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Technical Notes

Microtiter Plate-Format Optode

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Microtiter plate-format optodes could be assembled by casting bulk-response membranes into the standard 96-well polypropylene-based plate or by screen printing them on an optically transparent substrate with 96-well pattern. The compositions of thick optode membranes, especially the ratios of poly(vinyl chloride) (PVC) to plasticizer [bis-(2-ethylhexyl) sebacate (DOS)], were carefully optimized to provide reproducible and rapid response. Adjusting the ratio of PVC to DOS by 1:6, bulk-response membranes containing neutral carrier (4-*tert*-butyl calix[4]arene tetraacetic acid tetraethyl ester for sodium-selective membrane or valinomycin for potassium-selective membrane) and lipophilic pH indicator (ETH 5294) could exhibit equilibrium response in 5 min. The practical utility of microtiter plate-format optodes has been examined by determining clinically relevant electrolytes in serum samples. It was demonstrated that microtiter plate-format optodes can provide high sample throughput (∼100 samples in less than 5 min), analytical performance comparable to that of a potentiometric clinical analyzer, and additional information on electrolytes using the same samples prepared for other colorimetric measurements.

Introduced by Simon and co-workers in the late 1980s, optodes based on bulk-response membranes (bulk optodes) have attracted considerable interest as an alternative ion-sensing system to ion-selective electrodes (ISEs).[1,2] The chemical recognition process and design principles of bulk optodes have been intensively studied,[3,4] and their analytical utility has been well demonstrated with real samples.[5−12] However, bulk optode-based analytical systems has not been used beyond research laboratories. It may be attributable to the intrinsic limitations of bulk optodes, e.g., the requirement for precise pH control throughout the measurement, their liability to the interferences from sample color or turbidity, and their short lifetimes in flow-through analytical systems.[1,5−13]

Despite these drawbacks, there have been several attempts to design practically useful bulk optode-based analytical systems. For example, small light sources such as light-emitting diodes (LEDs) or diode lasers have been assembled with bulk optode membranes coated on the surface of photodiode, at the distal end of an optical fiber, or on a dielectric waveguide to make compact analytical systems.[14−18] Those novel devices, however, delivered no distinctive advantages over the corresponding electrochemical sensors, especially with respect to their practicability and analytical performance.

Instead of devising an elegant analytical system with bulk optodes, we considered a new approach that can improve their own utility: solvent polymeric bulk optode membranes are arranged in microtiter plate format. The microtiter plate, an injection-molded rectangular plastic component with rows and columns of honeycomb-like wells, is commonly used in biomedical laboratories to perform various types of immunoassays. Employing a contemporary microtiter plate reader, multiple colorimetric changes occurring in 96 (8 × 12) wells of a microtiter plate are simultaneously processed in few seconds. Hence, the microtiter plate-format optodes are designed to take such multiplexing and high sample throughput advantages of a microtiter plate reader, including the easy sample handling advantages of multiwell plate.[19] In this technical note, we discuss how to make microtiter plate-format optodes and demonstrate their utility by determining typical

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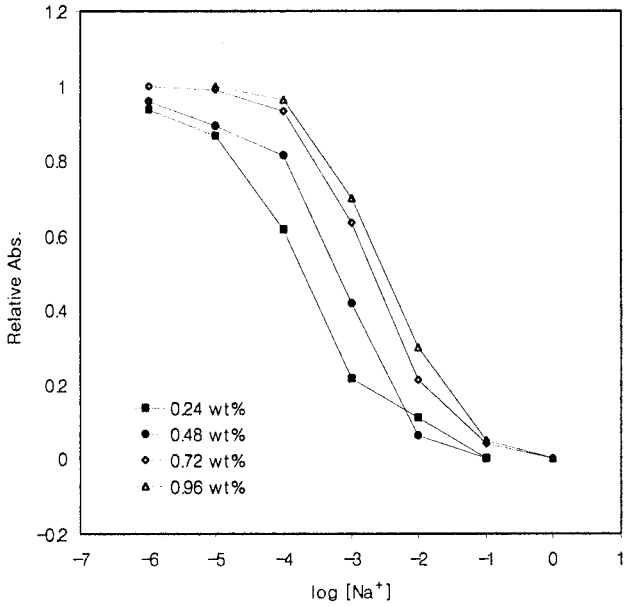

Figure 1. Variations in measuring range with increasing contents of chromoionophore (ETH 5294): (a) 0.24, (b) 0.48, (c) 0.72, and (d) 0.96 wt %.

