Development of a Reference Electrode Chip for Application in Ion Sensor Arrays

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A reliable screen-printing technique for fabricating an Ag/AgCl reference electrode on a solid substrate in a simple two-step procedure, which allows the incorporation of four different ion-selective electrodes on a chip, is presented. A 'sandwich' composite structure, comprising the reference electrode and a chip, was fabricated as a junction polymer membrane having an inner electrolyte layer and an Ag/AgCl electrode supported on a ceramic substrate. The junction polymer membrane was composed of chloroprene rubber, and the electrolyte layer was formed from agar gel. The electrochemical characteristics and performance of the reference electrode when integrated into a chip were investigated. From the results, it was shown that the potential change at the reference electrode chip as a function of the chloride ion concentration and pH can be kept reliably small. Its potential was also independent of the presence of other, physiologically significant, ions such as Ca^{2+}, K^+ and Li^+. The reference electrode, fabricated as a chip, can be used continuously for more than 60 h. Furthermore, its integration with four different ion-selective electrodes (potassium, sodium, ammonium, and calcium) on a substrate to form an ion sensor array chip was shown to be a facile process. The integration procedures and the sensing performances of the ion sensor array chip are also described.

Key words: Reference Electrode, Ion-selective Electrode, Sensors, Liquid Junction, Screen-printing

Introduction

In the history of electrochemistry, the reference electrode occupies a pivotal role. Its function is not only to complete the electrochemical measuring cell, but also to provide a stable constant potential during the course of the experiments [1]. Its characteristics should ensure that the responses of electrochemical sensors using potentiometric measurement systems, such as ion-selective electrodes (ISE), are meaningful and can be used to make quantifiable comparisons [2, 3]. Thus, ISEs have been used to determine physiologically important ions in extracellular fluids for diagnosing diverse health problems [4]. Furthermore, owing to the advancement of microfabrication technology, they can be miniaturized by using screen-printing or semiconductor-compatible fabrication technologies into potentiometric sensor chips and ion-sensitive field effect transistors (ISFETs) for environmental, clinical and biomedical applications [5, 6]. However, in the miniaturizing process, one important problem must be addressed, which is how to scale down a macroscopic commercial reference electrode with an inner filling solution.

Over the past few years, there has been a lot of effort focused on the fabrication of miniaturized reference electrodes. Reference electrodes used for electrochemically potentiometric sensor chips have been formed by directly modifying Ag/AgCl electrodes in various ways. Initial modification methods used electrolyte-doped polymeric materials to cover the Ag/AgCl surface in the form of Ag/AgCl/polymers [7 – 9]. Alternatively, Nafion [10, 11], or a metallic layer [12], can be used to protect the Ag/AgCl couple in planar reference electrodes. Other interesting approaches to producing miniaturized reference electrodes have included using a) two ion-selective membranes coated on Ag/AgCl electrodes in parallel [13, 14], b) an Ag/AgCl electrode covered with a polymer film doped with lipophilic salts together with ionophores [15, 16], or c) an Ag electrode coated with a polyion-responsive membrane to form a liquid junction-free reference electrode [17, 18]. There is also a particular way to manufacture a miniaturized reference electrode named the micro-

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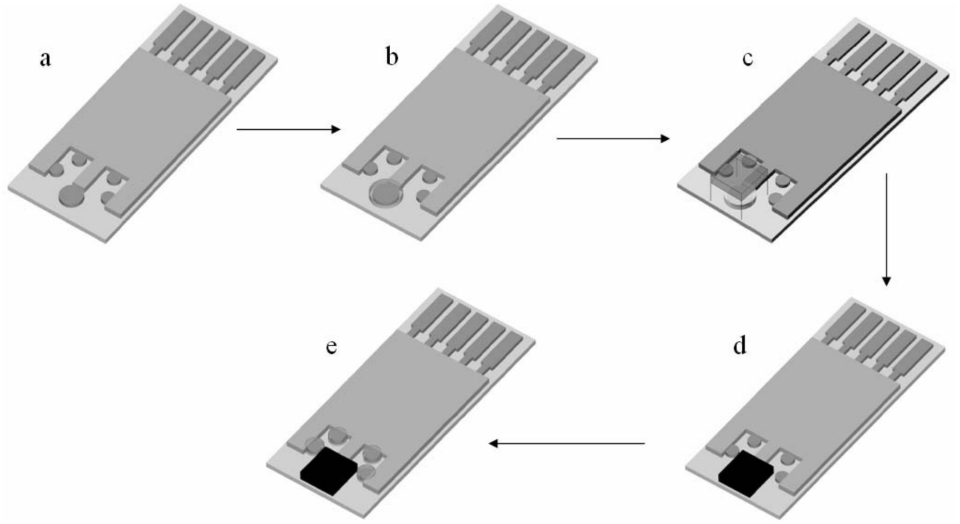


