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A novel surface acoustic wave-based biosensor for highly sensitive functional assays of olfactory receptors

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ABSTRACT

Olfactory receptors, which are responsible for sensing odor molecules, form the largest G protein-coupled receptor (GPCR) family in mammalian animals. These proteins play an important role in the detection of chemical signals and signal transduction to the brain. Currently, only a limited number of olfactory receptors have been characterized, which is mainly due to the lack of sensitive and efficient tools for performing functional assays of these receptors. This paper describes a novel surface acoustic wave (SAW)-based biosensor for highly sensitive functional assays of olfactory receptors. An olfactory receptor of *Caenorhabditis elegans*, ODR-10, was expressed on the plasma membrane of human breast cancer MCF-7 cells, which was used as a model system for this study. For specific odorant response assays, the membrane fraction of MCF-7 cells containing ODR-10 was extracted and integrated with our SAW sensors. The response of ODR-10 to various odorants was monitored by recording the resonance frequency shifts of SAWs applied to the sensor. Our results show that heterologously expressed ODR-10 receptors can specifically respond to diacetyl, its natural ligand. Dose-dependent responses were obtained by performing measurements using various concentrations of diacetyl. The sensitivity of this biosensor is 2 kHz/ng and can detect concentrations as low as 10^{-10} mM, which is 10× lower than what has previously been reported. This biosensor can be used to characterize odorant response profiles of olfactory receptors and provide information rich data for functional assays of olfactory receptors. In addition to providing a greater understanding of the biological mechanisms of GPCRs, such data holds great potential in many other fields such as food industry, biomedicine, and environmental protection.

1. Introduction

Olfactory systems can recognize and discriminate a large number of distinct odorants with extreme high sensitivity and specificity. Since the discovery of gene superfamily encoding olfactory receptors in 1991, great advances have been made in the research of olfactics [1,2]. Olfactory receptors have been identified from chemosensory cells of various species across phyla [3,4]. In mammalian, olfactory receptors form the largest G protein-coupled receptor (GPCR) family. It is a large family of membrane proteins which play important roles in the detection of chemical signals outside cells. To have a better understanding behind olfactory mechanisms, it is crucial to identify the cognate ligands that interact with the olfactory receptors. Various functional assays have been used to characterize odorant response profiles of olfactory receptors such as electroolfactogram [5], Ca^{2+} imaging [6,7], quartz crystal microbalance measurements [8–10], and semiconductor potentiometric sensing [11–13]. Currently, only a limited number of olfactory receptors have been functionally characterized mainly due to the lack of enough sensitivity and efficiency of above mentioned approaches for performing such assays.

Surface acoustic wave (SAW) sensors have several advantages over above mentioned sensors such as high sensitivity, small size, and uncomplicated integration. These sensors have been previously applied for high sensitivity, real-time detection of intermolecular interactions for functional assays [14,15]. SAW sensors generate and detect acoustic wave using interdigital transducers (IDTs) on the surface of a piezoelectric crystal. The acoustic energy is strongly confined at the surface of the crystal in the range of acoustic wavelength, and is independent of its thickness. SAWs are thus very sensitive and are capable of measuring minute changes in mass on the sensor's surface. These changes can be monitored by recording the resonance frequency shifts of the

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SAWs, which are proportional to the mass changes on the surface. To our best knowledge, no previous work has been reported on the utilization of SAWs for functional assays of olfactory receptors.

In this study, a novel SAW-based biosensor was developed for highly sensitive functional assays of olfactory receptors, which can provide information rich data characterizing the response profiles of olfactory receptors. An olfactory receptor of *Caenorhabditis elegances*, ODR-10 [16], was employed as a model of olfactory receptors and expressed on the plasma membrane of human breast cancer MCF-7 cells. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to verify the expression of ODR-10 at the mRNA level. Additionally, the distribution of expressed ODR-10 on the surface of MCF-7 cells was determined by fluorescent staining experiments. The expressed ODR-10 receptor was extracted and integrated onto the SAW sensor for odorant response assays. The response of ODR-10 to various odorants was then monitored by recording the resonance frequency shifts of SAWs. Various concentrations of diacetyl, which is a natural ligand of ODR-10 [17], were used to further characterize the response profile of ODR-10.

