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Real-time and noninvasive monitoring of respiration activity of fertilized ova using semiconductor-based biosensing devices

Toshiya Sakata · Izumi Makino · Sayaka Kita

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Abstract In this report, we propose a novel evaluation method of embryo activity, describing the real-time and noninvasive electrical monitoring of embryo activity, caused by fertilization of the sea urchin, using a biologically-coupled field-effect transistor (bio-FET) comprised of semiconductor-based biosensing devices. The detection principle of bio-FET is based on the potentiometric detection of charge density change at the gate insulator, which includes changes of hydrogen ion concentration corresponding to pH variation. The surface potential at the gate surface of the bio-FET increased after the introduction of sperms into the ova, resulting in fertilization on the gate sensing area. The positive shift of surface potential indicates the increase of positive charges of hydrogen ions generated by dissolved carbon dioxide in artificial sea water based on respiration activity of the embryo. Moreover, the electrical signal of embryo activity is suppressed due to the inhibition of cytokinesis by introduction of cytochalasin B. The platform based on the bio-FET is expected to be a real-time, label-free and noninvasive detection system, not only in fundamental studies of embryo activity but also in the evaluation of embryo quality for in vitro fertilization.

Keywords Fertilization · Embryo activity · Semiconductor · Respiration · Hydrogen ion

T. Sakata (✉)
Department of Materials Engineering,
Graduate School of Engineering, The University of Tokyo,
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
e-mail: sakata@biofet.t.u-tokyo.ac.jp

T. Sakata · I. Makino · S. Kita
Center for NanoBio Integration, The University of Tokyo,
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Introduction

Recently, assisted reproductive technology (ART) has been pursued as one of the therapeutic methods for treating sterility. The success of ART programs depends on advances in engineering. For one ART program, in vitro fertilization (IVF), an important factor is how to evaluate embryo quality and select embryos in good condition. Morphological evaluation has been widely used to rank embryo quality, because microscopic analysis is noninvasive and useful in predicting pregnancy rates (Veek 1991; Gardner et al. 2000). However, the standard classification of embryo quality is ambiguous among the operators, so the search continues for a new principle by which to evaluate embryo quality accurately and noninvasively.

Oxygen consumption is widely considered to be the parameter that provides the best indication of overall metabolic activity of a single embryo (Fridhandler et al. 1957; Magnusson et al. 1977; Overstrom et al. 1992; Houghton et al. 1996; Lopes et al. 2005), although embryo metabolism has also been assessed by measurement of nutrient consumption, such as glucose, pyruvate and amino acids (Wales 1986; Javed and Wright 1991; Rieger et al. 1992; Khurana and Nieman 2000). The electrochemical system is being developed as one of the detection methods for the evaluation of embryo quality. Shiku et al. presented the concept of detecting oxygen consumption based on the respiration activity of the embryo (Shiku et al. 2001). In this method, the oxygen reduction current was detected near the surface of a single embryo using the cyclic voltammetry technique.

In our previous work we proposed a new method for continuously and noninvasively detecting electrical phenomena at or near the cell membrane using a semiconductor device, a bio-coupled field-effect transistor

(bio-FET) (Sakata and Miyahara 2008a, b, c). The detection principle of bio-FET is based on the potentiometric detection of charge density changes at the gate insulator. Field-effect devices have been used to electrochemically detect nerve cell function and DNA hybridization events on a solid surface (Fromherz et al. 1991; Souteyrand et al. 1997; Fritz et al. 2002; Sakata et al. 2003, 2005; Pouthas et al. 2004; Uslu et al. 2004). Since the gate insulator usually consists of Si_3N_4 or Ta_2O_5 with a hydroxyl group at the surface in solutions. The platform supporting the bio-FET is suitable for a real-time and noninvasive detection of cell functional analysis,. Furthermore, the bio-FET is sensitive to hydrogen ions and is already utilized as a pH sensor. Therefore, the pH variation due to the respiration activity of embryo can be monitored noninvasively using the bio-FET, yielding significant information regarding embryo activity based on respiration. In this paper, we report the real-time and noninvasive electrical monitoring of sea urchin embryo activity based on respiration using the bio-FET and investigate the possibility of this evaluation method for the standardization of embryo quality in IVF.

