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A biomimetic olfactory-based biosensor with high efficiency immobilization of molecular detectors

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ABSTRACT

The immobilization efficiency of molecular detectors is of great importance with regard to the performances of biosensors such as the sensitivity, stability, and reproducibility. This paper presents a biomimetic olfactory receptor-based biosensor with better performances by improving the immobilization efficiency of molecular detectors for odorant sensing. A mixed self-assembled monolayers (SAMs) functionalized with specific olfactory receptors (ODR-10) was constructed on the sensitive area of surface acoustic wave (SAW) chip. The immobilization of ODR-10 was characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The responses of this biosensor to various odorants were recorded by monitoring the resonance frequency shifts of SAW, which is correlated to the mass loading on its sensitive area. All the results demonstrate this biosensor can specifically respond to the natural ligand of ODR-10, diacetyl, with high sensitivity and stability. The sensitivity is 4 kHz/ng, which is 2× higher than that of previous work. The detection limit is 1.2×10^{-11} mM. The major advances on immobilization efficiency of molecular detectors presented in this work could substantially promote and accelerate the researches and applications of olfactory receptor-based biosensors with different transducers, such as quartz crystal microbalance (QCM), surface plasma resonance (SPR), and field effect transistors (FET).

1. Introduction

The advances on the signal transduction mechanisms of biological olfactory systems (Buck and Axel, 1991; Ache and Young, 2005) have made it possible for mimicking nature's approach to develop novel biosensors for chemical sensing with extreme high sensitivity and specificity. Specifically, the unique ability of olfactory receptors interacting with the specific odorant molecules makes them ideal candidates as molecular detectors for biosensors towards odorant detection. In recent decades, olfactory receptors have been combined with various transducers to develop biomimetic olfactory-based biosensors (Wu et al., 2007), such as quartz crystal microbalances (QCM) (Wu, 1999; Ko and Park, 2005; Sung et al., 2006), Surface acoustic wave (SAW) (Wu et al., 2011), field effect transistors (FET) (Schütz et al., 2000), microelectrodes (Huotari, 2000), and light addressable potentiometric sensors (LAPS) (Wu et al., 2009). However, these olfactory-based biosensors suffer greatly from the unstable and low efficiency immobilization due to the direct combinations of olfactory receptors with transducers by physical absorption.

Olfactory receptors consist of 7 transmembrane domains and some domains are directly related to the specific binding with odorant molecules (Krautwurst et al., 1998; Dryer and Berghard, 1999). The hydrophobic environment of cell membrane is crucial to the natural structures and functions of olfactory receptors. Stable and high efficiency immobilization of functional olfactory receptors on the sensitive area of sensing chips is of great importance with regard to the performances of biosensors, such as the sensitivity, stability, and reproducibility. On the other hand, self-assembled monolayers (SAMs) can significantly improve the immobilization efficiency by providing a reproducible, ultrathin, and well-ordered functional layer for biological molecule immobilization (Qian et al., 2002; Su and Li, 2004). SAMs can provide anchoring sites for further attachments, which are prepared by spontaneous tethering of molecules with active chemical moieties onto reactive solid surfaces (Wong and Ho, 2009). In addition, the SAMs technique has unique advantages such as ease of preparation, low cost, and versatility (Ulman, 1996). Herein, we exploited the use of SAMs technique for improving the immobilization efficiency of olfactory receptors on the sensitive area of transducers.

