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Dual functional extracellular recording using a light-addressable potentiometric sensor for bitter signal transduction

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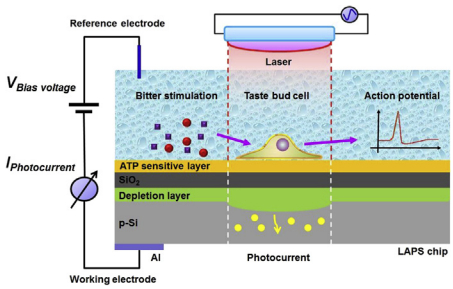
HIGHLIGHTS

- A biosensor was developed for extracellular recording of membrane potential changes and ATP release from single taste cell.
- Enhancive and inhibitory effects on the extracellular membrane potential changes and ATP release were efficiently recorded.
- Inhibitory effect of CBX also validates that LAPS extracellular recordings are originated from bitter signal transduction.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper presents a dual functional extracellular recording biosensor based on a light-addressable potentiometric sensor (LAPS). The design and fabrication of this biosensor make it possible to record both extracellular membrane potential changes and ATP release from a single taste bud cell for the first time. For detecting ATP release, LAPS chip was functionalized with ATP-sensitive DNA aptamer by covalent immobilization. Taste bud cells isolated from rat were cultured on LAPS surface. When the desired single taste bud cell was illuminated by modulated light, ATP release from single taste bud cells can be measured by recording the shifts of bias voltage-photocurrent curves (*I-V* curves) when the LAPS chip is working in discrete mode. On the other hand, extracellular membrane potential changes can be monitored by recording the fluctuation of LAPS photocurrent when the LAPS chip is working in continuous mode. The results show this biosensor can effectively record the enhancive effect of the bitter substance and inhibitory effect of the carbenoxolone (CBX) on the extracellular membrane potential changes and ATP release of single taste bud cells. In addition, the inhibitory effect of CBX also confirms LAPS extracellular recordings are originated from bitter signal transduction. It is proved this biosensor is suitable for extracellular recording of ATP release and membrane potential changes of single taste bud cells. It is suggested this biosensor could be applied to investigating taste signal transduction at the single-cell level as well as applied to other types of cells which have similar functions to taste bud cells.

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1. Introduction

Mammalian taste can detect and distinguish various taste signals presented by tastants using different kinds of taste bud cells, which play crucial roles in providing valuable information about living such as the nature and quality of food [1–3]. Taste bud is the taste sensation organ, which consists of four morphological types of taste bud cells [4]. Among them, type II cells are in charge of detecting sweet, bitter, and umami, which express the specific taste receptors and transduce the taste signals by a cascade of intracellular biochemical reactions [5–7]. It is proved that type II cells relay the taste signals to surrounding taste bud cells by ATP release in a cell membrane voltage-dependent manner, which plays a significant role in taste signal transduction [5,8]. The process of taste signal transduction is a complex and multistage process [9,10]. It is still far from complete understanding of mechanisms underlying taste sensation, especially the mechanisms of taste signal transduction. The main reason is attributable to the complications of taste signal transduction and the lack of powerful tools for the research of taste sensation. Specifically, the multiparameter measurement is highly desirable for the successful monitoring of taste signal transduction process. It is important to develop novel approaches for extracellular recording to investigate the signal transduction mechanisms of taste bud cells.

Light-addressable potentiometric sensor (LAPS) is a photoelectric semiconductor that is very sensitive to surface potential changes [11]. LAPS can measure any response that results in the changes of surface potential, such as cell membrane potential changes [12,13], ion concentrations [14,15], and charged molecules [16,17]. LAPS have two significant advantages compared to the field-effect transistor (FET) and microelectrode array (MEA). One is the large continuous surface, which makes it easy for the surface modification. LAPS chip can be functionalized with ultrathin sensitive membranes for detecting specific ions and charged molecules such as K^+, Na^+, and Ca^{2+} [18], 5-HT [19], and DNA [16,17]. The other one is the light-address ability, which can realize the single-spot measurement by illuminating the desired spot with a movable light. Versatile measurement principles of LAPS are capable of design and invent novel tools for multifunctional extracellular recording.

Various methods have been developed for ATP detection such as stand luciferin-luciferase assay [5], cell-based ATP-sensitive biosensor [20], cell surface attached firefly luciferase [21,22], myosin-functionalized cantilevers [23], and enzyme-functionalized Pt microelectrode [24]. However, these methods have some intrinsic limits such as tedious design and preparation of sensitive molecules, sophisticated responses of sensitive molecules to ATP, precise manipulation of measuring cells. Compared to methods mentioned above, aptamer-based methods have become more and more promising, which use ATP-sensitive aptamer as sensing elements [25,26]. Aptamers are nucleic acid ligands with high binding affinity to specific molecular targets [27,28]. It is thus tempting to combine the extracellular potential recording capability of LAPS with ATP-sensitive aptamer for the sake of providing novel solutions to the problems faced in developing multiparameter measurement biosensors for the research of taste signal transduction mechanisms.

