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Integrated olfaction, gustation and toxicity detection by a versatile bioengineered cell-based biomimetic sensor

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ABSTRACT

The biological olfactory and gustation system can discriminate thousands of odor and taste substances with high sensitivity and specificity, specific receptor proteins play an important role in this process. This study used the human neuroblastoma SH-SY5Y cell line endogenously expressing the human bitter receptor, T2R16. Meanwhile, an olfactory receptor, ODR-10, was transfected on the plasma membrane of SH-SY5Y cells. T2R16 could specifically respond to bitter compounds with the structure of β-glucopyranosides by activation of G protein coupled receptors (GPCRs) causing cell morphologic changes, which could be monitored using a cell-impedance sensor. ODR-10 could specifically respond to diacetyl by changing the extracellular potential of the cells, the resopnse was recorded by a microelectrode array (MEA). The cell index (CI) value and firing rates were extracted from the signals as the biosensor response characteristics. The results with the sensors indicated a dose-dependent response within a defined concentration range. Moreover, this cell-impedance biosensor enabled quick toxicity detection of salicin when the concentration was ≥6 mM. In conclusion, the biomimetic sensors integrated olfaction, gustation and toxicity detection using the same cell, and has showed great potential for use in both basic research and practical applications.

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1. Introduction

Biological olfactory and gustatory systems are natural chemical sensors that provide essential environmental chemical signals accurately related to food foraging, communication and general behavior of organisms, playing a crucial role in survival and evolution within the ecosystem [1–3]. To mimic these biological functions, various types of electronic noses and tongues employing optical, electrochemical and mass sensors have been created [4–7]. However, most electrochemical detection devices rely heavily on redox-active interactions, and many chemicals cannot be easily oxidized or reduced due to their high stability. Hence, to date, biological olfactory and gustatory systems have not been replaced by electrochemical instruments to identify complex chemicals. With advances in the research of olfactory and gustatory transduction mechanisms, more and more researchers are making efforts to develop biomimetic chemical sensors in many fields such as food, biomedicine and environmental monitoring [8].

At present, two kinds of cells, primary sensory neurons [9–11] and bioengineered cells [12,13] with olfactory and gustatory receptors have been used in the development of biomimetic chemical sensors. The primary sensory neuron is hard to culture, and experimental repeatability is low. Bioengineered cells based on tumor-derived cell lines are easy to acquire and can be transfected with the desired receptor protein, making for a more practical approach to developing biomimetic sensors.

Individuals are used to identify a substance from different features such as, colour, smell, and taste. In this paper, we have begun work on biosensors based on different kinds of receptors on the same cell to detect different substances with different characteristics. The bioengineered, cell-based, biomimetic sensors integrating olfaction, gustation and toxicity detection is the first such reported development. We conclude that this approach will be attractive for applications in many fields.

The human neuroblastoma cell line SH-SY5Y is a highly suitable model for developing biomimetic sensors with olfactory and gustatory sensing. Firstly, the human bitter receptor hTAS2R16 (T2R16) is endogenously expressed in the SH-SY5Y cells. It is reported that this human bitter receptor can be activated by β-glucopyranoside (salicin and related compounds) [14]. Salicin, isolated from willows, belongs to a family of bitter phytonutrients and is generally used for the treatment of pain and inflammation. However, at high concentration, salicin may damage the efficacy of other medicines and affect the health of patients. It is worth stressing that the bitter receptor regulates cell morphology by activating the GTP enzyme of the RHO family [15,16]. Thus, when salicin binds to a bitter receptor, cell impedance will be changed [17],

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which can be monitored with impedance sensing. Secondly, an olfactory receptor of *Caenorhabditis elegans*, ODR-10, can be expressed heterologously on the SH-SY5Y cell line, which can specifically respond specifically to diacetyl by opening of the innate Cl- channel on the cell membrane [10,18,19]. Moreover, trains of spikes can be generated in differentiated cells by treatment with differentiating agent retinoic acid (RA) [20,21] Diacetyl is an odor substance widely used as a food additive to inhibit the growth of some fungi. Also, due to its butter-like flavor, it is widely found in various foods and beverages [22]. In recent years, excess intake of diacetyl has been reported as a possible cause of certain lung diseases [23]. So detection of diacetyl in food has become necessary. SH-SY5Y cells expressing T2R16 and ODR-10 receptors can be used as a sensitive element to construct cell-based biosensors. The extracellular spike signals and cellular morphological changes, which are induced by receptor activation, can be recorded by a microelectrode array (MEA) (Fig. 1a) and cell-impedance sensors (Fig. 1b). In our experiments, the cell-impedance sensor could also detect the toxicity of salicin in high concentration. Immunofluorescent staining and calcium imaging were used to identify the successful expression of olactory receptors and the activity of the bitter receptor prior to measurement. The experimental use of the cell counting kit-8 in alternative detection experiments allowed the toxicity of salicin at high concentrations to be demonstrated.

2. Material and methods

2.1. Cell culture, adenovirus package and viral transfection of SH-SY5Y cells

The constructed AD5 adenovirus-based vector pHBAD-CMV-3flag/rho-tag/odr-10 was confirmed by DNA sequencing, and the rho-tag imported sequence was inserted into the N-terminus of ODR-10. A Flag-tag was inserted into the C-terminal of ODR-10 for functional immobilization. SH-SY5Y cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum (FBS) in 37 °C 5% CO2 incubator. After seeding on 24-wall plates, the transfection experiments were divided into two groups, adenovirus with the ODR-10 gene and control virus. The multiplicity of infection (MOI) ranged from 5 to 200, and the medium was replaced with fresh DMEM medium after 8 h. After culturing for 72 h, transfection was confirmed by green fluorescence protein (GFP) with fluorescence microscopy.