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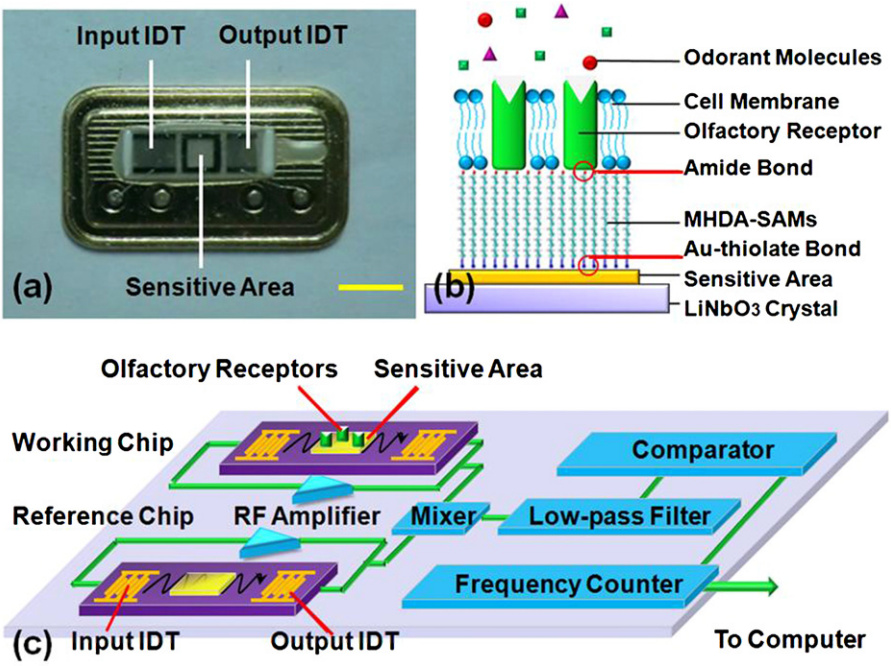

Fig. 1. (a) Photograph of a SAW chip. It shows the input and output interdigital transducers (IDTs) as well as a square sensitive area (scale bar = 50 mm). (b) Schematic diagram of functional immobilization of olfactory receptors on the sensitive area of SAW chips. (c) Schematics of the SAW measurement system.

Surface acoustic wave sensors permit a highly sensitive approach for the detection of interactions between biological molecules (Andle and Vetelino, 1994; Länge et al., 2008). Along our previous works (Wu et al., 2011), this work employed SAMs to improve the immobilization efficiency of olfactory receptors on the sensitive area of SAW chips for the sake of sensitivity enhancement. A bioengineered olfactory receptor of *C. elegances*, ODR-10, was served as molecular detectors for odorant detection, which was expressed on the plasma membrane of human breast cancer MCF-7 cells (Sengupta et al., 1996). SAMs with expressed ODR-10 in its membrane fraction was constructed on the sensitive area of SAW chips. The immobilization of ODR-10 was characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The responses of this olfactory receptor-based biosensor to various odorants were monitored by recording the resonance frequency shifts of SAW. In addition, heat-treated cell membrane fractions with ODR-10 were also tested to further characterize the responsive property of this biosensor.

2. Materials and methods

2.1. Materials

The olfactory receptor of *C. elegances*, ODR-10, was heterologously expressed in human breast cancer MCF-7 cells and the membrane fractions were prepared as previously reported (Wu et al., 2011). The membrane fractions containing ODR-10 were frozen and stored at −80 °C in order to maintain the biological activity of ODR-10. 16-Mercaptohexadecanoic acid (MHDA), 1-ethyl-3-(3-dimethylaminoprppyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and odorant molecules including diacetyl, butanone, and 2,3-pentanedione, were all purchased from Sigma–Aldrich (USA). All other chemicals were of analytical pure grade or better quality.

2.2. SAW chip fabrication and functionalization

SAW chip was designed and fabricated on the basis of ST-cut $LiNbO_3$ crystal using the conventional micro-fabrication techniques (Soluch, 1998; Chen et al., 2005). The input and output IDTs was fabricated on the surface of crystal, which consists of 22 and 31 finger pairs, respectively. There is a square area with gold surface located between input and output IDTs. It is 0.5 mm^2 and used as sensitive area for the functional immobilization of olfactory receptors. Fig. 1a is the photograph of the SAW chip, which shows the input and output IDTs as well as sensitive area. For sidelobe suppression, the periodicity and aperture are designed to be 38.5 μm and 0.5 mm, respectively. The central frequency of this SAW chip is 120.1 MHz. Its bandwidth is 0.9 MHz. Its insert loss and quality coefficient are 7.01 dB and 133.5, respectively. In our SAW measurement setup, the output power of SAW is estimated to be −10.11 dBm.

