation of the first enzymatic reaction, such as glucose detection by pH change,[17] or creatinine detection by ammonium concentration change.[18] Since it has proven to respond to changes in concentration, it can be integrated onto CAs-CHIP systems with other types of capillary biosensors for simultaneous multiple analyte sensing. Furthermore, the simple biosensor preparation with a commercially available square glass capillary made this technology industrially viable. This kind of microanalytical tool may be of great help in clinical diagnostics and drug discovery applications.

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