2.2. Immunofluorescence staining

After transfection for 72 h, cell immunofluorescence staining was operated to determine the Flag tag by fusing with the target protein in order to identify successful expression of the target protein. In this experiment, cells were washed with phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde at room temperature for 30 min. After being washed three times with PBS buffer, the fixed cells were permeabilized with 0.15% Triton X-100 for 15 min. The cells were washed three times again with PBS buffer, and then incubated with a blocking solution of 1% BSA for 30 min at 37 °C. After that, the cells were incubated with primary antibody anti-Flag at 4°Cfor 12 h. After washing three times with PBS, the cells were further incubated with AlexaFluor secondary antibody at room temperature for 2 h. The cells were then washed three times again with PBS, and the cell nucleus was finally stained by DAPI staining and the cells thenmounted on glass slides. Primary and AlexaFluor secondary antibodies were diluted with a solution containing 1% Triton X-100, 5% goat serum and 1% BSA with a dilution ratio of 1:100 and 1:500, respectively. Images were captured by Laser confocal fluorescence microscopy.

2.3. Fabrication of MEA chip and extracellular recording

The MEA chip was designed and fabricated by standard microfabrication technology as shown in Fig. 2a. At first, a layer of Ti (30 nm) was deposited onto the glass substrate as the adhesion layer, followed by a Au layer (300 nm) as the electrode. Then the metallic layer was coated with photoresist and exposed to ultraviolet light under a mask with a defined electrode pattern. The exposed metallic area was etched, and electrodes and interconnections were thus obtained. After removing the photoresist, a layer of Si3N4 with 1 μm was deposited by plasma enhanced chemical vapor deposition (PECVD). Finally, the electrodes and contact pads were exposed after Si3N4 etching in KOH solution. Contact pads were connected on a printed circuit board (PCB) through a gold wire. A detection chamber with a diameter of 15 mm was fixed to the chip using polydimethylsiloxane. MEA chips were designed with 8 × 8 arrays (see Fig. 2b). The diameter of each electrode was 20 μm, and the distance between two electrodes was 150 μm. Before the experiments, the MEA chips were plated with platinum black to reduce impedance through increase of the specific surface area and to further improve signal to noise ratio. AC impedance spectroscopy was used to evaluate the electrodes surface characteristics. As shown in

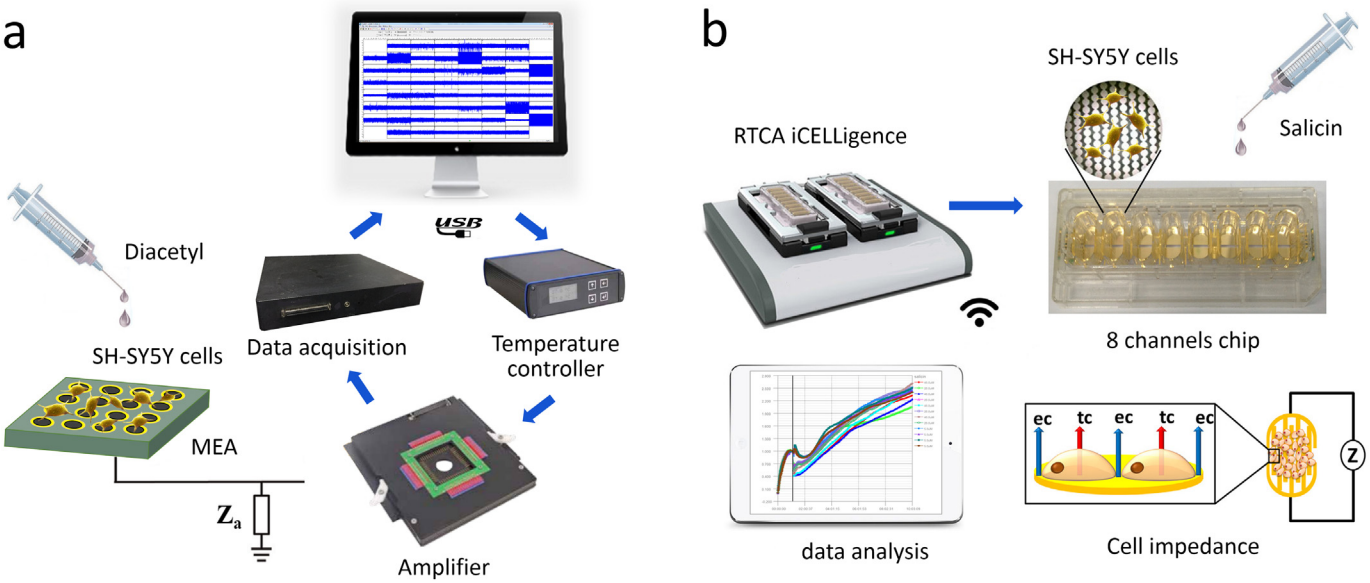

Fig. 1. Schematic of the biomimetic chemical sensors. (a) MEAs sensor system (b) Cell-impedance sensor system.

Supplementary Fig. 1a, the impedance amplitude of the electrode plated with platinum black decreased significantly compared with the bare gold electrode.