2. Materials and methods

2.1. Construction of odr-10 expression vector

PCR was performed to amplify the full-length cDNA of *odr-10* from *pBluescript SK⁻/odr-10* (provided by Professor Cornelia I. Bargmann at the Laboratory of Neural Circuits and Behavior, Howard Hughes Medical Institute, The Rockefeller University, NY, USA). The PCR primers were 5′-GAGTTGGAATTCATGTCGGGAGA ATTGTGG-3′ and 5′-CAGTAAGGATCCCGCGTCGGAACTTGAGACAA-ATT-3′. PCR was carried out in a 50 μL reaction volume using 2.0 μL of the plasmid DNA template, 0.2 μg of target primers, 25 mM dNTP-Mix, and 2.5 U Pyrobest™ polymerase (Takara, Japan). The thermo cycle conditions were 30 cycles of 94 °C, denaturation for 30 s, 55 °C annealing for 1 min, 72 °C extension for 1 min, followed by a final extension at 72 °C for 10 min. The *rho-tag* import sequence was inserted into the N-terminus of ODR-10 [18]. All the DNA sequences were purchased from Takara (Japan). The *rho-tag* sequence was inserted into multiple cloning sites (MCS) of *pFLAG-CMV-3* between Hind III and EcoR I. Then *odr-10* full-length sequence was then subcloned into *pFLAG-CMV-3/rho-tag* digested with EcoR I and BamH I. The sequence of constructed vector *pFLAG-CMV-3/rho-tag/odr-10* was confirmed by DNA sequencing.

2.2. ODR-10 expression and extraction

MCF-7 cells were maintained at 37 °C in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, UK) containing 10% fetal bovine serum (FBS) in 5% CO_2 incubator. One day before transfection, MCF-7 cells were seeded on 6-well plates. When 90% confluent, MCF-7 cells were transfected with 4 μg of expression vector (*pFLAG-CMV-3/rho-tag/odr-10*) for each well by using Lipofectamine 2000 (Gibco, UK) as the DNA carrier. The cells were incubated with the mixture for 6 h at 37 °C. The transfected cells were used for further experiments 24 h after transfection.

The transfected MCF-7 cells were washed with PBS and harvested from the 6-well plates. The concentration of cells was adjusted to 10^{11} cells/mL, which was counted using a hemacytometer (Gibco, UK). The cells were sonicated with a probe-type sonicator for 5 min and centrifuged at 16,000 g for 40 min at 4 °C. The membrane fraction was then separated from the resulting solution through centrifugation and 1 μL aliquot of the membrane fraction containing ODR-10 was evenly coated on the sensor's surface and set to dry at room temperature. The membrane fraction without ODR-10 was utilized as the negative control. For scanning electron microscopy (SEM) imaging, the surface of the SAW sensors were sputter coated with 10 nm of gold. The sensors were examined with SEM (Cambridge Stereoscan 260, UK) at an accelerating voltage of 20 kV.

2.3. RT-PCR and fluorescent staining

Total RNA was extracted using the Trizol Reagent (Invitrogen, USA). The reverse transcription (RT) was performed for 1 h at 42 °C with 200 U reverse transcript enzyme (PrimeScript™ RTase), 62.5 μmol random oligohexamer primers, 10 mmol dNTP-Mix (Takara, Japan), and 2 μg total RNA. PCR was carried out in a 50 μL reaction volume using 2 μg of the previous RT reaction product, 0.2 μg of target primers (5′-GTCGCAGGATCCTCGAGAT-GAACGGGACCGAGGGC-3′ and 5′-CAGTAAGGATCCCGCGTCGGAACT TGAGACAAATT-3′), 25 mmol dNTP-Mix, and 2.5 U Taq polymerase (Takara, Japan). PCR was performed under the conditions: 5 min, 94 °C preincubation, followed by 30 cycles of denaturation (1 min, 94 °C), annealing (50 s, 55 °C), and extension (80 s, 72 °C), followed by a final extension of 5 min at 72 °C. PCR products were analyzed by gel electrophoresis.

For fluorescent staining experiments, transfected MCF-7 cells were seeded on 6-well plates and fixed with a freshly prepared mixture of methanol:acetone (1:1) for 5 min at room temperature. After washing 4× with phosphate buffered saline (PBS) (pH 7.4), cells were incubated with anti-flag M2 monoclonal antibody-FITC conjugate at 10 μg/mL for 1 h at 4 °C. To visualize the surface expression of ODR-10, cells were washed twice with PBS and examined under a confocal fluorescence microscope (Leica TCS-SP, Germany) with an excitation wavelength of 492 nm and an emission wavelength of 520 nm.