Materials and methods

Electrical measurement using bio-coupled field-effect transistor (bio-FET)

Insulated gate field effect transistors (FETs) were fabricated using the standard integrated circuit technology, except for deposition of the gate electrode. Each *N*-channel depletion mode FET was made in a 2×5 mm chip (Sakata et al. 2003). The thicknesses of the Si_3N_4 layer and the SiO_2 layer were 140 and 35 nm, respectively. The channel width, W and the channel length, L were designed to be 340 and 10 μm, respectively, and the resulting ratio, W/L, was 34. The fabricated FET chip was mounted on a flexible polyimide film with patterned copper electrodes and wire-bonded. The FET chip was encapsulated with a glass ring, except for the sensing areas, using an epoxy resin (ZC-203, Nippon Pelnox). In addition to the fabricated FETs, commercial ion sensitive FETs (BAS Inc.) of which the gate insulator consisted of Ta_2O_5/SiO_2 double layer, were used for the experiments in the present study. The bio-FET was immersed in an artificial seawater (ROHTOMARINE by REI-SEA Co. Ltd.) of pH 8.1 at room temperature, with an Ag/AgCl reference electrode with saturated KCl solution. Ova of sea urchin were seeded on the gate area of the bio-FET, then sperms were added to ova on the gate surface (Fig. 1a, b). The electrical characteristics of the bio-FET such as the gate voltage (V_G)-drain current (I_D) characteristics and the surface potential at the gate surface were measured in the artificial seawater at room temperature using a semiconductor parameter analyzer (4155C, Agilent) and a custom-made potentiometric analyzer (Ryokusei M.E.S. Co. Ltd.), respectively. As the basic electrical characteristic, the threshold voltage shift ΔV_T was determined after introduction of ova and sperms into the bio-FET. The ΔV_T was defined as a difference of the V_G-I_D characteristics at a constant drain current of 700 μA. The time course of the surface potential at the gate surface was monitored using a circuit, as shown in Fig. 1c (Sakata et al. 2005) with which the potential change at the interface between an aqueous solution and the gate insulator could be read out directly at a constant drain current. In the present study, the drain voltage and the drain current were set to be 1 V and 700 μA, respectively.

Preparation of sea urchin ovum and sperm

Sea urchins were purchased from Misaki Marine Biological Station, The University of Tokyo in Japan. The injection of 0.5 M KCl into the mouth of a female or male sea urchin induced numerous ova or sperms to be expelled from the anus into the artificial seawater. The obtained ova or sperms were turned into the fresh artificial seawater for cleaning. The controlled ova ($1x10^4$/ml) were introduced into the gate surface of a bio-FET, while the number of sperms could not be counted. Since many ova had been added on the device, a few ova were directly arranged on the gate sensing spots, because the size of ovum was about 100 μm and the size of gate sensing area was 10×340 μm. Larger embryos and closer adhesion would result in stronger electrical signals because the detection ability of bio-FET depends on the charge density changes at the gate sensing area. The temperature was controlled at 20°C while injecting ova and sperms and measuring electrical characteristics.

Results and discussion

The detection principle of bio-FET is based on the potentiometric detection of charge density changes at the gate insulator, on which specific binding between target and probe molecules allows for molecular recognition. Figure 1 shows the conceptual structure of the bio-FET intended to detect embryo activity after fertilization. Sea urchin ova were seeded on the surface of the gate insulator of the bio-FET. The gate surface of the bio-FET was immersed in a measurement solution together with an Ag/AgCl reference electrode with a saturated KCl solution (Fig. 1a). The charge density changes based on hydrogen ions could be detected as the shift of surface potential of the bio-FET (Fig. 1b). Basically, the ion or molecular charges at the