This study presents a dual functional extracellular recording biosensor based on a light-addressable potentiometric sensor (LAPS) functionalized with ATP-sensitive aptamer for the first time, which can record both extracellular membrane potentials and ATP release from single taste bud cells. DNA competitor (with a full complementary sequence of ATP-sensitive aptamer) was covalently immobilized on the LAPS surface, which can capture ATP-sensitive aptamer by DNA hybridization. Taste bud cells prepared from rat were cultured on the LAPS surface. When the desired single taste bud cell was illuminated by a modulated light, ATP release can be measured by recording the shifts of bias voltage-photocurrent curves (*I-V* curves) when the LAPS chip is working in discrete mode, while extracellular potential can be monitored by recording the fluctuation of LAPS photocurrent when the LAPS chip is working in continuous mode. Further, a hemichannel blocker, carbenoxolone (CBX), which can dramatically inhibit the membrane current and ATP release of taste bud cells, was utilized to confirm the LAPS extracellular recordings are originated from bitter signal transduction. This dual functional extracellular recording biosensor cannot only be applied to taste bud cells for the taste signal transduction, but also be applied to other types of cells which have similar functions to taste bud cells, which can be accommodated by current devices or methods.

2. Methods and materials

2.1. LAPS chip fabrication and functionalized with ATP-sensitive aptamer

For LAPS chip fabrication, the silicon wafer (p-type, <100>, 3–8 Ωcm) was extensively cleaned and then was thermally grown a layer of SiO_2 on the surface under 1180 °C with a thickness of 50 nm. A thin film of titanium dioxide (TiO_2) was spin-coated onto the upper side of the silicon wafer. The silicon wafer was then dried in a furnace at 120 °C for 20 min and sintered at 500 °C for 60 min in the air with the temperature raised at 10 °C/min. Hydrofluoric acid was used to treat the back side of the silicon wafer to remove the SiO_2 layer. Afterward, 1 μm thickness aluminum layer was evaporated on the back side of the silicon wafer to create an ohmic contact and served as the working electrode. UV was employed to treat the upper side of the silicon wafer for the sake of improving the following silanization level. For silanization, the silicon wafer was dipped into the toluene solutions with 3-aminopropyltriethoxysilane (APTES) concentration of 0.1% (v/v) for 1 h and sealed at room temperature. The silicon wafer was then cleaned completely by rinsing in toluene and ethanol and cured at 120 °C for 1 h. Finally, the upper side of the silicon wafer was attached to a detection chamber with a 5 mm-diameter hole in the bottom center.

ATP-sensitive aptamer (5′-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3′) and DNA competitor (5′-ACC TGG GAA TAC TCC CCC AGG T -3′, 5′-end with C6-NH_2 modified) were purchased from Takara Biotechnology Co. Ltd., Dalian, China. For LAPS chip functionalization, DNA competitor was covalently linked to LAPS surface by a crosslinking method. Amine residues were introduced to LAPS surface by silanization. Glutaraldehyde (Sigma-Aldrich, USA) was used to treat LAPS surface for cross-link reaction. DNA competitor was covalently immobilized by reaction between amine residues of DNA competitor with aldehyde residues of TiO_2 film. Bovine serum albumin (BSA) (1% (v/v), Sino-American Biotechnology Co. Ltd., China) was used to block unreacted aldehyde residues of LAPS surface to avoid nonspecific binding. The ATP-sensitive aptamer was then added and hybridized with DNA competitor. PBS was used to wash the LAPS chip to get rid of the excessive aptamer and avoid nonspecific adsorption. LAPS chips functionalized with ATP-sensitive aptamers were stored at 4 °C for further experiments.

2.2. Preparation of taste bud cells and fluorescent imaging

Preparation of taste bud cells was according to previously described method [29]. Briefly, Sprague-Dawley rat in a 5–7 days old was sacrificed. The entire tongue was later dissected and used for harvesting taste bud cells. For this, the lingual epithelium was

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peeled from the tongue after injection of 1–1.5 ml Tyrode's solution (pH = 7.4) containing collagenase (2 mg/ml) and elastase (0.25 mg/ml) under the epithelium and 30 min incubation with divalent-free Tyrode's solution. Taste bud cells were harvested from the dissected lingual epithelium by gentle suction using a fire-polished glass pipette. Cells were seeded on LAPS surface at the density of 1×10^5 cells/cm^2 and cultured for 2 h at 37 °C under humidified air with 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, UK). After cells were attached to the surface of LAPS chip, it is ready for extracellular recording experiments. Tyrode's solution was homemade and consisted of 126 mM NaCl, 5 mM KCl, 5 mM NaHEPES, 1.25 mM $NaH_2PO_4 \cdot H_2O$, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$ (pH = 7.4, adapted with NaOH). All chemicals used throughout this study were of analytical pure grade or better quality, which were used without further purification.