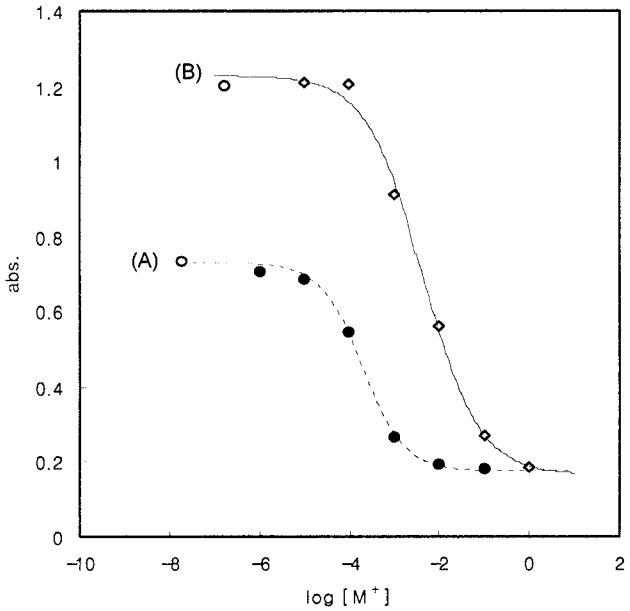

Figure 2. Calibration plots of (A) potassium- and (B) sodium-selective membranes cast in polypropylene-based microtiter plate. (Open circles in the plot indicate the absorbance due to 0.05 M Tris-H_2SO_4 buffer solution.)

electrolytes such as sodium and potassium in serum and macromolecular polyionic species such as heparin.[20,21]

EXPERIMENTAL SECTION

Reagent. The sources of reagents used in this experiment were as follows: valinomycin, 4-*tert*-butylcalix[4]arenetetraacetic acid tetraethyl ester (calixarene ester), bis(2-ethylhexyl) sebacate (DOS), potassium tetrakis(*p*-chlorophenyl)borate (K-TpClPB), tridodecylmethylammonium chloride (TDMAC), high-molecular-weight poly(vinyl chloride) (PVC), 9-(diethylamino)-5-(octadecanoylimino)-5*H*-benzo[α]phenoxazine (ETH 5294), 3-hydroxy-4-(4-nitrophenylazo)phenyl octadecanoate (ETH 2412), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) from Fluka (Buch, Switzerland); human serums from Nissui Pharmaceutical Co. (Tokyo, Japan); horse serums from Pel-freez Biologicals (Rogers, AR); and heparin sodium salt 150 units/mg (Sigma Chemical Co., St. Louis, MO). All other solvents and chemicals employed were of analytical grade. All aqueous solutions were prepared with deionized water (18 MΩ·cm).

Preparation of Microtiter Plate-Format Optodes. Potassium- and sodium-selective bulk-response membranes were prepared from the mixtures of 14.0 wt % PVC, 83.3 wt % DOS, 0.6 wt % ETH 5294, 0.5 wt % K-TpClPB, and 1.7 wt % ionophore (valinomycin for potassium and calixarene ester for sodium). Heparin-responsive optode membranes were formulated with 20.2 wt % PVC, 77.1 wt % DOS, 1.7 wt % ETH 2412, and 1.0 wt % TDMAC. The membrane compositions, especially the ratios of DOS/PVC, were carefully optimized to obtain rapid and reproducible responses. The mixtures (~200 mg) were dissolved in 700 μL of freshly distilled THF and loaded into the dispensing syringe connected to a pneumatic dispenser system (air fluid dispenser 1000XL, EFD Inc.). The membrane cocktails were uniformly dispensed (~5 μL/drop) into each U-bottomed microwell of the polypropylene-based plate (96-well; model M 4404, Sigma Chemical Co.) and air-dried in a dust-free vessel for 1 day. Another type of bulk optode plate was fabricated by screen-printing the membrane pastes (the membrane mixtures dissolved in 500 μL of THF) onto a glass plate (12.6 cm × 8.4 cm) with a 96-well pattern. It was air-dried for 1 day and affixed to a specially designed slab that has 96 wells.

Apparatus. Absorption profiles of bulk-response membranes were measured with an MPDS-1024 spectrophotometer (MJL Crystek Inc., Taejeon, Korea). Absorbance measurements for the microtiter plate-format optodes were performed with an Emax precision microplate reader (Molecular Devices Co., Sunnyvale, CA), selecting 650- (for ETH 5294-based membranes) or 540-nm (for ETH 2412-based membranes) band-pass filter. The results obtained from the optode plates were compared with those from an automated clinical analyzer (Stat Profile Ultra L, Nova Biomedical, Waltham, MA).