2.4. SAW sensor and measurement system

The SAW sensor and measurement system used in this work are similar to that reported in prior work [19]. Briefly, the SAW sensor is developed on a ST-cut $LiNbO_3$ crystal [20] and is comprised of a pair of IDTs and a sensitive area. The detection region is 0.5 mm^2 and is located between the IDTs. Fig. S1(a) is the photograph of SAW sensor. Fig. S1(b) shows the transmission response of the SAW sensor, which indicates its central frequency is 120.1 MHz. The bandwidth is 0.9 MHz. The quality coefficient is 133.5. The schematic diagram of the SAW measurement system is shown in Fig. S1(c). It is comprised of a pair of SAW sensors; one is coated with olfactory receptors, which is the working sensor, while the other sensor has a bare surface, which serves as the reference. Both SAW sensors are mounted on a semiconductor-refrigerating chip. Cascade RF amplifiers, which drive the sensors, and a mixer to output their frequency difference are also fixed on the semiconductor-refrigerating chip. A custom circuit, consisting of a low-pass filter and a comparator, is utilized to generate a square wave. Another circuit is utilized as a frequency counter, which consists of a microprocessor and a trigger flip-flop. RS232 port is used to transport the experimental data to a computer.

The resonance frequency shifts of SAWs were recorded to monitor changes in mass on the sensor's surface. Once the resonant frequency of the sensor reached a steady baseline, various odorants were applied to the sensor's surface. In order to detect the odorants, the SAW sensor was capsulated in a sealed hexagonal flask with inlet and outlet ports. All the odorants used in this study were purchased from Sigma–Aldrich (USA). The concentrations of odorants were freshly prepared in Tedlar bags (SKC Inc., USA) by a liquid organic gas blender (MF-3B,

Huanchen Instruments, China) and were injected into the detection chamber by syringe pump. The final vapor concentration of each odorant was 1 nM. After each measurement, the detection chamber was refreshed by injecting a stream of pure N_2. To minimize influences from environmental factors, all the measurements were carried out at the same environmental conditions with the humidity at 10% and temperature at 25 °C. All the chemicals used in this study were of analytical pure grade or better quality. Ultrapure deionized water (18.2 MΩ/cm) was obtained from a Milli-Q system (Bedford, USA).

2.5. Statistical analysis

The experimental data was expressed as means ± standard deviation (SD). The significance was carried out by performing student's *t*-test of the data. $p < 0.05$ was considered as statistical significant.

3. Results and discussion

3.1. Expression of ODR-10 on the plasma membranes of MCF-7 cells

Olfactory receptors are members of the superfamily of GPCRs, which contain 7 transmembrane domains [21]. The hydrophobicity of the cell membrane is essential for maintaining the natural structure and function of the olfactory receptors. In this study, ODR-10 was expressed on the plasma membrane of MCF-7 cells. Fig. 1(A) shows the structure of the constructed expression vector. A *flag-tag* was fused on the N-terminal of ODR-10 to visualize its expression on the cell's plasma membrane. To improve the expression of ODR-10 on cell plasma membrane, a *rho-tag* import sequence was inserted immediately following the *flag-tag* [18]. In Fig. 1(B), a bright band appears at 1 kb which verifies the results of RT-PCR. This result is consistent with the gene length of *odr-10* and also indicates the expression of ODR-10