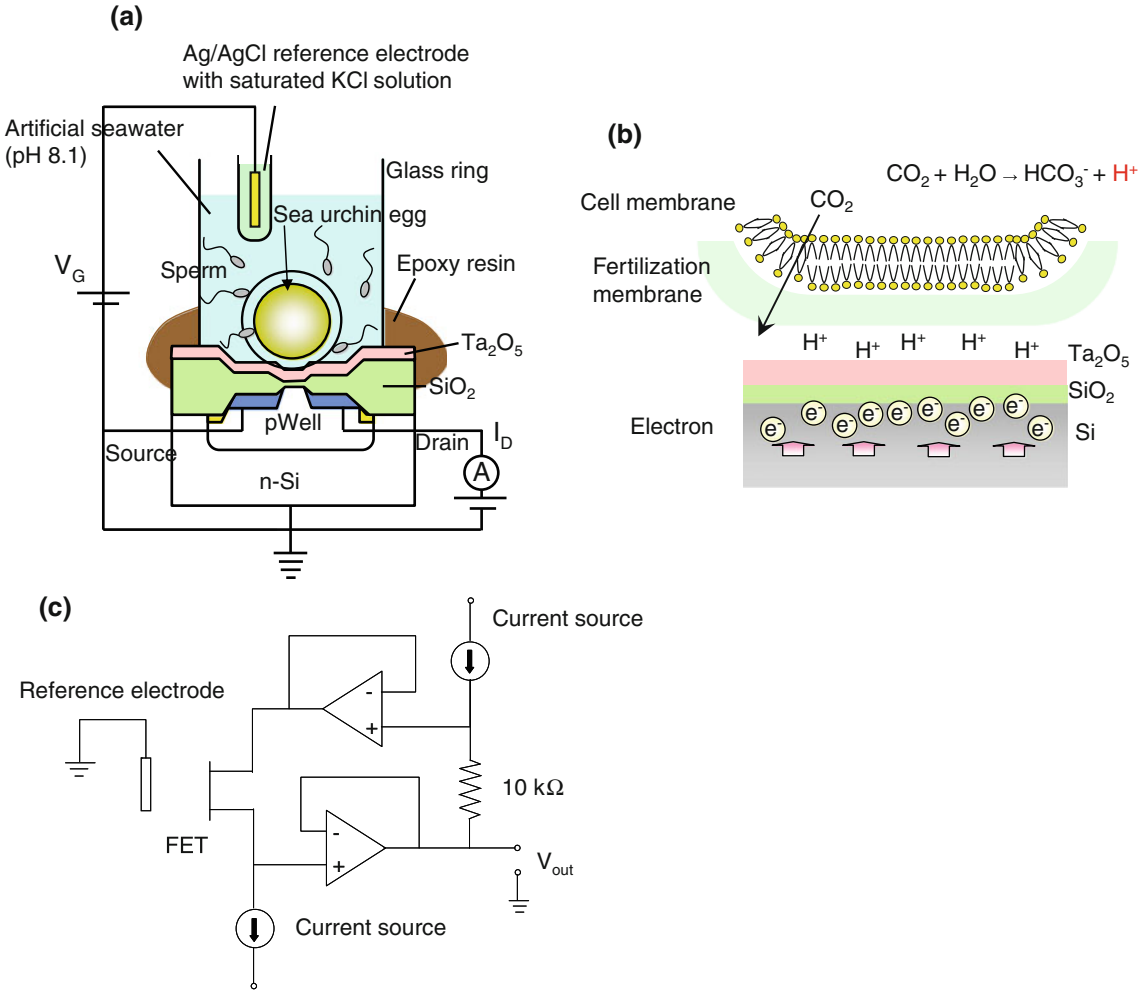

Fig. 1 Schematic illustration for measurement of surface potential of bio-FET. A shift of the threshold voltage V_T can be determined from the gate voltage (V_G)-drain current (I_D) characteristics in artificial sea water (pH 8.1). The V_G was controlled through the Ag/AgCl reference electrode with the saturated KCl solution. Sea urchin ova or sperms were introduced to the gate sensing area during the measurement of the electrical signal (**a**). The Ta_2O_5 gate surface with hydroxyl group in solutions is sensitive to pH based on the concentration of hydrogen ions. The charge density changes at the gate surface induce electrostatic interaction with electrons at the channel in silicon crystal resulting in a change of drain current (**b**). Circuit for measuring surface potential of field effect transistor (FET) (**c**)