Fig. 1. Schematic diagram of the fabrication processes for a reference electrode chip and an ion sensor array chip. Stages (a) to (d) show the preparation procedures for the reference electrode chip, the final stage (e) shows the formation of the ion sensor array chip.

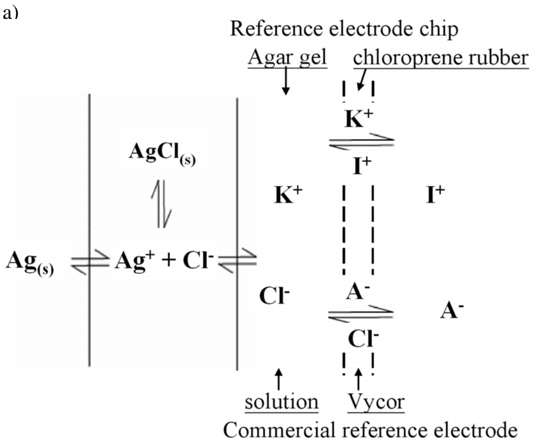
machined liquid-junction Ag/AgCl reference electrode created by Suzuki by using MEMS (Micro-Electro-Mechanical Systems) technology [19–22].

Reference electrodes for ISFETs, called reference field effect transistors (REFETs), were formed by modifying FETs with polymer membranes. These polymer membranes were hydrophobic, isolative, and ion-blocking films such as polyacrylates or polybutadienes. The modifications were achieved in different ways: vacuum deposition, ion beam sputtering, plasma polymerization, photo-polymerization, or chemically grafting an alkyl- or chloroalkylsilane [23–27]. REFETs can also be formed by coating the FETs with bilayer or multilayer films. The bilayer films consist of an electro-polymerized inner layer and a photo-polymerized outer layer [28, 29]. The multilayer films have the structure of a perchlorate-sensitive acrylate/electrolyte/polyethylene and a polyester sandwich multilayer to form the RFET [30].

The question that must be addressed when designing miniaturized reference electrodes is how to provide a stable junction between the sample solution and the Ag/AgCl electrode. This is an important factor contributing to the electrode's constant potential. A simple but novel Ag/AgCl reference electrode formed on a chip (reference electrode chip) which can satisfy this criterion has been developed in a previous study [31]. It was manufactured by a screen-printing method in a simple two-step procedure, using low-cost and readily commercially available products as the inner electrolyte and junction function polymer layers. Here we focus on the electrochemical characterization to establish the mechanism of the reference electrode chip by potentiometry using a two-electrode system. The stability of the reference electrode chip was also studied by continuously measuring its potential drift *vs.* a commercial reference electrode (model MF-2079, Bioanalytical system Inc. USA) in different pH buffer solutions and physiologically important ionic solutions, *e. g.* those containing sodium, potassium, lithium, calcium, ammonium, chloride, nitrate, acetate, phosphate, and sulfate ions. In the final part of this study, the fabricated chip containing the reference electrode was integrated with four different ion-selective electrodes to form a chip-type ion sensor array (Fig. 1), the performance of which is also discussed.

