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Response enhancement of olfactory sensory neurons-based biosensors for odorant detection*

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Abstract: This paper presents a novel strategy for the response enhancement of olfactory sensory neurons (OSNs)-based biosensors by monitoring the enhancive responses of OSNs to odorants. An OSNs-based biosensor was developed on the basis of the light addressable potentiometric sensor (LAPS), in which rat OSNs were cultured on the surface of LAPS chip and served as sensing elements. LY294002, the specific inhibitor of phosphatidylinositol 3-kinase (PI3K), was used to enhance the responses of OSNs to odorants. The responses of OSNs to odorants with and without the treatment of LY294002 were recorded by LAPS. The results show that the enhancive effect of LY294002 was recorded efficiently by LAPS and the responses of this OSNs-LAPS hybrid biosensor were enhanced by LY294002 by about 1.5-fold. We conclude that this method can enhance the responses of OSNs-LAPS hybrid biosensors, which may provide a novel strategy for the bioelectrical signal monitor of OSNs in biosensors. It is also suggested that this strategy may be applicable to other kinds of OSNs-based biosensors for cellular activity detection, such as microelectrode array (MEA) and field effect transistor (FET).

Key words: Olfactory sensory neurons (OSNs), Response enhancement, Light addressable potentiometric sensor (LAPS), Olfactory-based biosensor

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INTRODUCTION

The mammalian olfactory system can recognize and discriminate a large number of distinct odorants and shows a very high sensitivity. Odor detection via the olfactory system relies on the olfactory sensory neurons (OSNs). OSNs can respond to odorants and convert the chemical signals of odorant molecules into action potentials (Buck and Axel, 1991). This feature makes them suitable to serve as sensitive elements in biosensors for odorant detection. Nowadays, more and more efforts have been devoted to the research of biomimetic olfactory-based biosensors due to their potential commercial prospects and promising industrial applications. Based on olfactory transduction mechanisms, many types of biomimetic olfactory-based biosensors have been developed by combining OSNs with various secondary sensors (Wu *et al.*, 2007), such as field effect transistor (FET) (Schütz *et al*., 2000) and microelectrode (Huotari, 2000). However, the sensitivity and signal-to-noise ratio (SNR) of these biosensors are limited (Schöning *et al*., 2000), which affects their practical applications. So, there is an urgent need to find novel approaches to improve the sensitivity or SNR of these biosensors. To achieve a higher sensitivity or SNR, it is necessary to enhance the responses of sensitive elements to the corresponding stimulations. In the cases of OSNs-based biosensors, it means to enhance the responses of OSNs to odorants.

It has been demonstrated that blocking the

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phosphatidylinositol 3-kinase (PI3K) activity can enhance the responses of rat OSNs to a complex odorant, and that LY294002, which is the specific inhibitor of PI3K (Vlahos *et al*., 1994), can be used to enhance the responses of rat OSNs to odorants (Spehr *et al*., 2002). Inspired by this biological mechanism, we thus attempted to apply this enhancive effect to improve the responses of OSNs-based biosensors. By the use of LY294002, the responses of OSNs-based biosensors are expected to be enhanced due to the enhancive effect of LY294002 on the responses of OSNs to odorants.

Light addressable potentiometric sensor (LAPS) is a surface potential detector and can be used to monitor extracellular potential by culturing excitable cells on the chip (Hafeman *et al*., 1988). LAPS has many advantages such as convenience, simplicity, low cost, and addressable measurement. There are several reports on the research of using LAPS as a cell-semiconductor hybrid system to monitor the extracellular potentials of cells cultured on the surface of LAPS chip (Ismail *et al*., 2003; Stein *et al*., 2004; Xu *et al*., 2005; Liu *et al*., 2006; Zhang *et al*., 2008). Fig.1 shows the basic principle of LAPS detecting extracellular potential of OSNs cultured on it. When the LAPS is biased in depletion and with proper light illuminated on it, the width of the depletion layer is a function of the local value of the surface potential. The local value of the bias voltage can be read out with ac photocurrent, which can generate corresponding fluctuation when the extracellular potential of cells cultured on LAPS is changed. In this way, it is possible to record the changes in the extracellular potential by measuring the local surface potential at the illuminated region. As the light source is able to move arbitrarily above the LAPS chip, cells can be cultured randomly and the changes of the extracellular potentials can be detected addressably.