Before receptor transfection, 10 μM retinoic acid (RA) was used to induce SH-SY5Y cells for 5 days. At the beginning of the odor identification test, transfected cells were cultured on the MEA chips as shown in Fig. 2c. Before odor stimulation, cells activity was recorded for 1 min. Diacetyl, as the odor stimulus to the cells, was diluted to 1 mM, 10 mM and 100 mM, and 1 mM 3-isobutyl -1-methyl xanthine (IBMX) was added to increase membrane conductivity. The electrophysiological signals were recorded using stimulation with diacetyl from low to high concentration. Each group was tested for 120 s, and finally the potential changes were recorded after the diacetyl was removed by washing twice with culture medium. As shown in Fig. 1a, the MEA-1060 system was utilized for simultaneous recording, and MC-Rack software applied for real-time monitoring and data recording. All experiments were conducted at 37 °C.

2.4. Cell-impedance measurement

The cell-based impedance chip consisted of 8 wells in each plate, and the electrode in each well was made of thin gold film (Fig. 2d). We also used AC impedance spectroscopy to evaluate the characteristics of the impedance chip with and without cells (Supplementary Fig. 1b). In the sampling frequency range of 100 Hz-10 MHz, the difference between the two kinds of impedance curve was most obvious at a frequency of 10 kHz, indicating that the chip was most sensitive at 10 kHz, so we chose 10 kHz as the working frequency.

The cell index (CI) was used to represent the status of cell based on electrical impedance. In the iCELLigence system, CI was calculated according to the formula:

$$\max_{i=1,\ldots,N}\left(\frac{R_{cell}(f_i)}{R_0(f_i)}-1\right) \quad (1)$$

N is the number of the frequency points at which the impedance is measured, and $R_0(f_i)$ and $R_{cell}(f_i)$ is, respectively, the frequency-dependent electrode resistance without cells or with cells present in the wells. When cells are present in a well, changing cell status will lead to a change in CI. For example, an increase in cell adhesion, or cell spread leading to larger cell/electrode contact area, will lead to an increase in $R_{cell}(f_i)$ and a larger CI. Alternatively, a cell death or toxicity induced cell detachment, will lead to smaller $R_{cell}(f_i)$ and thus smaller CI.

At first, no cells were present on the electrodes, CI = 0. After the cell resuspension solution was added, cells attached well to the electrodes within an hour; cells adhesion increased, leading to a large cell/electrode contact area. This also leads to an increase in $R_{cell}(f_i)$ and a larger CI. After the cells were all well attached to the electrodes, there was a lag time between the attachment phase and the exponential growth phase. In this phase, the CI values ceased to increase and came to a plateau phase. We chose this phase as the stimulation point. At this time, cells in each well should achieve 90%–100% confluent, we performed impedance tests on cells at different densities (Fig. 2d), and found that 1.5×10^5 cells in each well achieved 90%–100% confluence under microscopy imaging which could be considered the optimum planting density (Fig. 2e). The iCELLigence system is shown in Fig. 1b, and an iPad (Apple Inc. USA) was used to record data using Wi-Fi. Salicin, as the stimulating solution, was freshly prepared with distilled water at 10-fold concentration. Prior to the experiments, all the solutions were kept at 37 °C. Cells with 180 μL fresh medium were cultured in a well with 90 min incubation time. After that, a 20 μL salicin solution with