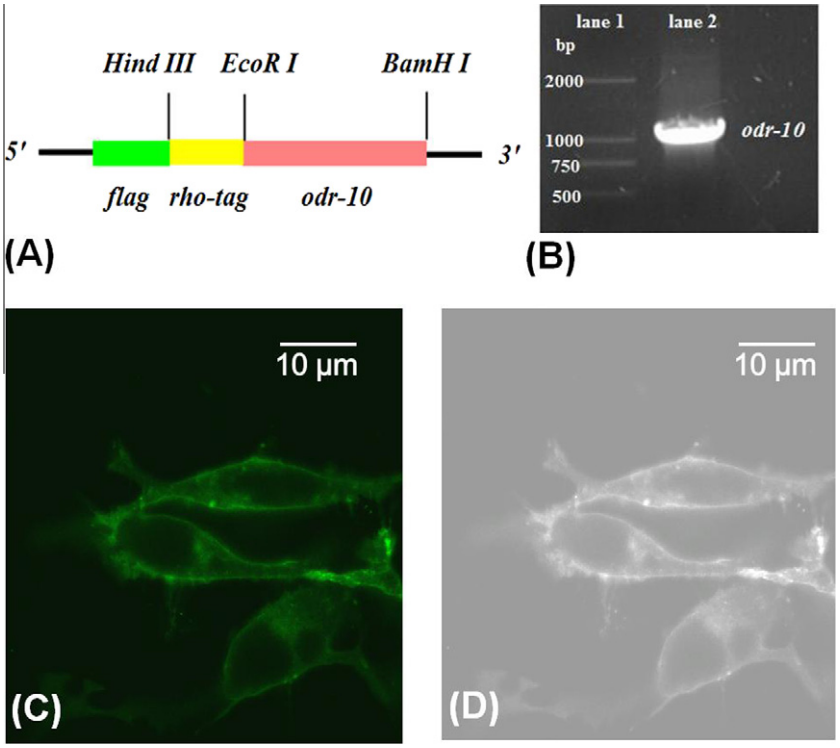

Fig. 1. (A) Schematic diagram of constructed mammalian ODR-10 expression vector *pFLAG-CMV-3/rho-tag/odr-10*. (B) An argose gel of RT-PCR products. Lane 1 is the DNA size marker; lane 2 is the products of RT-PCR. Confocal microscope images of transfected MCF-7 cells taken under (C) fluorescent field and (D) optical field. Anti-flag M2 monoclonal antibody-FITC conjugate was used to visualize the expressed ODR-10. The excitation and emission wavelength were 492 and 520 nm, respectively.

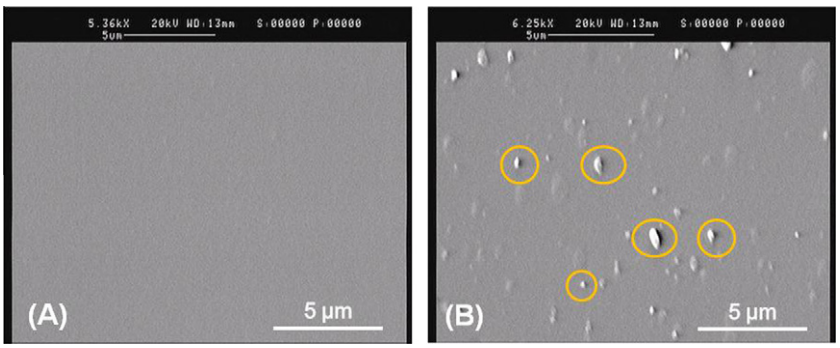

Fig. 2. SEM images of SAW sensors (A) with a bare surface and (B) with cell membrane fractions containing ODR-10 coated on the surface.

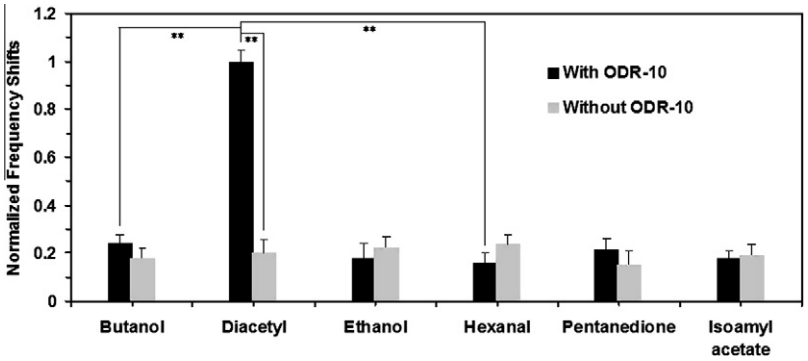

Fig. 3. Response assays of ODR-10 to various odorants. ODR-10 specifically responds to diacetyl at a concentration of 10^{-6} mM. The responses of various odorants were normalized to that of diacetyl. All the data are represented by means ± SD. $**p < 0.05$, Student's *t*-test. The mean and SD of 12 experiments are shown.