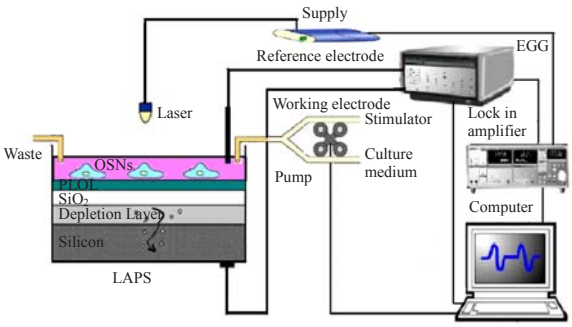


Fig.1 Schematic diagram of basic extracellular potential detection mechanism of LAPS and LAPS test setup

Due to the advantages of LAPS mentioned above, in the present study we designed and built the structure of OSNs-based biosensor on the ground of LAPS to validate the efficiency of this novel strategy for enhancing the responses of OSNs-based biosensors. Rat OSNs were cultured on the surface of LAPS and served as sensing elements. The responses of OSNs to odorants with and without the treatment of LY294002 were recorded by LAPS. If the responses of this OSNs-LAPS hybrid biosensor can be enhanced by the use of LY294002, this strategy may also be applicable to other kinds of OSNs-based biosensors for cellular potential detection, such as microelectrode array (MEA) and FET.

MATERIALS AND METHODS

Materials

Silicon wafer (*n*-doped, <100>) with a specific resistance of 10~15 Ω·cm was thermal oxidized with a layer of 30 nm SiO_2. Then the back of silicon wafer was grinded to 100-μm thickness and a layer of Al was evaporated on the wafer to create an ohmic contact, which was used as working electrode. Via a Pt wire as reference electrode in the electrolyte, a bias voltage could be applied between the backside of the chip and the electrolyte. LAPS chip was fixed on the bottom of the chamber made by polydimethylsiloxane for cell culture and measurement.

Mammalian Ringer's solution consisted of 138 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mmol/L glucose (pH 7.4). Phosphate buffered saline (PBS) solution consisted of 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4 (pH 7.4). Poly-L-ornithine and laminin mixture included 100 μg/ml poly-L-ornithine PBS and 8 μg/ml laminin PBS (1:1, v/v).

Odorants were prepared as 0.5 mol/L solution in dimethyl sulfoxide (DMSO) and stored at −20 °C. LY294002 was prepared in DMSO at 32.2 mmol/L. Final solutions were prepared fresh on the day of use in mammalian Ringer's solution, the buffering capacity of which was sufficient to maintain pH 7.4. The

ultimate concentrations of odorants and LY294002 applied to OSNs were 0.1 mmol/L and 10 μmol/L, respectively. The odorants contained 5 compounds: acetic acid, octanal, cineole, hexanal, and 2-heptatone. LY294002 and odorant compounds, except acetic acid, were purchased from Sigma-Aldrich (USA). Dulbecco's Modified Eager's Medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL (USA). All other chemicals were of analytically pure grade or better quality.

Cell culture

Rat OSNs were harvested from Sprague-Dawley rats (1~3 d old) (Wu *et al*., 2009). Briefly, after decapitation, the rat nose was removed from the head and the olfactory neuroepithelium was tenderly dissected from the nose. The olfactory neuroepithelium was subsequently scissored into pieces and dissociated by 0.25% (w/v) trypsin (pH 7.4~7.8) at 37 °C for 20 min. Then the solution was changed to mammalian Ringer's solution and blew repeatedly with pipette. After filtered with a 200-mesh sieve into fresh DMEM with 10% (v/v) FBS, the suspension was prepared. Finally, the prepared cells were added into the cell culture chamber and allowed to seed on the LAPS surface in 5% CO_2 atmosphere at 37 °C. Before cell seeding, the surface of LAPS chip was modified by the poly-L-ornithine and laminin mixture to facilitate cell attachment (Ismail *et al*., 2003). On the 3rd day, OSNs were mature and available for further experiments.