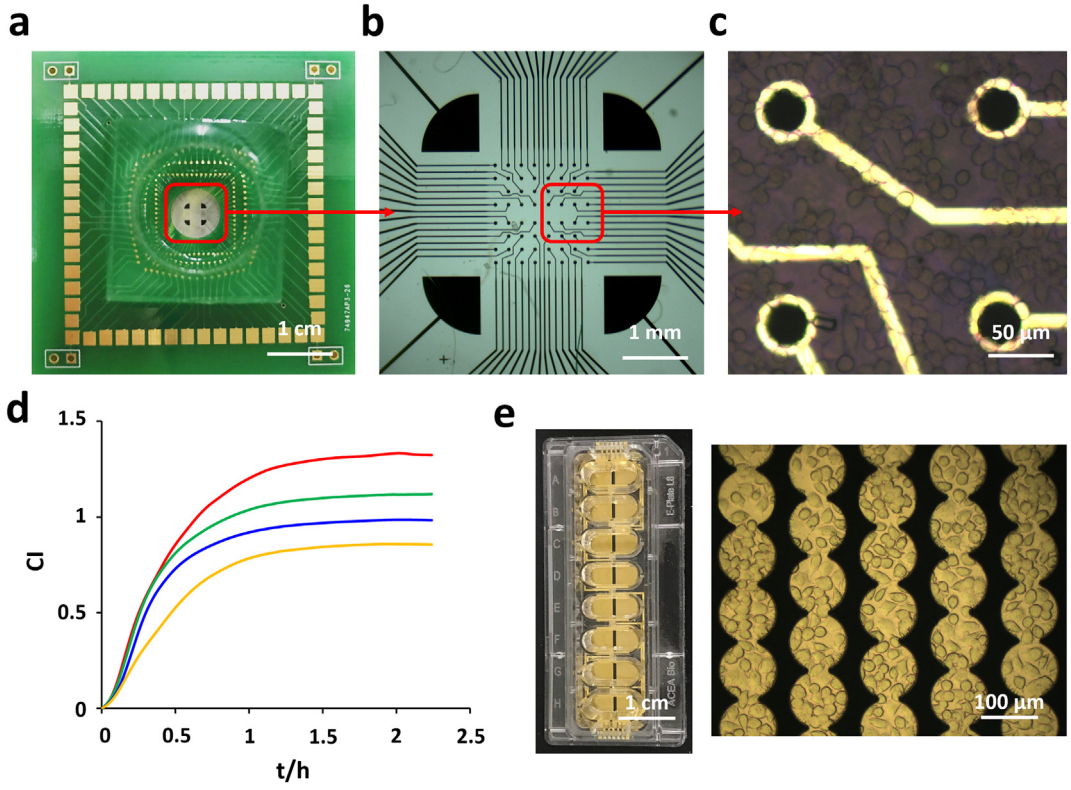

Fig. 2. (a) The MEA chip was designed and fabricated by standard microfabrication technology. (b) The layout of 64 electrode points on the surface of MEA chip. (c) The surface of electrode points was electroplated with platinum black and the SH-SY5Y cells were cultured on the chips. (d) Impedance detection curve of different densities of cells (red line: 1.75×10^5 cells/well; green line: 1.5×10^5 cells/well; blue line: 1.25×10^5 cells/well; yellow line: 1×10^5 cells/well). (e) The SH-SY5Y cells were cultured on 8-well and the density of cells was 90%–100% confluent in each well.

different concentrations was added to each well. The impedance response to the bitter stimulation was recorded for at least 60 min.

2.5. Calcium responses of SH-SY5Y cells to bitter compounds

A calcium imaging technique was used to validate the function of T2R16 in the SH-SY5Y cells. The cells were cultured on a 35 mm 4-wells plate with a glass bottom. HBSS medium was used to wash the cells three times, then the cells were loaded with 4 μM Fura-4/AM and 80 μg/mL Pluronic F127 for 30 min at 37 °C. The dye solution was replaced with HBSS medium containing 1% FBS for 20 min, and calcium signals were recorded by laser confocal fluorescence microscopy. The duration of stimulation by 10 mM salicin was 30 s. The cells excited by salicin were monitored at 340 nm and 380 nm excitation wavelengths, and fluorescent signals captured at 510 nm. All the procedures were also operated without light.

2.6. CCK8 assay for cytotoxicity evaluation

The principle of CCK8 assay in living cells is based on WST-8 which can be reduced by dehydrogenases to an orange-colored formazan. Firstly, cells were seeded in 96-well plates for 2.5 h with 180 μL DMEM medium. At the same time, the same volume of cell-free medium was added in the blank group. Cells were treated with 4 mM, 6 mM, 8 mM and 10 mM of salicin for 3 h, respectively. The "blank" and "control" were added the same volume of cell-free medium. Then, 20 μL CCK8 solution was added to each well, and 200 μL medium was finally collected and read at 450 nm by microplate reader. The optical density (OD) value was used as the parameter for cytotoxicity evaluation.

2.7. Data processing and analysis

The experimental data for odor detection was recorded and displayed in the MC Rack software. The raw signals were filtered by Off-line Sorter software using a 4-pole Bessel low-cut filter (cut-off frequency 250 Hz). Then, the detection threshold was set based on using three standard deviations from the mean value of the peak height histogram (3 sigma), and spike waveforms sorted out by a Valley Seeking Scan. The spike signals of each channel were analyzed by calculating firing rates which were represented by the mean ± SD of n samples (n is the number of valid electrodes). Each group of data was normalized to the mean value of the highest response [24]. The Student's *t*-test was used to evaluate significant differences between different diacetyl concentration treatments. The baselines of the CI values were normalized to quantify the CI response under various concentrations before the bitter stimulation, and the other CI values were adjusted to the normalized value. When the CI value reached its peak, response intensity, namely maximum CI, was calculated by subtracting the CI value of the control group from the CI values of the various concentrations of the bitter treated group. All data were acquired from at least three independent experiments.

3. Results and discussion

3.1. Differentiation of SH-SY5Y cells with RA treatment and expression of olfactory receptor

With treatment with RA, the human neuroblastoma cell line SH-SY5Y can be induced to acquire a neuronal phenotype. The cells have a relatively small size without extension as axons and dendrites before being induced (Fig. 3a), after treatment with 10 μM RA for 5 days in complete medium, the cells show a higher level of differentiation (Fig. 3b). The expression of ODR-10 was validated by immunofluorescent staining. The control group is shown in Fig. 3c. When the cells were transfected with adenovirus without the gene for the olfactory receptor, the cell membranes could not be marked with anti-marker for the target protein. The cell nucleus was labeled using DAPI (blue fluorescence) which indicated the location of the cells. The results in Fig. 3d demonstrate the successful expression of ODR-10 in SH-SY5Y cells which is mainly distributed on the plasma membrane of the cells and is marked by a red fluorescence.

3.2. Performance of olfactory receptor-based biosensors

Fig. 4 shows that the odorant receptor ODR-10 belongs to the superfamily of G-protein-coupled receptors with seven putative transmembrane helices. When the odor diacetyl was bound to the receptor, the binding reaction led to the activation of an endogenous G-protein and stimulation of adenylate cyclase, resulting in the increase of intracellular cAMP. cAMP activates cAMP dependent PKA, which phosphorylates the CFTR protein. The PKA-mediated phosphorylation of the CFTR protein in the presence of intracellular ATP finally leads to opening of the innate Cl^- channel and a corresponding increase of the membrane conductance [16]. Previous reports confirmed that the action potential repolarization rate in differentiated cells is about threefold faster than in undifferentiated cells under current-clamp conditions [17]. Furthermore, trains of spikes can be generated in some differentiated cells with prolonged stimulation. To further amplify the signals, the enzymatic breakdown of cAMP was blocked by the application of the membrane-permeable phosphodiesterase inhibitor IBMX. Superfusion of the SH-SY5Y cells with IBMX (1 mM) and the presumptive ligand diacetyl increased the membrane conductance.