gate interact electrostatically with electrons in silicon crystal through the thin gate insulator and induce electrical signals by the field effect, and are monitored using the bio-FET system. The details of the bio-FET device and the fabrication process have been reported previously (Sakata et al. 2005). Furthermore, two types of bio-FETs were prepared for differential measurements in this study; one was the bio-FET on which sea urchin ova were kept and fertilization was accomplished by induction of sperms, and the other was the control bio-FET on which there were no ova, but sperms were introduced. Using these bio-FETs, differential measurements were performed in order to eliminate the common background noise such as temperature change, non-specific adsorption and change in ion concentration.

The change in the surface potential at the gate surface of the bio-FET was monitored after adding sea urchin ova and sperms in turn (Fig. 2). First of all, the surface potential showed a negative shift after introducing ova at t_1 on the gate because of negative charges of the ovum membrane in contact with the gate surface. The negative charges of the ovum membrane derived from mainly sialic acids and sulphated saccharides of its jelly coat (Sakata et al. 2008c). After ova were kept on the gate area, sperms were added there at t_2 but little surface potential change of bio-FET could be detected, although some noise based on temperature and ion concentration changes could be found. The size of a sperm is smaller than that of an ovum (about 100 μm) and the diameter of the head is about 5 μm. They were running around above or on the gate surface. Since