Experimental Procedures. To evaluate the analytical performance of potassium- and sodium-selective microtiter plate-format optodes, standard solutions containing the respective primary ion from 10^{-6} to 10^{-1} M in 0.05 M Tris-H_2SO_4 buffer (pH 7.4) were simultaneously pipetted into a row of eight wells in the plate. The membrane compositions and the dispensing volumes of the membrane cocktails for polypropylene-based plate have been adjusted by measuring the absorbance changes (ΔA) with respect to the concentrations (C) of the standards, response times, and sensitivities (i.e., $\Delta A/\Delta C$). The same compositions have been used to fabricate the glass-bottomed plate.

To determine the sodium and potassium contents in human and horse serums, two rows of wells in the plate were filled with the calibration solutions (S1585 and S1590 calibration solutions for ABL505 Blood Gas & Electrolyte System, Radiometer A/S, Copenhagen, Denmark) and the rest with the samples. The concentrations of electrolytes contained in S1585 and S1590

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Table 1. Sodium and Potassium Concentrations in Commercial Quality Control Human Serum and Horse Serum Samples[a]

	sample type	supplier's specification	clinical analyzer[b] ($n = 10$)[c]	polypropylene plate ($n = 8$)[c]	glass-bottom plate ($n = 8$)[c]
potassium	human (normal)	3.88 ± 0.13	3.97 ± 0.10	4.27 ± 0.20	4.10 ± 0.21
	human (abnormal)	6.18 ± 0.42	6.32 ± 0.08	5.90 ± 0.24	6.13 ± 0.37
	horse		1.89 ± 0.07	1.96 ± 0.25	1.80 ± 0.10
sodium	human (normal)	137 ± 1	137 ± 1	143 ± 6	145 ± 3
	human (abnormal)	151 ± 2	155 ± 1	151 ± 5	152 ± 3
	horse		70 ± 1	69 ± 4	71 ± 5
sample throughput			120 s/sample	300 s/plate	120 s/plate

[a] Listed values (mean ±2 SD in mM unit) were determined with microtiter plate-format optodes and an automated clinical analyzer. [b] Stat Profile Ultra L blood electrolyte/gas analyzer (Nova Biomedical). [c] Number of samples averaged.

calibration solutions were 145.0, 4.0, 1.25, and 106.24 mM, and 20.0, 40.0, 5.0, and 52.70 mM, respectively, in the order of Na^+, K^+, Ca^{2+}, and Cl^-. All samples and calibration solutions were diluted by 20 times with Tris-H_2SO_4 buffer (pH 7.4) before the measurement to maintain constant pH and maximum sensitivity.

Heparin solutions in the 0−10 units/mL concentration range were simultaneously loaded into the wells of microtiter plate-format optodes using a multichannel pipet. The absorbance changes of heparin-responsive membranes were recorded in 1-min interval.

RESULTS AND DISCUSSION

To develop microtiter plate-format optodes, we initially chose commercially available polypropylene-based microtiter plates, which are resistant to most organic solvents, as the optical substrate of bulk optode membranes. Bulk optode membranes were cast by dispensing a controlled volume (∼5 μL) of the membrane cocktail dissolved in THF to the center of U-bottomed wells of the plate. The U-shaped bottom helped form more uniform membranes throughout the plate than the flat bottom. The compositions of the sodium- and potassium-selective membranes have been optimized by adjusting their measuring ranges and response times.

As Figure 1 shows, the measuring ranges of bulk optode membranes are largely dependent on the relative amount of chromoionophore incorporated; it was found that the membranes containing ∼0.6 wt % ETH 5294 exhibited the most sensitive response below 10^{-2} M range for sodium (below 10^{-4} M for potassium). Considering that the serum or plasma samples are prediluted by 10−20 times for bulk optode measurements,[5−9] the optimized measuring ranges are appropriate for clinical analysis. To reduce the response times of thick membranes cast in the wells of the microtiter plate, it was necessary to increase the ratio of plasticizer to PVC.[22] The response times of the optodes were decreased from 30 to 5 min by varying the ratio from 2:1 to 6:1. The calibration plots shown in Figure 2 were obtained simultaneously from a microtiter plate-format optode with optimized membranes.