Our results were in agreement with this mechanism as shown in Fig. 5a. On the basis of spontaneous electrophysiological signals, the responses of transfected SH-SY5Y cells to diacetyl at various concentrations were recorded to determine the response characteristics of the biosensor. The original signals with diacetyl stimulation are shown in Fig. 5a with concentrations ranging from 1 to 100 mM. The firing rate of the spikes became higher with the increase of the odor concentration. After washing out the odor compound, the firing rate decreased to the baseline approximating to that recorded before giving the stimulus. The corresponding peri-stimulus time histograms (PSTHs, mean firing rates/bin, bin = 1 s) of spike trains were used to quantify the data shown in Fig. 5b. The normalized firing rate is shown in Fig. 5c, and the results show that the firing events obviously increase with increasing concentrations. The analysis of firing rates showed a significant difference between PBS and different concentrations of odors. The firing activities almost completely recovered when the diacetyl solution was washed out and replaced with medium. Fig. 5d shows the dose-dependent response of the biomimetic chemical sensor with the correlation coefficient (R2) of 0.9904.

3.3. Calcium responses of SH-SY5Y cells to bitter compound

Cell impedance was used to detect GPCR activation which has been reported in many studies [25]. SH-SY5Y cells endogenously express the TAS2R16 bitter receptor, and as shown in Fig. 4, the bitter receptor also activates phospholipase (PLC), which then hydrolyzes and converts phosphatidyl inositol 1,4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3). IP3 opens Ca^{2+} channels on the surface of the endoplasmic reticulum which leads to a calcium release [26–28]. In order to further determine whether salicin could activate bitter taste receptor-mediated signaling pathways in SH-SY5Y cells, the calcium response was measured. Fig. 6a shows that salicin can increase intracellular calcium release in SH-SY5Y cells. Thus calcium imaging demonstrate that SH-SY5Y cells are able to respond to salicin by activating TAS2R16, which is the biological base for the biomimetic chemical sensor.

3.4. Bitter receptor activation detected by biomimetic chemical sensor

In this experiment, we found that the bitter receptor activation response was not induced by the toxicity reaction of the cell. The results

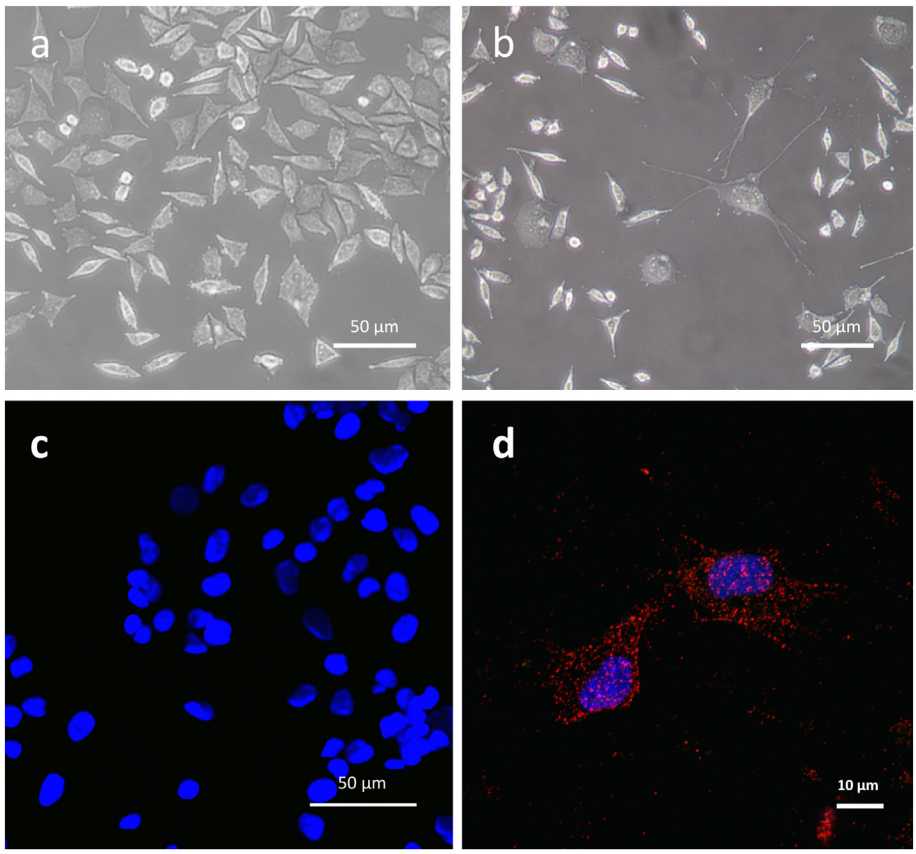

Fig. 3. (a) The human neuroblastoma cell line SH-SY5Ys were showed under microscope in complete medium without differentiated. (b) The differentiated SH-SY5Y cells with treatment of RA, which resulted in neurite outgrowth. (c) The cells transfected with the control adenovirus without olfactory receptor, blue: cell nucleus. (d) The cells transfected with olfactory receptor and the expression of receptor protein with red fluorescence.