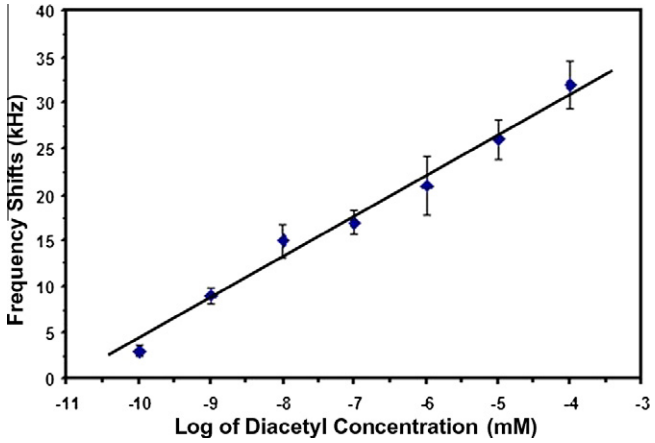

Fig. 4. Dose-dependent responses of ODR-10 to serial concentrations of diacetyl. The mean and SD of 6 experiments are shown.

in MCF-7 cells at the mRNA level. Fluorescent staining experiments were performed to determine whether the ODR-10 receptors were present on the surfaces of the MCF-7 cells. Our results, Fig. 1(C) and (D), show that ODR-10 was expressed at high levels with 40% of the cells being transfected. Specifically, Fig. 1(C) reveals that the fluorescence is mainly distributed on the cell membrane, which indicate that the ODR-10 receptors were effectively expressed in the cells and evenly distributed across the plasma membrane.

3.2. ODR-10 coupled with SAW sensors

In this study, a membrane fraction of MCF-7 cells containing ODR-10 was extracted and evenly coated on a SAW sensor for odorant response assays. By this method, the expressed ODR-10 receptors can still remain embedded in the cell membrane, which provides a hydrophobic environments for maintaining its natural structure and function.

The resonance frequency shifts of SAWs were measured before and after coating the membrane fraction onto the sensor's surface. To accurately control the quantity of cell membrane fractions coated onto the sensor's surface, the concentration of cells was adjusted to 10^{11} cells/mL for each iteration of membrane extraction. The frequency shifts of SAWs resulting from the membrane coating corresponded to the concentration of membrane fraction on the sensor's surface. Our measurements show that the differences of frequency shifts between each iteration were less than 2 kHz (n = 14). In addition, SEM was employed to characterize the membrane fraction coating on the sensor surface. Fig. 2(A) and (B) are SEM images of a bare sensor and a sensors coated with membrane fraction respectively. By comparing Fig. 2(A) and (B), we can see that the membrane fraction is evenly coated on the sensor surface. Some clusters of proteins can be observed in Fig. 2(B) as indicated by the yellow round circles, which contain the ODR-10 receptors.

3.3. Odorant response assays of ODR-10

To characterize the odorant response profiles of ODR-10, various odorants including diacetyl, ethanol, butanol, pentanedione, hexanal, and isoamyl acetate were tested using our SAW biosensor. The resonance frequency shifts of SAW were recorded to monitor the responses of ODR-10 to these various odorants.

Fig. 3 are the results of ODR-10 response to various odorants. The frequency shifts were normalized to that of diacetyl. The membrane fraction of MCF-7 cells without ODR-10 was used as a negative control. This data clearly shows that ODR-10 specifically responds to its natural ligand, diacetyl [16], where the frequency shift was 4× larger for the ODR-10 coated sensor compared with the uncoated sensor. Other odorants such as ethanol, butanol, pentanedione, hexanal, and isoamyl acetate, resulted in significantly smaller responses compared with that of diacetyl. These frequency shifts could result from non-specific interactions between the odorant molecules and the membrane fraction of the MCF-7 cells.

The sensitivity of this biosensor was determined by measuring various concentrations of diacetyl. Fig. 4 shows the dose-dependent response of ODR-10 to diacetyl in the range of 10^{-10} to 10^{-4} mM. Based on this data, the sensitivity of the sensor was determined to be 2 kHz/ng and the lowest concentration that could be detected was 10^{-10} mM, which is 10× lower than what has previously been reported [10]. These results demonstrate that this SAW-based biosensor can be used as an efficient tool for highly sensitive functional assays of olfactory receptors. It could make contributions to the progress of odorant response assays of olfactory receptors. This SAW-based biosensor also holds great potential in many other fields such as food industry, biomedicine, and environmental protection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.073.

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