Results and Discussion

The underlying mechanistic basis of the fabricated solid-state reference electrode chip is shown in Fig. 2a. In order for this design to be able to function as a commercial reference electrode, its performance should approximate to, or preferentially exceed, that of current commercially available designs. This was achieved by using an agar gel, containing KCl, to replace the commercial electrode's 'unshapable' liquid inner filling solution. A chloroprene rubber polymer film, which was a)



b)

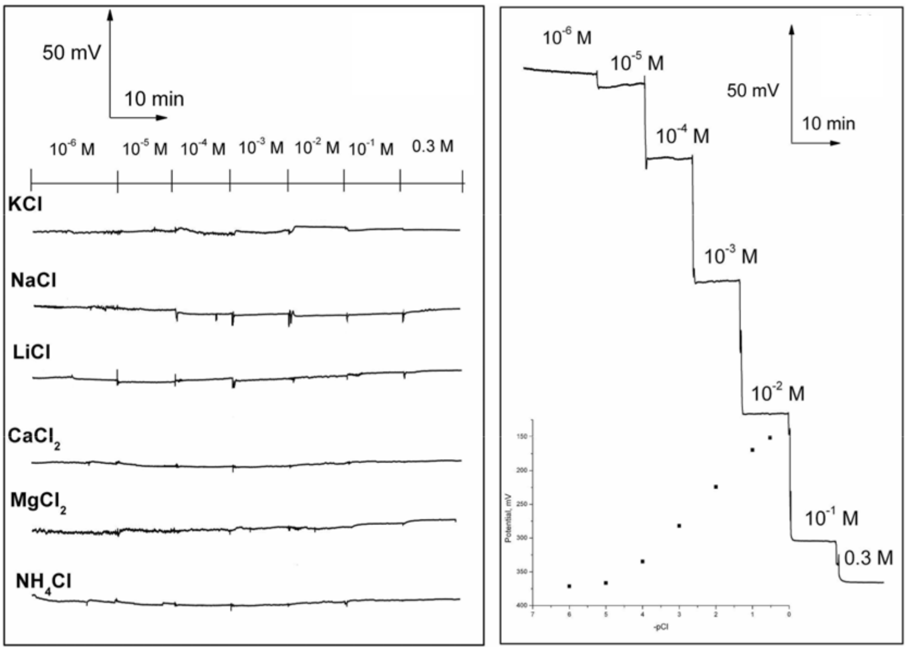


Fig. 2. a) Schematic drawing of the reference electrode chip and a commercial reference electrode. b) The dynamic emf response curves of the reference electrode chip and its Ag/AgCl electrode *vs.* a commercial Ag/AgCl reference electrode in various chloride ion solutions.

used both as a junction and also to form the pathway for ion transfer from the agar gel into the sample solution additionally served to prevent the sample solution from mixing with agar gel. As shown in Fig. 2a, the reference electrode chip consists of an Ag/AgCl electrode covered by an agar gel containing a constant chloride ion concentration, which is able to generate a constant potential as determined by the Nernst equation. In this way the chloroprene rubber polymer has the important function of establishing a stable junction potential between the electrolyte gel and the sample solution, thereby ensuring that the Ag/AgCl electrode has an unchanging potential in a chloride ion solution with a constant concentration.

In order to demonstrate the merit of the proposed design, it was tested by measuring its potential shift with respect to a commercial reference electrode in a KCl solution, the concentration of which was increased in a stepwise manner from 10^{-6} M to 0.3 M. From the results, which are shown on the left hand side of Fig. 2b, it is apparent that the potential shift was less than 15 mV. These results confirm that the chloroprene rubber provided a similar function as porous Vycor, by not only dividing the inner electrolyte from the sample solution but also by connecting the elements of the circuit, thereby enabling the Ag/AgCl electrode on the chip to give a constant potential in the aqueous system. As an extension of this approach to testing the electrode, different chloride salt solutions were used to evaluate the reference electrode chip. These results, shown on the left hand side of Fig. 2b, demonstrate that the reference electrode chip can maintain a constant potential without being influenced by cations, such as K^+, Na^+, Li^+, $NH_4{}^+$, Ca^{2+} and Mg^{2+}. Taken together, the results clearly show the potential utility of this approach to forming a miniaturized commercial reference electrode. In contrast, an unmodified Ag/AgCl electrode, when incorporated into the chip exhibited clear potential shifts when the KCl concentration changed, in a stepwise manner, from 10^{-6} M to 0.3 M, revealing Nernstian behavior with a sensitivity of 54 mV (–pCl^{-1} as shown on the right-hand side of Fig. 2b. These results highlight the fact that a bare Ag/AgCl electrode cannot perform as a reference electrode in a chloride ion solution where the concentration is not maintained at a fixed value.