Scanning electron microscope (SEM)

For SEM observation, rat OSNs were prepared according to standard protocol (Braet *et al*., 1997). OSNs were cultured on the surface of LAPS chip for 3 d. After washed twice with PBS, OSNs were fixed with 2% (v/v) glutaraldehyde at 4 °C for 12 h. Then glutaraldehyde was blotted up and OSNs were washed twice with PBS and post-fixed with 1% (v/v) osmium at 4 °C for 1 h. The samples were then washed twice with PBS and dehydrated in ethanol (once in 70%, 80%, and 90%, thrice in 100%; 10 min each). The samples were immersed for 3 min in amyl acetate and the excess amyl acetate was blotted away with filter paper. After hexamethyldisilazane drying, the samples were mounted on stubs and sputter coated with 10 nm gold. Samples were examined with an SEM (Cambridge Stereoscan 260, Cambridge, UK) at an accelerating voltage of 20 kV.

Test setup and measurement

LAPS test setup is similar to the system we previously reported (Xu *et al*., 2005; Liu *et al*., 2006; Zhang *et al*., 2008). Fig.1 shows the schematic diagram of the LAPS test setup. When the OSNs cultured on the surface of LAPS were ready to be measured, the detection chamber with LAPS chip was fixed under a microscope objective in the setup. The light generated by a He-Ne semiconductor laser (Coherent Co., USA) was modulated and focused to less than 10 μm. Then the light with 543.5 nm wavelength and 5 mW power was used to highlight on the desired neuron. The responses of OSNs to stimulations were monitored by LAPS chip, which can generate corresponding photocurrent fluctuations at once. The photocurrent fluctuations were subsequently transmitted into peripheral equipments via the electrodes of potentiostat (EG & G Princeton Applied Research, M273A, USA). The data were collected, analyzed and stored by the use of a 16-bit data collection card and the software of LABVIEW. A peristaltic pump was used to pump culture medium or stimulator alternatively into the detection chamber through a degasser and a selective valve. A personal computer controlled the on-off of pump and the temperature of chip. The whole setup was shield with a copper box to exclude ambient light and to minimize background noise. All measurements were performed at (37±0.2) °C.

Statistical analysis

Data are expressed as the mean±*SEM*. Student's *t*-test was performed and $P<0.01$ was considered statistically significant.

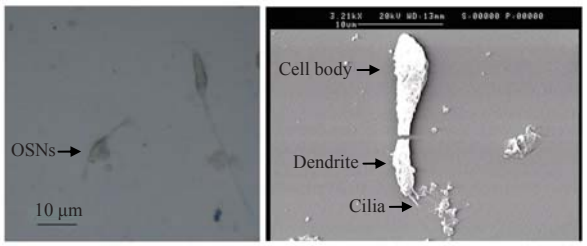
RESULTS AND DISCUSSION

Cells cultured on LAPS chip

OSNs in all vertebrates are characterized by cycles of birth, maturation, and death (Ache and Young, 2005). OSNs have the ability to turn over and replace themselves throughout the life of the animal. To use OSNs as sensitive elements, it is necessary to integrate OSNs with secondary transduce sensors properly and keep them functionally alive. In this study,

poly-L-ornithine and laminin mixture was used to modify the surface of LAPS chip to improve the biocompatibility of the silicon device (Ismail *et al*., 2003). SD rat postnatal within 1~3 d was used to prepare for OSNs. Because the olfactory system of these rats is immature, OSNs prepared from them can grow more efficiently on the LAPS chip than those from adult rats (data not shown).

OSNs are slender and bipolar neurons, having dendrites that extend toward the surface of olfactory epithelium. Dendrites terminate in a swelling called the olfactory knob, where they give rise to a number of specialized cilia. The membrane of cilia is the location of the olfactory receptors and assumed to provide an extensive, receptive surface for the interactions of odorant molecules with the olfactory receptors. The axon of OSNs typically extends to the olfactory bulb to establish contact with the secondary neurons, such as mitral cells and tufted cells, which are the output neurons of the olfactory bulb and can relay olfactory signals to the olfactory cortex (Mori *et al*., 1999). Fig.2a shows the result of OSNs cultured on the surface of LAPS chip for 3 d. The dendrites of OSNs can be seen clearly under microscope. This indicates that OSNs grew well on the LAPS chip and became mature. To further confirm the formation of olfactory cilia, we used an SEM to observe the OSNs cultured on the LAPS chip. From Fig.2b, some olfactory cilia can be seen extended from the end of dendrite. The gap between the cell body and dendrite may due to the hurt effect during the preparation process for SEM observation. These results demonstrate that OSNs can be efficiently cultured on the LAPS chip and maintain their native morphology to some extent.