The schematic diagram of SAW chip functionalized with ODR-10 by SAMs is shown in Fig. 1b. Firstly, the SAW chip was fixed in a chamber allowing the only exposure of gold sensitive area to the solution added to the chamber. In order to remove the inorganic and organic contaminants, the sensitive surface of the SAW chip was cleaned thoroughly with 1 mol/L NaOH for 20 min, 1 mol/L HCl for 5 min, and Pirannha solution (30% H_2O_2:H_2SO_4 = 2:3, v/v) for 1 min, in sequence. Then it was rinsed with absolute ethanol and directly blown dried with a stream of nitrogen gas. To obtain a well-organized monolayer, 10 mmol/L MHDA in absolute ethanol solution was used to treat the gold sensitive surface of the SAW chip for 24 h. Then they were thoroughly rinsed with ethanol and successively treated with a mixed solution of 75 mmol/L EDC and 15 mmol/L NHS for 1 h. Finally, ODR-10 membrane fractions were added and immobilized on the SAM. Then phosphate buffered saline (PBS, NaCl 8.00 g/L, KCl 0.20 g/L, Na_2HPO_4 1.38 g/L, KH_2PO_4 0.2 g/L, pH 7.4) was used to remove the excessive ODR-10 membrane fractions and the non-specific adsorption. After dried with a stream of nitrogen gas,

the functionalized SAW chips can be used for further experiments.

2.3. SEM and AFM

SEM and AFM were employed to characterize the immobilization of ODR-10 on the sensitive surface of SAW chips. For SEM, the sensitive surface with and without immobilization of ODR-10 was sputter coated with a 10 nm gold layer and then examined with an accelerating voltage of 20 kV under SEM (Cambridge Stereoscan 260, UK). For AFM, the sensitive surface immobilized with ODR-10 was observed under a MultiMode scanning probe microscopes (Veeco Instruments Inc.) system. AFM images were acquired in tapping mode with Veeco tips which cantilever frequency is 318 kHz.

2.4. Experimental setup and measurements

Fig. 1c is the schematic diagram of the SAW measurement system, which consists of a pair of SAW chip, a low-pass filter, a comparator, a frequency counter, and a computer. A pair of SAW chips is utilized as working chip and reference chip, respectively. On the sensitive area of working SAW chip, olfactory receptors was functionally immobilized and served as molecular detectors for odorant detection. While the sensitive area of reference SAW chip was kept bare. In order to minimize the influences of environmental temperature on the measurements, the pair of SAW chips is mounted on a semiconductor-refrigerating chip. The SAW chips were driven by their own Cascade RF amplifiers. A mixer was employed to mix the signals from working SAW chip and reference SAW chip and output their frequency difference. Then the frequency difference was filtered by a low-pass filter. A comparator is utilized to generate a square wave. A frequency counter, which consists of a microprocessor, trigger flip-flop was used to count frequencies. Finally, the data was transported to a computer by RS232 port.

In this study, the responses of olfactory-based biosensors to various odorants were monitored by recording the resonance frequency shifts of SAW, which can reflect the changes of mass loading on the sensitive area of SAW chips. The SAW chips were fixed in a sealed chamber with input and output pipes. During measurements, the odorants were applied to the detection chamber through input pipe. The measurements started once the resonance frequency of SAW reaches a steady baseline. After each measurement, the detection chamber was refreshed by a stream of nitrogen gas. Odorants were prepared as 0.5 M solution in dimethyl sulfoxide (DMSO) and stored at −20 °C. Saturated vapor of odorants was used as stimuli after they were diluted to the desire concentrations with nitrogen by a liquid organic gas blender (MF-3B, Huanchen Instruments, China). All the measurements were carried out at the same environmental conditions with humidity at 10% and temperature at 25 °C to minimize the influences of environmental factors to the measurements.