The stability and durability of the reference electrode chip

The long-term stability of the reference electrode chip was evaluated by continuous measurement of the potential shift *vs.* the commercial reference electrode in unstirred KCl solutions for more than 60 h. The potential shift was recorded at 0.1 second intervals for each data point in order to show the potential variation. In Fig. 3, it is shown that the reference electrode chip keeps a constant potential *vs.* the commercial reference electrode in 3 M, 10^{-1} M, and 10^{-2} M KCl solutions and shows only a slight potential fluctuation in 10^{-4} M and 10^{-6} M KCl solutions. The reference electrode chip showed both negligible potential drift in any concentration of KCl and also protracted stability when used for long-term measurements.

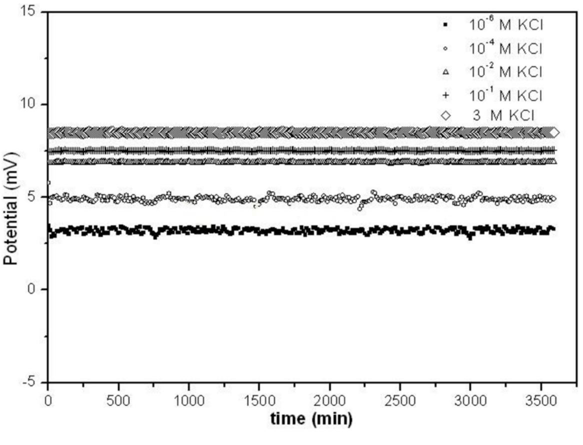


Fig. 3. Stability tests for the reference electrode chip in unstirred 10^{-6} M (■), 10^{-4} M (○), 10^{-2} M (△), 1 M (+), and 3 M (◇) KCl solutions.

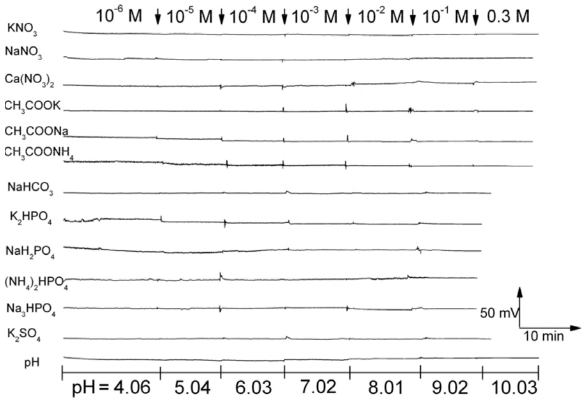


Fig. 4. The dynamic emf response curves of the reference electrode chip *vs.* a commercial reference electrode with continuously varying concentrations of nitrate, acetate, carbonate, phosphate, and sulfate and in different pH buffer solutions.

Ideally, the potential of a reference electrode chip should not be affected by other substances present in the analytical matrix. To evaluate the interference of various ions with respect to the reference electrode chip, its potential *vs.* the commercial reference electrode was measured in stepwise concentration changes with various kinds of salts in the test solution. The results of this investigation, presented in Fig. 4, show that the potential drifts of the reference electrode chip and the commercial reference electrode were within 10 mV of each other with nitrate, acetate, carbonate, phosphate, and sulfate ions present in the solutions, and also at various pH values. In other words, the developed reference electrode chip had a potential shift within 1.5 ∼ 2 mV for one magnitude change of the concentration of various ions in the test solution.

These results have confirmed that the reference electrode chip worked as well as commercial reference electrodes and could be regarded as a potential standard for potentiometric sensors in aqueous solutions.

Fabrication reproducibility of the reference electrode chip

The same fabrication procedure was employed to manufacture 10 similar reference electrode chips in

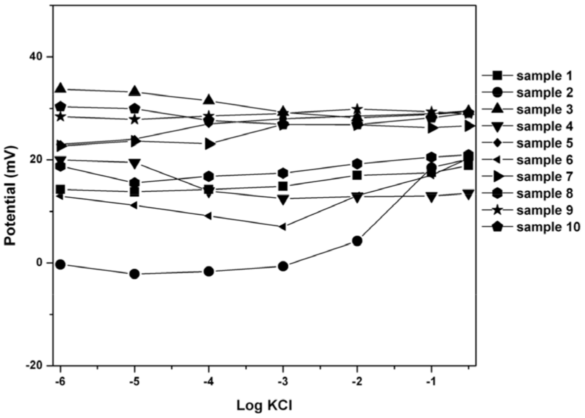


Fig. 5. The dynamic emf response curves of 10 reference electrode chips *vs.* a commercial reference electrode in continuously changing KCl concentrations.