of CCK8 in Fig. 6b show that the salicin under the concentration of 4 mM has no toxicity effort on the cells. The typical cell-impedance responses of the biosensor to salicin areshown in Fig. 6c. Impedance rapidly increases and reaches a peak value at around 90 min after addition of the salicin solution. The CI values at the peak increased with the higher concentrations. Fig. 6d shows that the impedance is dose-dependent manner, and accords with the calcium response observations. The detection limit of this response to salicin was calculated as 1.96 μM. Meanwhile, in order to determine whether this cell-based biosensor is specific to salicin, which is a bitter taste among five basic tastes, several representative compounds were added for taste detection, including sweet (glucose), umami (MSG), salty (NaCl) and sour (HCl). Relatively low concentrations of those compounds were chosen, and the results show that the biosensor gives negligible responses to these compounds. However, under the same concentration range, the salicin was still able to evoke responses in a dose-dependent manner (Fig. 6e). Thus, the biosensor can specifically detect bitter compounds among five taste stimuli.

3.5. Toxicity assessment of salicin to SH-SY5Y cells

The cell-impedance sensor has been reported earlier to detect paralytic shellfish poisoning (PSP) toxins with a neuroblastoma cell; here the growth and proliferation of the cells were inhibited and cell impedance significantly decreased [29]. The results in Fig. 7a indicate that treatment with higher concentrations of salicin can have an adverse effect on the growth of SH-SY5Y cells. The CI value decreased rapidly in 2 h and recovered with slow proliferation when the concentration of

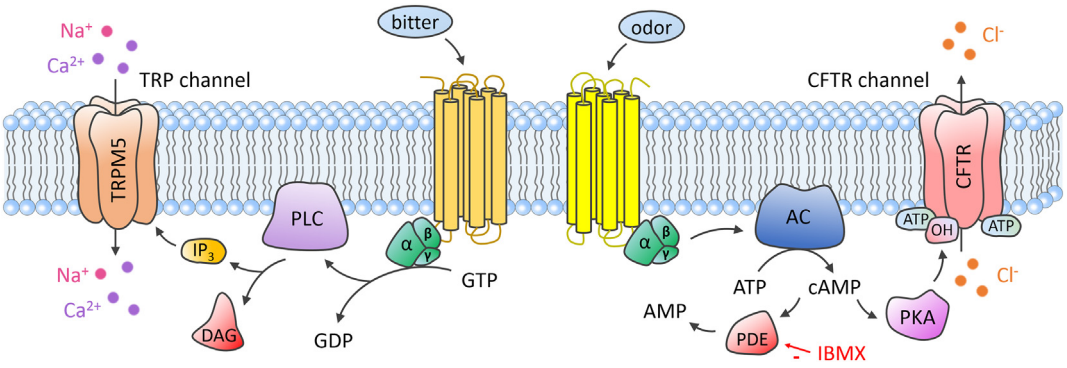

Fig. 4. The signal transduction pathway in SH-SY5Y cells of olfactory and bitter receptors activated by diacetyl and salicin.