(a) (b)

Fig.2 Results of rat OSNs cultured on the surface of LAPS chip for 3 d examined by microscopy (a) and SEM (b)

Under microscopy, OSNs were slender and bipolar and the dendrites can be seen clearly. In SEM, some olfactory cilia can be observed extended from the end of dendrite

Responses of OSNs to odorants

Mammalian OSNs are interesting, challenging objects of investigation, because of their very low thresholds to ambient odorants and their ability to convert the chemical signals of odorant molecules into electrical signals. In odorant exposure, odorant molecules firstly interact with olfactory receptors located in the cilia of OSNs. These interactions can activate specific G proteins, which then activate adenylyl cyclase (AC). AC can increase the level of intracellular cyclic adenosine monophosphate (cAMP), which subsequently initiates a cascade of intracellular enzymatic reactions and ultimately leads to the generation of all-or-none electrical signals called action potentials. In vivo, the information of odorant molecules is first received by OSNs and transduced to action potential. In vitro, OSNs may still maintain their capacity of responses to odorants and encoding the odorant information. To test the function of OSNs cultured on the LAPS chip, the mixed odorants which contained 5 distinct odorant molecules were used to stimulate OSNs. The mixed odorants were diluted by Ringer's solution (0.1 mmol/L). The responses of OSNs to the mixed odorants were recorded by the LAPS test system.

Fig.3 shows the typical extracellular recording of OSNs by the LAPS measurement setup. Fig.3a shows the baseline of the LAPS measurement setup; Fig.3b is the recorded signals in the status of silence, when OSNs were treated without any odorant or drug; and Fig.3c is recorded signals in the status of stimulation, when OSNs were stimulated by the mixed odorants. During the period of silence, some spontaneous spikes were recorded, which were isolated and appeared randomly. This may be due to the spontaneous action potential produced by OSNs. When stimulated by the mixed odorants, the rate of spikes increased dramatically and some continuous spikes were recorded, indicating the responses of OSNs to stimulations.

These results confirm that OSNs cultured on the surface of LAPS still maintain their response capacity to odorants and this hybrid biosensor can monitor the extracellular potential of OSNs efficiently. In this study, about 1/3 of OSNs showed responses to the mixed odorants. We speculated that the reason that not all the OSNs responded to the mixed odorants may be due to the fact that the mixed odorants we

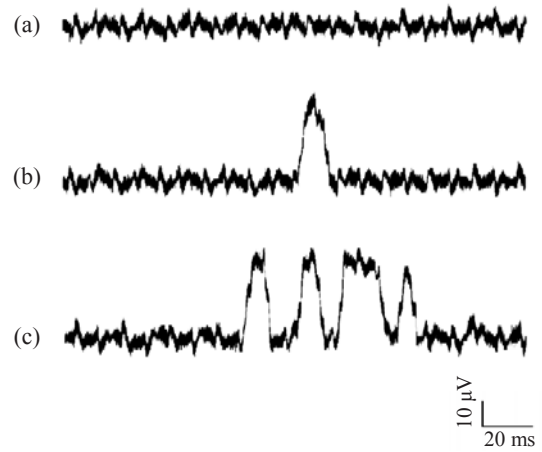


Fig.3 Typical extracellular recordings of OSNs by the LAPS measurement system. (a) Baseline, the baseline of the LAPS measurement system; (b) Silence, when OSNs were treated without any odorant or drug; (c) Stimulation, when OSNs were stimulated by the mixed odorants

used to stimulate OSNs were insufficient to cover the chemical space of all these OSNs. In the mammalian olfactory system, each OSN expresses only one type of olfactory receptor from the large olfactory receptor family encoded by more than 1000 genes scattered throughout the genome (Ache and Young, 2005; Lewcock and Reed, 2003). Each kind of odorant molecule can excite a few kinds of the olfactory receptors and each kind of olfactory receptor can respond to a few distinct odorant molecules (Ressler *et al*., 1994).