order to determine the consistency of this fabrication method. These 10 electrodes were evaluated by the same method as described in the Experimental Section, and the results are shown in Fig. 5. The criterion used to judge the quality of a good reference electrode chip is the potential shift of each electrode chip *vs.* that of a commercial reference electrode. This was required to be within 10 mV in continuously changing chloride ion concentrations from 10^{-6} M to 0.3 M in the test solution. As shown in Fig. 5, the potential shifts of those samples *vs.* the commercial reference electrode which tallied with the above criterion were for samples 1, 3, 4, 5, 7, 8, 9 and 10. The other samples, namely 2 and 6 had dramatic potential shifts of 20 to 30 mV at chloride ion concentrations from 10^{-6} M to 0.3 M. Therefore, the probability of successfully manufacturing the reference electrode chip to perform as well as a commercial reference electrode is about 80 %.

Analytical applications of the reference electrode chip in ion sensor array measurements

Our reference electrode chip was integrated with four ion sensors as an ion sensor array on a chip as shown in Fig. 1. The potentiometric responses of the four ISEs with respect to the commercial reference electrode and our electrode chip are shown in Fig. 6. The four ISEs showed near Nernstian behavior with slopes equal to 55.35 mV $(\mathrm{pK}^+)^{-1}$, 56.82 mV $(\mathrm{pNa}^+)^{-1}$, 55.95 mV $(\mathrm{pNH_4}^+)^{-1}$, and 21.89 mV $(\mathrm{pCa}^{2+})^{-1}$ by using a commercial reference electrode with a stepwise increase in the K^+, Na^+, $\mathrm{NH_4}^+$, and Ca^{2+} ion concentrations from 10^{-8} M to 0.2 M. Additionally, proportional potential responses of the ISEs *vs.* the developed reference electrode chip were also observed in the region of Nernstian behavior, with sensitivities of 55.01 mV $(\mathrm{pK}^+)^{-1}$, 55.93 mV $(\mathrm{pNa}^+)^{-1}$, 54.89 mV $(\mathrm{pNH_4}^+)^{-1}$, and 20.93 mV $(\mathrm{pCa}^{2+})^{-1}$ being determined during the same evaluation processes. These results can be regarded as typical of the performance of the ion sensor array. From Fig. 6, it is apparent that the sensing character of the ISEs has only a slight influence when used with different standard electrodes, a commercial reference electrode or a reference electrode chip. In other words, the developed reference electrode chip could be used for ISEs for the detection of ion concentrations. The developed reference electrode was formed on a chip by using a screen-printing method, and this fabrication technique provided a way for miniaturizing the electrochemical sensors on the chip.

Conclusion

A reference electrode on a chip was formed by using commercially available and low-cost materials, *viz.* agar and chloroprene rubber in uncomplicated procedures. Agar gel made with an aqueous 1 M KCl solution was used as the electrolyte layer to provide sufficient chloride ions to establish equilibrium between AgCl and Cl^- resulting in a stable potential. The chloroprene rubber membrane material prevents the mixing of the sample solution and the electrolyte layer and thereby forms a stable junction. Thus, the potential produced by the presence of Ag/AgCl in a constant KCl concentration agar layer can be maintained. From the results obtained, it is apparent that the potential of the reference electrode chip is not influenced by common physiological ions. The device can be used continuously for more than 60 h in an electrochemical system. Moreover, ion-selective electrodes are fully compatible with it, their sensing ability of ion concentrations being fully maintained. In conclusion, a miniaturized solid state reference electrode, formed by microfabrication technology, can be integrated into an ion sensing array, which allows for the stable determination of several ion concentrations.