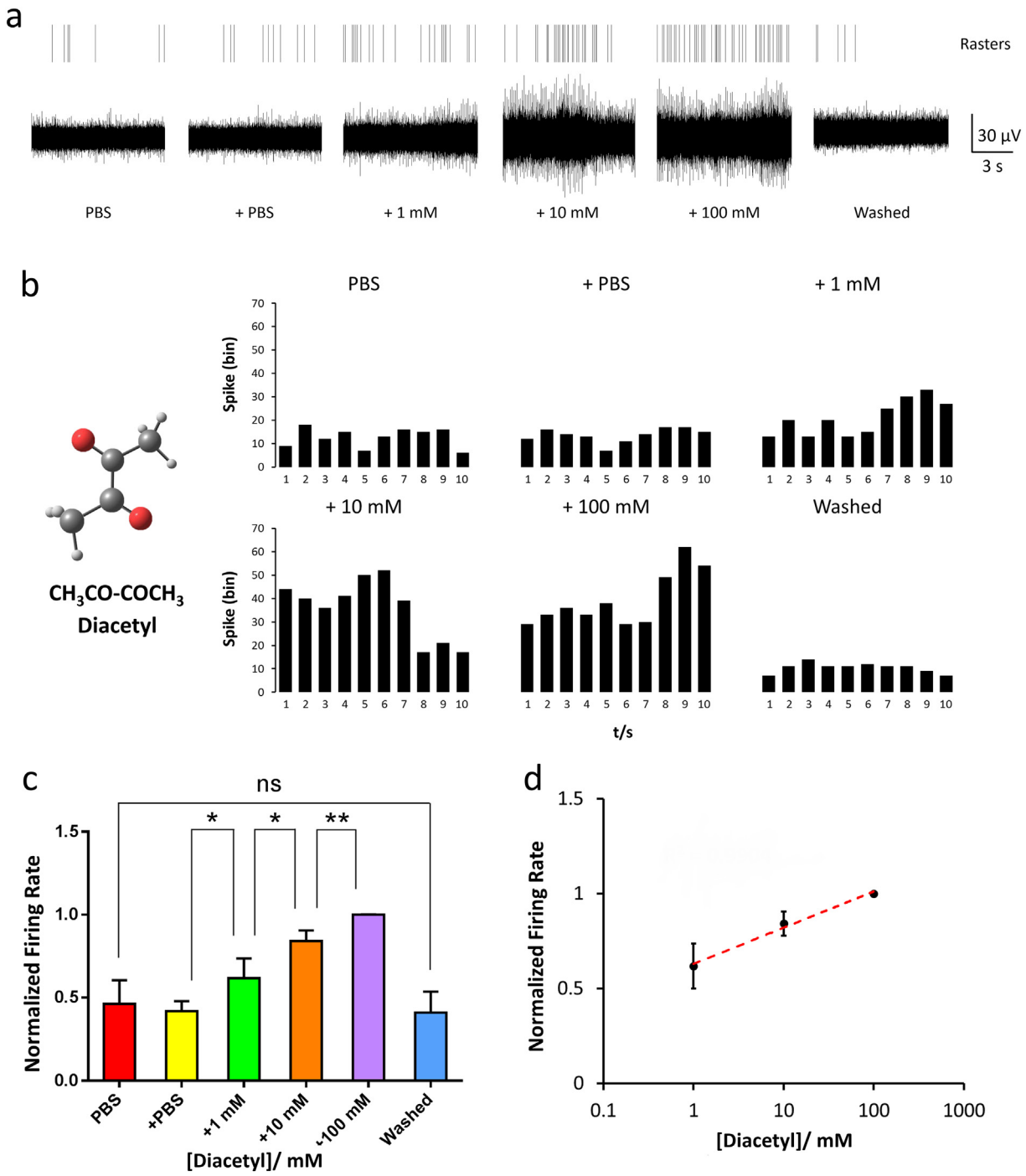

Fig. 5. (a) The original signals of cells with phosphate buffer saline (PBS) and diacetyl stimulation, upper panel shows raster plots of spike trains, below panel indicates an epoch of the raw spike recording from each channel of the electrode points on the MEA chip, PBS represents the control state and wash indicates the state after diacetyl washed out. (b) The left shows the chemical molecular structure of odor, diacetyl, and each graph on the right is the corresponding peristimulus time histograms of spike trains, (mean firing rates of a bin, bin = 1 s). The concentrations of each odor are 1 mM, 10 mM and 100 mM. (c) Each group of data was normalized to the mean value of 100 mM, the normalized firing rates of odor-evoked activities in (a) were calculated and student's t-test (**$P < .01$) was used to indicate the significant differences. (d) Standard curves of bioengineered cell-based sensor responses to diacetyl based on firing rate ($R^2 = 0.9904$). All data in (c) and (d) presented by the mean ± SD.

salicin were increased to 6 mM. It is highly probable that salicin at high concentration had toxicity effect on the cells, causing a decline in CI values.

Meanwhile, the results of CCK8 assay in Figs. 6b and 7b confirm that salicin has no toxicity effect on cells ≤4 mM, but some toxicity to cells can be observed at ≥4 mM. It can be concluded that the cell-based biosensor presents good specificity for bitter toxin detection.

4. Conclusions

In this study, the human neuroblastoma cell line SH-SY5Y was used for the first time as a sensing element to identify odor, bitter and toxic compounds. We used this cell with two receptors as the sensing element, MEAs and cell-impedance sensors were used as the secondary transducer through recording the extracellular signal and cellular