To demonstrate the practical utility of microtiter plate-format optodes, the sodium and potassium contents in three different serum samples were determined with a single 96-well plate. For this purpose, a microtiter plate-format optode was fabricated by casting bulk optode membranes in 10 rows of microwells (each 5 rows (or 40 wells) for sodium- and potassium-selective membranes, respectively). Two commercial calibration solutions, two types of human (normal and abnormal) serums, and a horse serum were prediluted by 20 times with Tris-H_2SO_4 buffer (pH 7.4) and applied to each five rows of the plate. At ∼5 min, the absorbance changes of 80 samples in the plate were read in few seconds.

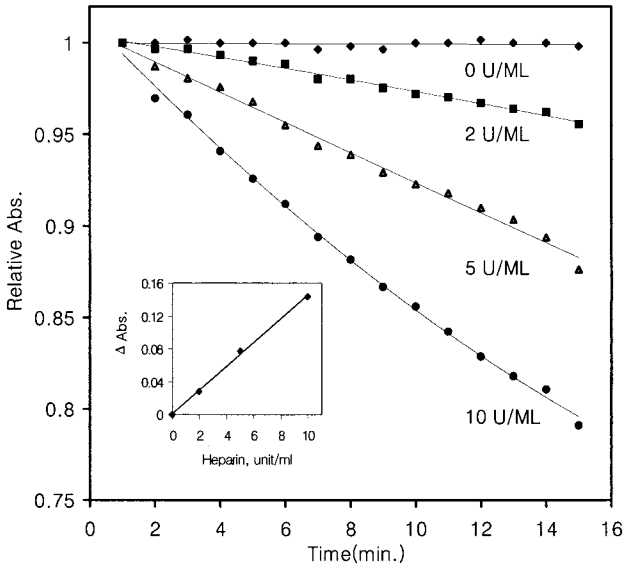

Figure 3. Absorbance variations of heparin-responsive microtiter plate-format optode with time. Samples of 0, 2, 5, and 10 units/mL heparin dissolved in 0.05 M Tris-H_2SO_4 buffer (pH 7.4) were measured simultaneously. Inset shows the absorbance changes vs initial concentrations of heparin measured after 10 min of exposure.

As listed in Table 1, the average values of sodium and potassium in each serum sample match well with those obtained with the ISE-type blood electrolyte/gas analyzer. However, the standard deviations of the optode plate measurements (±2 SD) were about 4−10 times larger than those of ISE measurements. It may be attributable to the lack of uniformity in optical densities of the polypropylene-based microtiter plate itself and the thick membranes manually cast on it. It could be improved by developing an advanced and automated manufacturing process. A large deviation often resulted from the irregular surface of aqueous samples in microwells. Such phenomena are usually observed when aqueous samples are contained in small hydrophobic cells due to the high surface tension of water. In this case,

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a neutral surfactant (e.g., 0.05 wt % Triton X-100) added to the diluting buffer greatly reduced the magnitude of the deviations.

Bulk-response membranes could be screen-printed on an optically transparent plate and assembled to a slab that has a standard 96-well pattern. Since the thickness of bulk-response membranes screen-printed on glass plate is much thinner than those cast in a polypropylene-based microtiter plate, the microtiter plate-format optodes with screen-printed membranes exhibit faster responses (<120 s).

The microtiter plate-format optode is particularly useful for measuring macromolecular polyionic species such as heparin. Meyerhoff et al.[20] have shown that the PVC-based optode membranes with TDMAC and ETH 2412 sensitively respond to heparin in the 0.2−5.0 units/mL range. Measuring the absorbance changes vs the initial concentrations at a given time, it is possible to determine the level of heparin contained in the sample. However, this is very time-consuming procedure if the calibrations are made separately at several different levels. Furthermore, the optode membranes exposed to macromolecular polyanionic species are not reversible because of their high partition coefficients. Hence, the use of a disposable microtiter plate-format optode is highly recommended in this case. Figure 3 shows the changes in absorbances with time of heparin-responsive PVC films cast in a polypropylene-based microtiter plate. It can be seen that the whole calibration plot is obtained with a single experiment.

In summary, microtiter plate-format optodes provide high sample throughput, analytical performance comparable to that of a potentiometric clinical analyzer, and additional information on electrolytes using the same samples prepared for other biomedical assays. Considering that most biomedical analyses are based on colorimetric methods, the use of microtiter plate-format optodes may become an important addition to standard potentiometric analysis in the determination of various clinically relevant electrolytes.

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