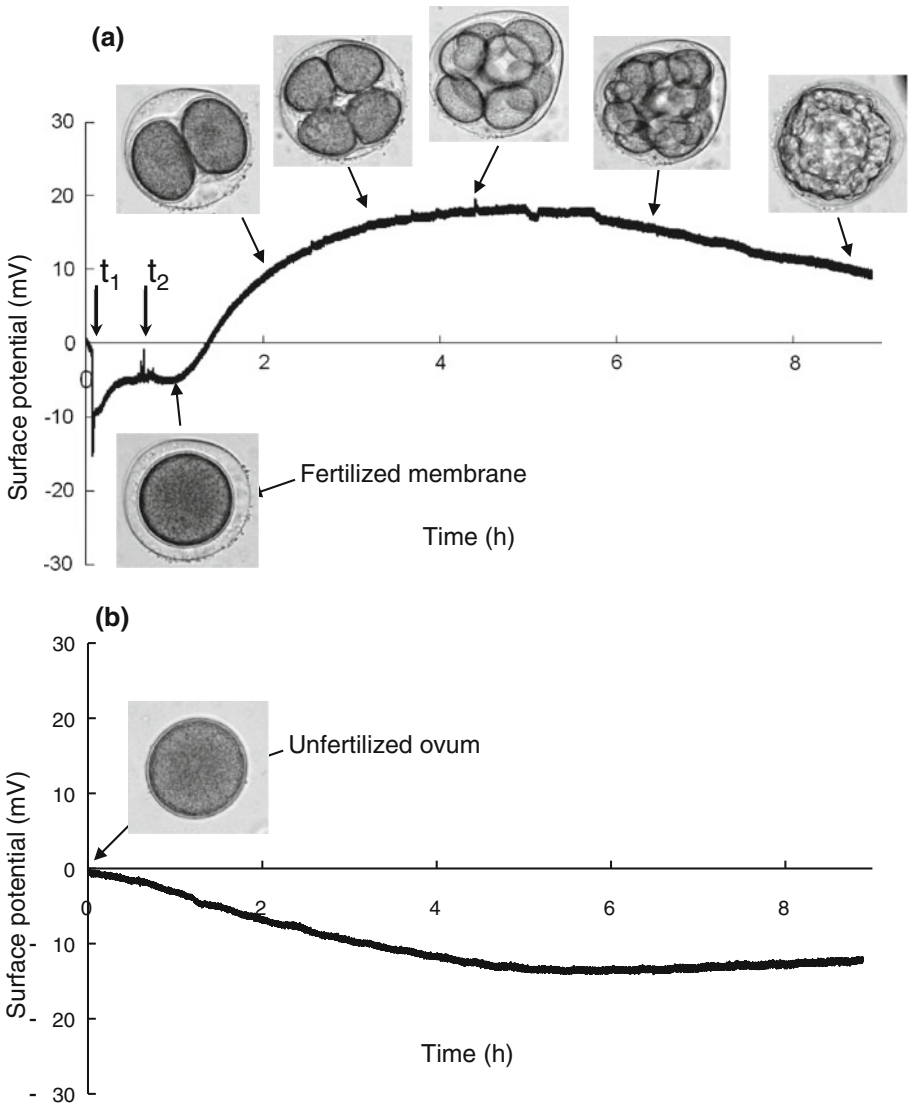

Fig. 2 Noninvasive and real-time monitoring of embryo activity of sea urchin using bio-FET. t_1 and t_2 shows the introduction times of ova and sperms, respectively. The fertilization occurred a few minutes after the induction of sperms to ova on the gate surface (t_2) (**a**). Electrical monitoring of unfertilized ova on the gate surface. They were added on the gate at 0 h (**b**)

most of small sperms attached and detached on the gate repeatedly, a continuing change of surface potential would not be detected after the introduction of sperms. Moreover, the fertilization occurred on the gate surface a few minutes after introducing the sperms, which was confirmed by the formation of a fertilization membrane. Cell division occurred on the gate area to 2-cell at about 1 h, 4-cell at about 2.5 h, 8-cell at about 3.5 h, 16-cell at about 5.5 h and morula at about 8 h after fertilization. The surface potential of the bio-FET increased gradually to to the 8-cell stage, then decreased after that. The increase of surface potential at the beginning of cell division indicates an increase of hydrogen ions induced by dissolved carbon dioxide created by respiration. Since little surface potential change was measured before fertilization, when virginal ova were kept on the gate surface, the shift of surface potential based on the increase of hydrogen ion might reveal the change of function of the respiration system due to fertilization. Mitochondria play an important role to respiration inside cells. Pyruvate molecules produced by glycolysis are transported into the mitochondrion matrix through the inner membrane, where they are oxidized and combined with coenzyme A to form carbon dioxide, acetyl-CoA, and NADH. The fertilized ova would become activated, accompanied by cell division, resulting in changes of shape and amount of mitochondria (Mills and Brinster 1967), which produce ATP and are closely related to the metabolism of the embryo. Cell division progressed to the 8-cell phase in a relatively short time, so the surface potential shifted increasingly due to the increase of hydrogen ions. On the other hand, the surface potential decreased gradually after reaching the 8-cell phase. The cell division of each blastomere occurred at random, and each step of cell division took longer than the previous step. For example, the transition from 8-cell to 16-cell took longer than that from 2-cell to 4-cell, resulting in the degeneration of

respiration for one fertilized ovum in a constant period. This explains why the concentration of hydrogen ions would decrease based on the diffusion at the interface between the embryo and the gate surface after the 8-cell phase. This means the effect of diffusion of hydrogen ions on the electrical signal was stronger than that of soluble carbon dioxide due to respiration. Thus, the embryo activity at the beginning of development can be easily detected in a real-time and noninvasive manner by use of the bio-FET.

The inhibition of cytokinesis may make it clear that the bio-FET detected embryo activity based on the cell division. The drug cytochalasin B is utilized to evaluate the functional role of contractile ring filaments in cleavage activity (Schroeder 1972). Figure 3 shows the effect of cytochalasin B on the electrical signal of the bio-FET after fertilization. Since the sperms were introduced to the ova on the gate surface resulting in fertilization, the cytochalasin B was added to the embryo on the gate after 30 min. The positive shift of surface potential due to cell division was inhibited by the introduction of cytochalasin B, as shown in Fig. 3a. The photograph of an embryo affected by cytochalasin B showed the inhibition of cytokinesis (Fig. 3b). From this result, the electrical signals of the bio-FET revealed that the embryo activity could be estimated by respiration activity based on cell division.