Experimental Section

Materials and reagents

The suppliers of the chemicals used to fabricate the Ag/AgCl reference electrode on the chip were as follows: agar (Lancaster), potassium chloride (Aldrich), iron(III)

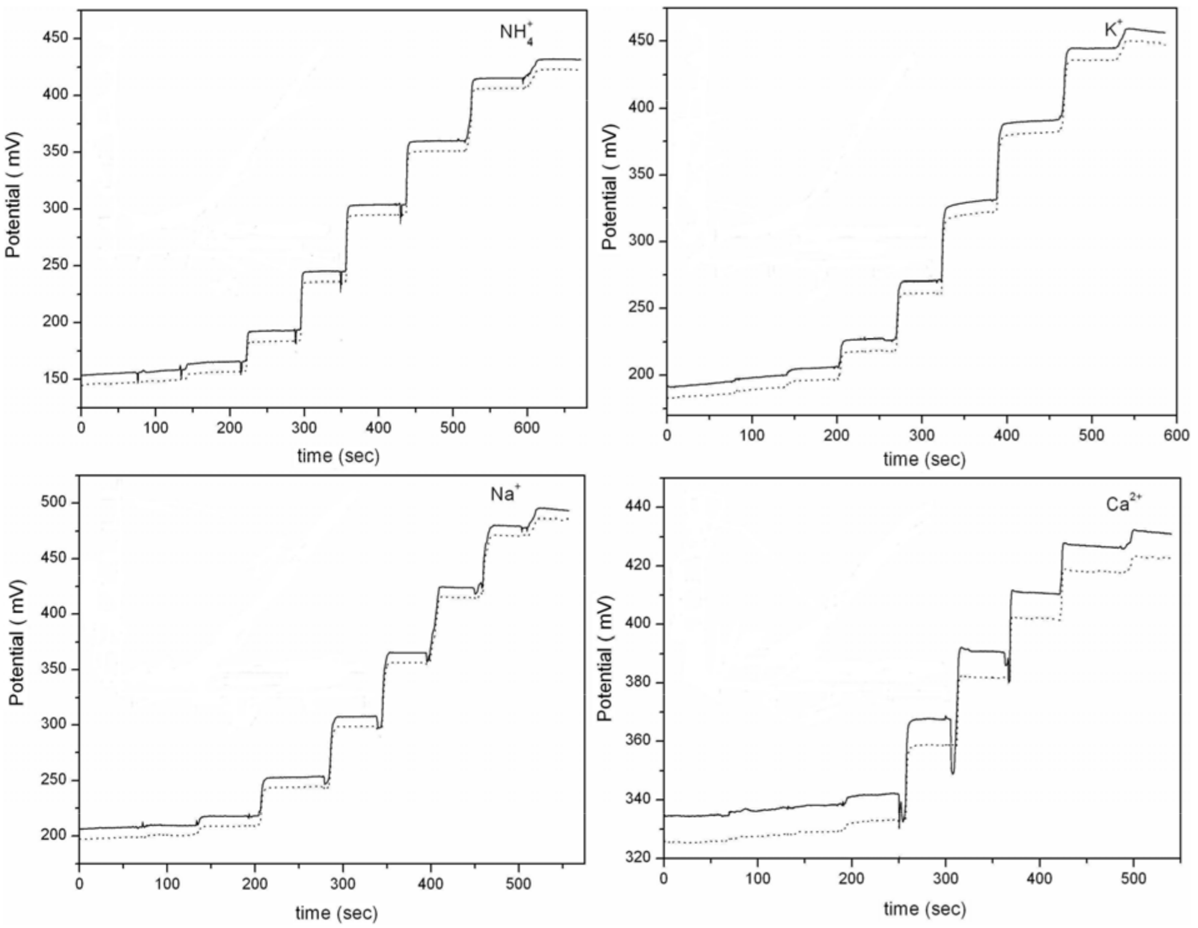


Fig. 6. The reference electrode chip integrated with four different ion-selective electrodes. The solid lines in each figure were emf responses of ion-selective electrodes *vs.* a commercial reference electrode with stepwise concentration changes of NH_4Cl, KCl, NaCl, $CaCl_2$ solutions from 10^{-8} M to 0.3 M. The dashed lines show the potential responses of ion-selective electrodes *vs.* the reference electrode chip in the ion sensor array, with stepwise concentration changes in the NH_4Cl, KCl, NaCl, and $CaCl_2$ solutions.

chloride (SHOWA), silver and platinum pastes (Heraeus Inc.), insulating ink (DuPont, Encapsulation paste, catalog number 7137), chloroprene rubber (Kuo Sen enterprise, Taiwan) and alumina substrate (Laser Tek Taiwan Co.). Chloride salts, nitrate salts, and sodium hydrogen-carbonate used for evaluating the reference electrode chip and the ion sensor array chip were of analytical grade and were purchased from Aldrich. The plasticizer for the ion-selective sensors was bis(2-ethylhexyl)adipate (DOA), obtained from Fluka Inc. All solutions prepared in this study were made using deionized (DI) water (Millipore Water System) with a resistance of 18.3 MΩ.