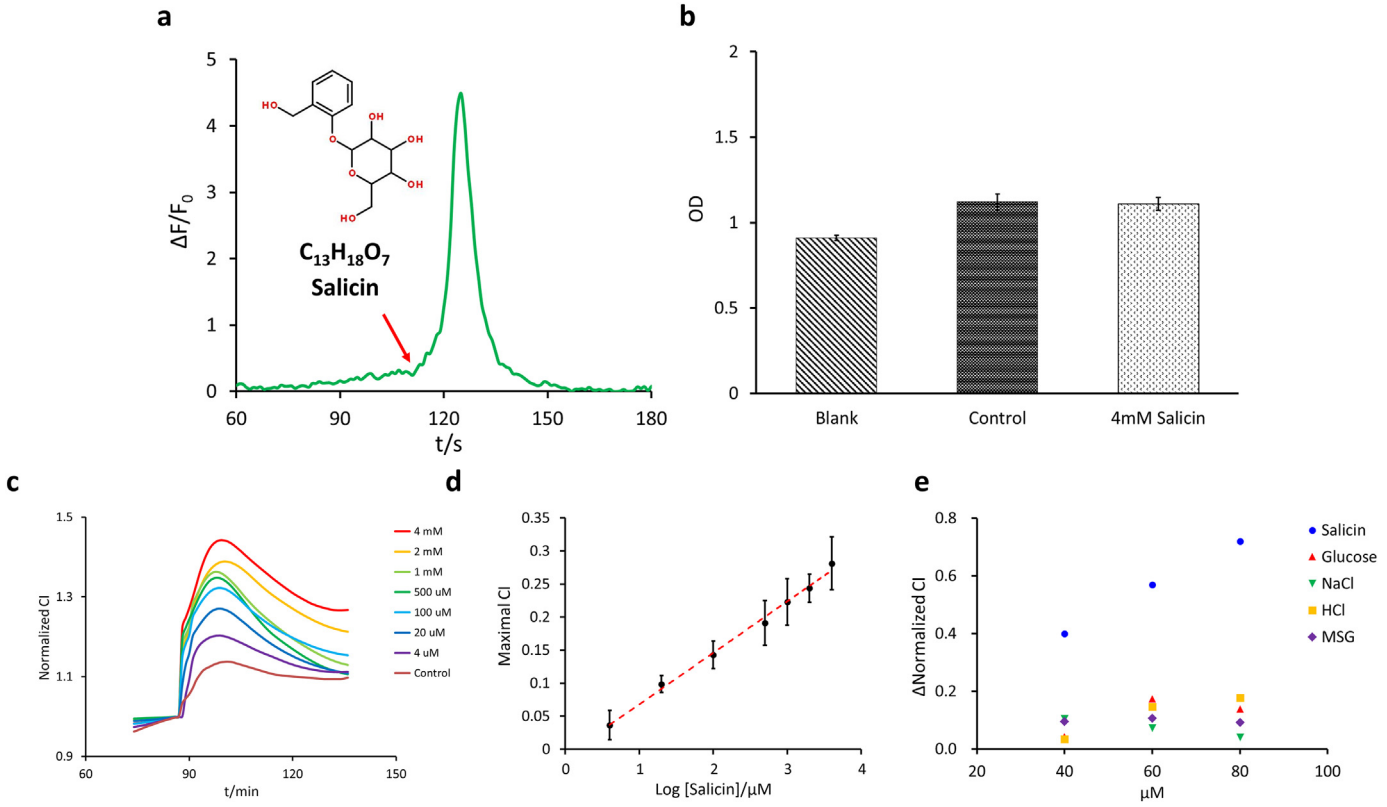

Fig. 6. (a) The typical calcium response curve of SH-SY5Y cells with the chemical molecular structure of bitter compound, salicin. The cells were loaded with calcium-sensitive dye Fura-4/AM, and the salicin (10 μM) was used to identify the response. (b) CCK8 results show the toxicity of salicin to SH-SY5Y at the concentrations below 4 mM. "Blank" means the group without cells on the 96-cell plate and only adding the CCK8 reagents at the same time. "Control" means the group with same number cells but without adding salicin. (c) The typical responses of cell-impedance to salicin at different concentrations with error bars (standard deviations). (d) Response intensity of SH-SY5Y cells to salicin stimuli presented dose-dependent relations. (e) This cell-impedance sensor specifically responds to bitter detection. "Glucose" represents taste of sweet. "NaCl" represents taste of salty. "HCl" represents taste of sour. "MSG" represents taste of umani. Only salicin (bitter) can cause obvious dose-dependent responses. All data in (c) and (e) were acquired from 3 independent experiments at least.

morphological changes caused by the activation of receptors. Moreover, the cell-impedance biosensor can specifically detect salicin at low concentrations demonstrated by the rise of CI values and the toxicity effect of salicin at high concentrations demonstrated by the decline of CI values.

We use two kinds of sensors based on different responses to detect different substances just to avoid the effect. The signaling pathways caused by cell responses are different, if we only use an EIS system, qualitative analysis between odor and bitter will be hard to realize. We speculate that the major reasons for the dramatic differences in detection threshold between MEAs and cell-impedance sensors are following: Firstly, trains of spikes could be generated in some, but not all, differentiated cells. Secondly, some of the low-concentration signals are lost through external noise interference. So, in order to improve MEA chip conductivity and to reduce external noise interference, effective methods focused in this direction are needed. Additionally, an integration of monitoring systems is also needed in future research. We conclude that the results from this study will provide a novel and promising approach for the research into multi-function, integrated, detection methods.

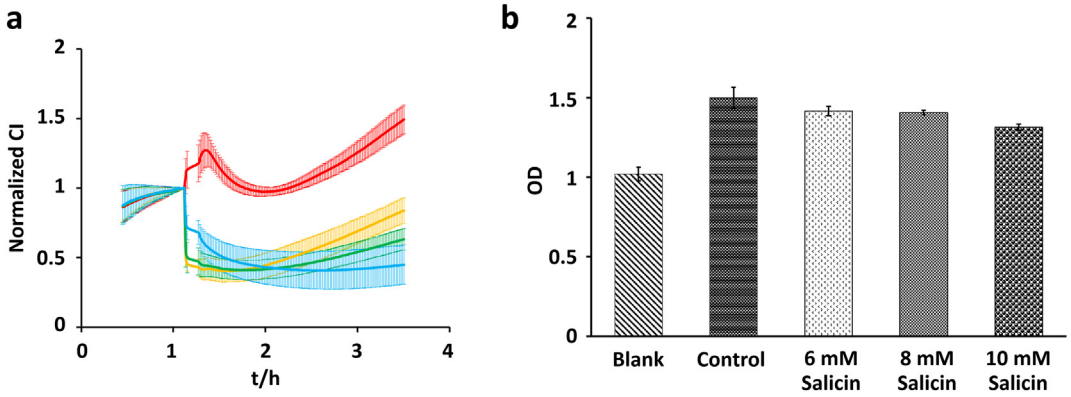

Fig. 7. (a) Various concentrations of 6 mM (yellow line), 8 mM (green line) and 10 mM (blue line) were added as stimuli to cells. Cell impedance measurement shows that the bitter has an inhibitory effect on the growth of cells. Activity and proliferation of cells indicate a gradual recovery after about 60 min. (b) CCK8 result indicates that salicin with the concentrations higher than 6 mM has a weak toxicity to SH-SY5Y cells. All data were acquired from 3 independent experiments at least.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2019.02.009.

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