The maximum of surface potential change was 23.8 mV at 4.1 h after fertilization in Fig. 2. Basically, the FET chip utilized in this measurement showed a change of gate voltage of 53.6 mV/pH for pH variation. The average change of surface potentials after fertilization was about 22.2 mV with standard error ±1.6 mV for three bio-FETs. Therefore, respiration activity triggered by fertilization caused the change of about pH 0.4 at the interface between the embryo and the gate surface. Strictly, the shift of pH from 8.1 to 7.7 was detected at the interface between the embryo and the gate by the bio-FET. The eliminated carbon dioxide was calculated as about 6.0×10^{-9} M based on the concentration change of hydrogen ions corresponding to pH variation, according to the equilibrium of carbon dioxide in solution. In the calculation, the oxygen consumption, the ATP synthesis and so on in the citric acid cycle inside the mitochondrion could be estimated from the amount of eliminated carbon dioxide, which is worked out from the electrical signal of the bio-FET. However, the diffusion of carbon dioxide at the interface between the embryo and the gate has to be considered in order to accurately estimate the consumption and the generation based on respiration activity. The biochemical information obtained by the electrical signals of the bio-FET, however, is significant for the convenient and noninvasive evaluation of embryo activity and quality.

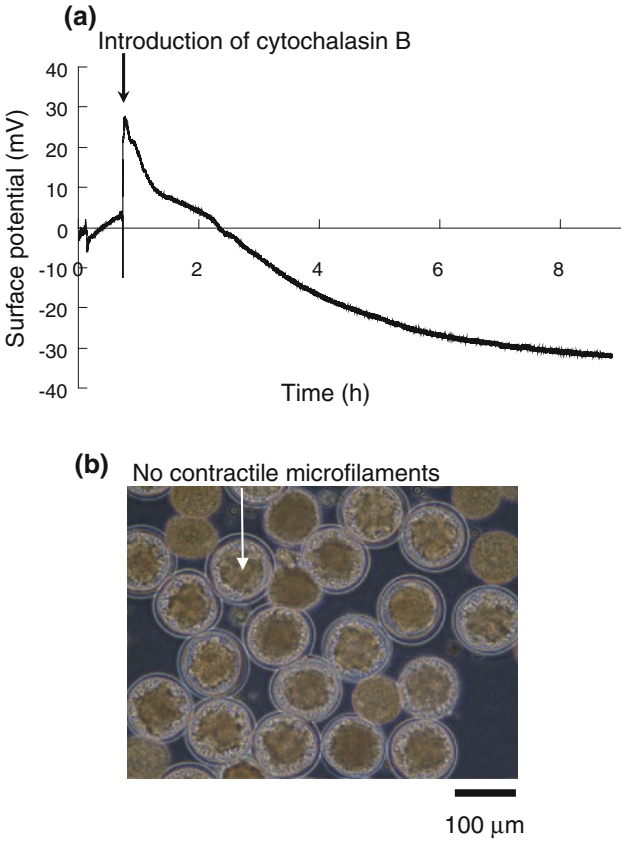

Fig. 3 Effect of inhibition of cytokinesis on electrical signal of the bio-FET. The drug cytochalasin B was added to the fertilized ova at 30 min after fertilization. The cytokinesis of most ova was not found after introducing the drug (**a**). Photograph of sea urchin embryo at 4 h after introduction of cytochalasin B to the fertilized ova. It inhibited cytoplasmic division by blocking the formation of contractile microfilaments (**b**)

Conclusions

In this study, we demonstrated real-time and noninvasive electrical monitoring of sea urchin embryo activity by use of the bio-FET. The electrical signal of the bio-FET was based on the charge density changes of hydrogen ion at the gate sensing surface. The concentration of hydrogen ions at the interface between the embryo and the gate surface was varied by dissolving carbon dioxide in artificial sea water based on the respiration activity of embryos. Since the output of the bio-FET is an electrical signal, the embryo activity could be quantitatively estimated using the bio-FET. The platform based on the bio-FET is suitable for a simple, real-time, label-free and noninvasive detection system of embryo activity, and could be applied in embryo diagnosis for in vitro fertilization (IVF) of mammals, such as humans, in the future.

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