The compositions of the selective polymer membranes of the ion-selective sensor array in this study were as follows: the lipophilic additive was potassium tetrakis-(4-chlorophenyl)borate (KClTPB) (0.6 wt.-%); the ionophores (all present at 1 wt.-%), for sensing potassium, sodium, ammonium, and calcium ions were valinomycin, calix[4]arenecrown-4, nonactin, and 3,6-dioxa-octanediamide (ETH 1001), respectively. The polymer matrix, comprising 33 wt.-% of the ion-selective membranes, was silicon rubber 3104 (3140-RTV sealant) purchased from Dow Corning (Midland, MI, USA). The plasticizers, present at 65.4 wt.-%, for the ion-selective sensors, were KClTPB and DOA. The chemical components of the ion-selective membrane were dissolved in tetrahydrofuran (THF) to form a solution for easy coating onto the sensor chip.

Ion sensor array chip structure and fabrication

The structure and fabrication processes of the reference electrode and ion sensor array chips are shown in Fig. 1. In this study, the chip was designed to sense four ions by using a two-electrode system. The dimensions of the alumina substrates for each chip were 30 mm × 10 mm (length × width). As shown in Fig. 1, the reference electrode was in the middle of the chip (0.35 mm in diameter), surrounded by the ion sensing electrodes (0.2 mm in diameter).

The fabrication process of the ion sensor array chip was as follows: initially [Fig. 1 stage (a)], platinum and silver pastes were printed onto the substrate to form the conducting paths for four ion-selective electrodes and one reference electrode. The electrodes were formed, the insulating ink was printed over the substrate surface except for the sensor regions (round shaped areas in Fig. 1) and the conducting pads. The calibration procedures of metal pastes and insulation inks were the same as described in a previous study [31].

Reference electrode and ion-selective electrodes on the chip

The chip-type reference electrode was obtained by modifying the silver electrode through several modification steps as shown in Figs. 1 (b) to (d). Firstly, step (a), iron(III) chloride solution (1 M, 50 μL) was used to form an AgCl layer on the silver surface. Then, step (b), an agar solution containing KCl was used to form a KCl gel layer on the AgCl surface. The last step, from (c) to (d), was to print a polymer membrane (chloroprene rubber) over the KCl gel layer to form the liquid junction functional part of a reference electrode. The complete chip-type reference electrode generated by this procedure is depicted in Fig. 1 (d).

The four ion sensors were based on an ion-selective membrane coated onto a conductive material generating so-called coated-wire electrodes. This final step in the fabrication procedure is shown in Fig. 1 (e). Four membrane solutions with different ionophores (3 μL) were dropped onto the four different platinum electrode surfaces, with the procedure being repeated again after 15 min, thereby generating ion-selective electrodes on the chip.

Finally, the integrated chip was left for 2 d at r. t. to allow stabilization of the reference electrode chip and to allow the evaporation of solvent from the ion-selective membranes.

Electrochemical measurements

The ion sensor array chip created in this study comprised two sections, a reference electrode and an ion-selective electrode. The performance of the ion-selective electrodes on the chip was also investigated by using an open-circuit potential method where the working electrode was an ion-selective electrode, and the reference electrode was a commercial electrode. The whole sensing system, *i. e.* the ion sensor array chip, was tested by the same method, and the results were compared to an ion-selective electrode with a commercial reference electrode in order to verify the chip's responses.

All the electrochemical examinations were made using a conventional two-electrode system for measuring potential shifts. The tests in this study were carried out using an electrochemical workstation, model 1000, CH Instruments, Austin TX. All experiments were performed at an ambient temperature of approximately 25 °C.

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