Responses enhanced by PI3K inhibitor

PI3K-dependent phosphoinositide signaling has been implicated in diverse cellular systems coupled to receptors for many different ligands, including olfactory transduction (Spehr *et al*., 2002). In vertebrates, odorants excite OSNs through cyclic nucleotide signaling. Spehr's findings suggest that 3-phosphoinositide signaling modulates cyclic nucleotide signaling in rat OSNs, and that blocking PI3K can enhance the response of rat OSNs to a complex odorant. With LY294002 pretreatment, the average odorant-evoked increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of OSNs was (1.85±0.30) times that evoked by the odorants alone (Spehr *et al*., 2002). The increase in $[Ca^{2+}]_i$ can subsequently enhance the electrical responses of OSNs to odorants.

Fig.4 shows the enhancive effect of LY294002 on the responses of OSNs to odorants. In Fig.4a, stimulation is recorded when OSNs were stimulated by the mixed odorants, and in Fig.4b, enhancement is recorded when LY294002 was used to enhance the responses of OSNs to odorants. We can see from Fig.4c that the spike rate of enhancement increased drastically compared with that of stimulation. However, there was no significant increase in the amplitude of the spikes. This phenomenon may be explained by the character of action potential, which is a kind of "all-or-none" signal and all the action potentials of one neuron have the similar shape and amplitude. Thus, the increase in action potential rate should not result in the amplitude increase of recorded spikes. The same paradigm mentioned above was applied to many OSNs. Although not all the OSNs we had tested responded to the mixed odorants, the enhancive effect could be observed in all the responsive OSNs. The statistical results in Fig.4c show that the spike rate of enhancement was significantly higher than that of stimulation. The reason for this

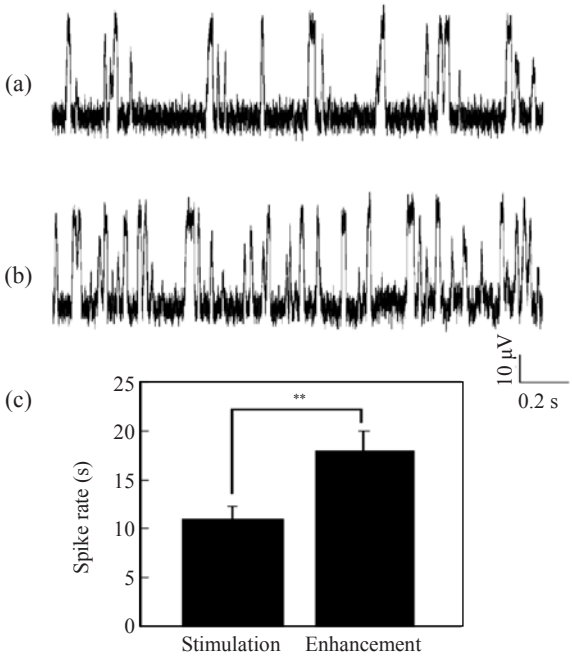


Fig.4 Enhancive effect of LY294002 on the responses of OSNs to odorants recorded by LAPS. (a) Stimulation, when OSNs were stimulated by the mixed odorants; (b) Enhancement, when OSNs were treated with the mixed odorants and LY294002; (c) Statistical results of the the spike rate recorded from the responses of OSNs. **The spike rate of enhancement is significant higher than that of stimulation with *P*<0.01 in *t*-test. All data are represented by the mean±*SEM* (the number of measured cells is 16)

phenomenon may be that the responses of OSNs to odorants were enhanced by LY294002 and the action potential rate increased, subsequently leading to the increase of the recorded spike rate. By the use of LY294002 and the calculation of the recorded spike rates, the responses of ORNs to the same concentration of odorants were (1.55±0.20) times those of the untreated. As to this OSNs-LAPS hybrid biosensor, it means that the responses of this biosensor were enhanced by LY294002 and the response enhancement was about 1.5-fold. These results are consistent with the previous results obtained by calcium imaging (Spehr *et al*., 2002).

CONCLUSION

We demonstrated a novel method for monitoring the enhancive effect of the OSNs-based biosensor. An OSNs-LAPS hybrid biosensor was developed, in which OSNs were used as sensitive elements. The responses of this hybrid biosensor can be enhanced about 1.5-fold by a PI3K inhibitor. It is also suggested that this OSNs-LAPS hybrid biosensor can be a potential tool for the research of olfactory transduction and that this novel strategy may also be applicable to other kinds of OSNs-based biosensors, such as MEA and FET.

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