3. Results and discussion

3.1. Functional immobilization of ODR-10

Functional immobilization of molecular detectors on the sensitive area of transducers is of great importance with regards to the performances of biosensors. In this study, an olfactory receptor of *C. elegances*, ODR-10, was used as molecular detectors, which was expressed on plasma membrane of human breast cancer MCF-7 cells. ODR-10 in its membrane fraction was extracted and immobilized on the sensitive surface of SAW chips by SAMs technique. In contrast to directly physical absorption, SAMs technique can immobilize the molecular detectors covalently, which is much more stable and effectively. Furthermore, the hydrophobic environments of SAMs facilitate the olfactory receptors to maintain their native conformation and functionality. Consequently, the stability and efficiency of immobilization can be improved by the use of SAMs technique. In this study, a compact SAMs of MHDA with EDC and NHS ester was employed to functionalize the sensitive area of SAW chips. The schematics of SAMs functionalized with ODR-10 is shown in Fig. 1b. Basically, MHDA is a long-chain carboxylic acid-terminating alkanethiol, which can form a well-organized and oriented monolayer on the gold surface via the strong Au–thiolate bond. On the other hand, the monolayer of MHDA can be activated by the mixed solution of EDC and NHS to improve the stability of the linker compounds. The ODR-10 can be immobilized by replacing the active NHS esters via the amide bonds. Compared with directly coating, SAMs technique can immobilize ODR-10 covalently on the sensitive surface of SAW chips with higher efficiency and stability.

In this study, SEM was employed to characterize the functional immobilization of ODR-10 on the sensitive area of SAW chips. Fig. 2a and b shows the SEM images of SAW chips with a bare sensitive area and ODR-10 functionalized sensitive area by SAMs, respectively. By the comparison, we can see that ODR-10 functionalized surface is rough, while the bare surface is pretty smooth. In addition, from Fig. 2b, we can see some dots are distributed on the surface in a pretty uniform manner. The diameter of these dots is pretty similar, which is around hundreds of nm. We anticipated these dots could be the immobilized ODR-10 probably in a cluster form. AFM was used to further characterize the surface morphology of the sensitive surface immobilized with ORD-10. Fig. 2c shows the AFM scans of the sensitive surface immobilized with ODR-10. We can see that similar dot distributions can be observed on the surface. It is indicated the height of the dots is approximately 10–15 nm.

As compared with directly physical absorption reported in our previous works (Wu et al., 2011), the SAMs could improve the immobilization efficiency significantly with regard to the size and distribution of dots on the sensitive surface. Moreover, the covalent immobilization is much stable than physical absorption due to the much stronger forces between molecular detectors and sensitive surface. All the results indicated that SAMs could significantly improve the stability and efficiency of immobilization, which could improve the sensitivity and stability of olfactory receptor-based biosensors. In addition, the regeneration of SAW chip can be easily achieved for some sequential experiments since the immobilized materials can be completely removed by the cleaning process as described in Section 2.

3.2. Detection of odorant molecules

We carried out odorant assays to examine the performances of this biosensor including sensitivity, specificity, and stability. The specific odorant ligand of ODR-10 has been well documented, which is diacetyl (Sengupta et al., 1996). In this study, serial concentrations of diacetyl were applied to the biosensor with SAW chip functionalized with ODR-10. The response signals were recorded by monitoring the resonance frequency shifts of SAW during the application of diacetyl in a certain concentration. Fig. 3 is the statistical results of resonance frequency shifts of SAW in response to diacetyl in the range from 10^{-4} to 10^{-10} mM. The responses of various concentrations of diacetyl were normalized to that of diacetyl at 10^{-4} mM. It is indicated a linear response can be obtained in a dose-dependent manner in the above-mentioned concentration range. The sensitivity of this biosensor is calculated by the following equation:

$$S = \frac{\Delta F}{\Delta M}$$

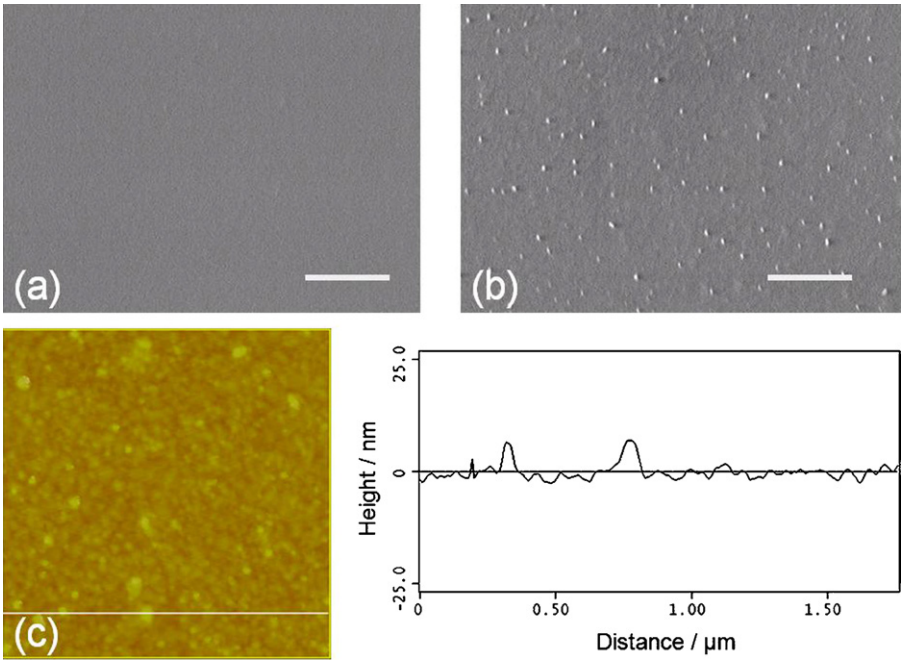

Fig. 2. SEM images of SAW chips (a) with a bare sensitive surface and (b) with a sensitive surface immobilized with ODR-10 by SAMs (scale bar = 5 μm). (c) AFM scans of the SAW chips with a sensitive surface immobilized with ODR-10 by SAMs.

where S denotes the sensitivity of this biosensor. As indicated in Fig. 3, ΔM denotes the mass differences of two different diacetyl concentrations. ΔF denotes the differences of resonance frequency shifts resulting from the corresponding two different diacetyl concentrations. Caculated by the above-mentioned equation, the sensitivity of this biosensor is 4 kHz/ng, which is 2× higher than that of previous works (Wu et al., 2011). The detection limit of this biosensor is 1.2×10^{-11} mM, which was calculated by the intersection point of the extrapolated linear region of the calibration curve with abscissa.

In this study, two related odorants, butanone and 2,3-pentanedione, which have similar chemical structures to diacetyl, were selected to test the specificity of this biosensor. Plasma membrane fractions of MCF-7 cells without ODR-10 were employed as the negative control. In addition, heat-treated (by thermal treatment in an oven at 150 °C for 1 h) membrane fractions of MCF-7 cells with ODR-10 were also tested to further examine the odorant detection function of ODR-10. Fig. 4 shows the responses of SAW chips immobilized with various membrane fractions to different odorant molecules. SAW chip with bare sensitive surface was used as control sensor. When diacetyl is applied, the response of membrane fraction with ODR-10 is significantly higher than that of membrane without ODR-10 and heat-treated membrane with ODR-10. When other odorants were applied, the responses were significant smaller than that of diacetyl. These much smaller responses are probably due to the non-specific binding of cell membrane fractions to the odorant molecules. For the control sensor, although its sensitive surface was kept bare, resonance frequency shifts are still existed but very small, which is probably due to the influence of gas flow on the sensitive surface.

The stability of this biosensor was also tested on the same samples. This biosensor was stored at 4 °C in the dried chamber for 2 weeks and its responses to diacetyl were tested everyday. The results indicated that the responses of this biosensor to diacetyl within 7 days show no significant decrease (data not shown). It is suggested that the performances of this biosensor is stable within 7 days. It is more stable than directly coating, which is uasually stable within 2 days. However, afer 7 days, the responses of this biosen-

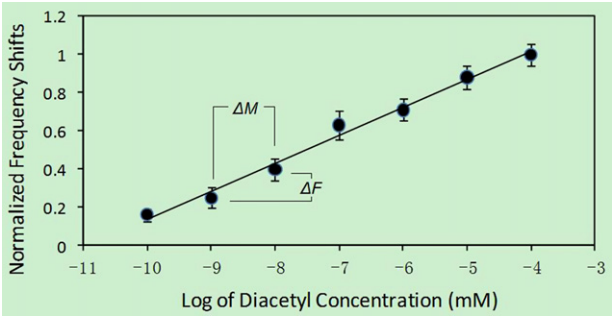

Fig. 3. Responses of SAW chips functionalized with ODR-10 to serial concentrations of diacetyl. The responses of various odorant molecules were normalized to that of diacetyl at the concentration of 10^{-4} mM. The mean and stand error of the mean of 5 experiments are shown.

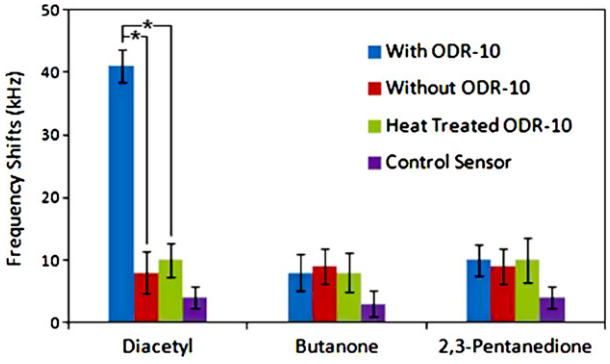

Fig. 4. Statistical results of SAW chips immobilized with various membrane fractions in response to different odorants at concentration of 10^{-4} mM. In the cases of diacetyl tested, the frequency shifts of membrane fraction with ODR-10 (41.3 ± 2.52 kHz) is significantly higher than that of membrane fraction without ODR-10 (8.25 ± 3.61 kHz, $p = 8.56 \times 10^{-9}$, $n = 6$). The frequency shifts of membrane fraction with ODR-10 (41.3 ± 2.52 kHz) is significantly higher than that of membrane fraction with ODR-10 treated by heat (10.57 ± 2.88 kHz, $p = 4.31 \times 10^{-5}$, $n = 6$). All data are represented by the mean ± SEM. *$p < 0.01$, Student's t-test.

sor to diacetyl show significant decrease. We anticipated the reason may be due to the change of natural sturature of ODR-10, which will result in the loss of odorant detection function. The function stability of molecular detectors for a longer time is a big challenge for the development and applications of biosensors, which is also the focus of our near future research works. In addition, the reproducibility over the different samples also need to be improved for potential practical applications. For this purpose, the methods that can precisely control the ammount of olfactory receptors immobilized on the sensing surface are deisrable.

4. Conclusion

This study developed a biomimetic olfactory-based biosensor with high efficiency immobilization of molecular detectors. The performances of this biosensor for odorant detection, such as the sensitivity and stability, can be improved significantly by the use of SAMs technique. All the results indicate the new method used in this study for achieving higher immobilization efficiency have great potential to be applied in many other olfactory receptor-based biosensors to promote and accelerate the related researches and applications.

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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.bios.2011.09.037.

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