For fluorescent imaging, taste bud cells attached to LAPS surface were incubated with primary antibody, mouse monoclonal anti-IP_3R3 (inositol 1,4,5-trisphosphate receptor type 3, diluted to 1:50, BD), in 0.1 M PBS overnight at 4 °C. After incubation, taste bud cells were washed 3 times with PBS (pH 7.4) and then incubated with secondary antibodies, FITC conjugated to goat anti-mouse IgG (diluted to 1:100, Jackson Lab), in 0.1 M PBS (pH 7.3) for 2 h at room temperature. After washing 3 times with PBS (pH 7.4), cells were fixed with 90% glycerin in PBS and examined under a confocal fluorescence microscope (Leica TCS-SP, Germany).

2.3. LAPS measurement setup

An image of LAPS measurement setup was shown in Fig. S1. During experiments, LAPS chip cultured with taste bud cells was mounted under a microscope objective (Jiangnan Optic Instrument Co., GAILEM). The volume of the measurement solution in the detection chamber is of two ml. Then the modulated light was focused to a diameter of less than 10 μm by a lens and highlighted on the desired single cell. The light source is a He-Ne semiconductor laser (Coherent Co.) with a wavelength of 543.5 nm and the power of 5 mW. A chopper (Stanford Research Systems, SR540) modulates the beam of the laser ($\varphi = 2$ mm) and generates modulated light at the frequency of 4 kHz. When cell produces action potentials or ATP release under stimulations of bitter substances, LAPS can detect the changes in cell membrane potential or local ATP concentration by giving corresponding changes in photocurrent. For the extracellular recording of membrane potential changes, LAPS worked in the continuous mode by applying bias voltage at the working point and recording the changes in photocurrent continuously. For extracellular recording of ATP release, the LAPS worked in the discrete mode by applying a series of the bias voltage ranging from −2 V to 2 V to LAPS chip to achieve the shift of the LAPS *I-V* curves. The elapsed time between baseline and after bitter exposure *I-Vs* is around 1 min. This is the primary work to combine extracellular recording of membrane potential changes and ATP release on one chip. For this, the software of the measurement setup was rebuilt to make it easy to transfer the working modes of LAPS. These signals will be transmitted into peripheral circuits allows for signal amplification and processing through the working electrode. The three electrodes of potentiostat (EG & G Princeton Applied Research , M273A) are used to detect the current and transfer the current into voltage. We utilized a 16-bit data collection card and the LabVIEW to control the collection, analyze and store data. A homemade LabVIEW software was employed to control the on-off of the peristaltic pump and the surrounding temperature. All measurements were performed at 37 ± 0.2 °C.

3. Results and discussion

3.1. Taste bud cell culture on LAPS surface

Among taste bud cells, type II cells are in charge of the detection and transduction of bitter signals. To perform extracellular recording of single taste bud cell under bitter stimulation, it is necessary to achieve good coupling between type II taste bud cells and LAPS chip with proper surface distribution density as low as 1×10^4 cm^2, it is calculated the mean distance between cells is around 100 μm. This distance makes it possible to perform single-cell measurement and avoid the influences of neighboring cells. Therefore, mouse monoclonal anti-IP_3R3 antibody, which is specific to IP_3 that was reported existed in type II TRCs but not in type III TRCs [30], was utilized as the primary antibody to show the distribution of type II taste bud cell on LAPS surface. Fig. 1 shows taste bud cells attached to the surface of LAPS after 2-h incubation. It is shown type II taste bud cells grow well on LAPS surface and preserve the morphological features of native taste cells to some extent, which looks like a spindle. In addition, taste bud cells spread on LAPS surface with suitable distances allowing for the extracellular recording of single taste bud cell.

Type II taste bud cells can convert bitter signals by an intracellular signal transduction pathway into cellular responses including cell membrane potential changes and release of ATP that served as the neurotransmitter for taste signal transduction. In this study, LAPS measurement setup was employed to realize the dual functional extracellular recordings, which include the extracellular electrical recording of cell membrane potential changes and the extracellular recording of local ATP concentration from a single taste bud cell.

3.2. Extracellular electrical recordings of single taste bud cell

The basic mechanism of LAPS chip for the extracellular electrical recording of cell membrane potential changes was presented in Fig. 2. Taste bud cells were cultured on the surface of the LAPS chip with electrolyte-insulator [SiO_2]-semiconductor [Si] (EIS) structure. A movable laser with a diameter around 10 μm was utilized to select the desirable single taste bud cell for measurement by local illumination. When the LAPS chip was supplied with a bias voltage, membrane potential changes of illuminated single taste bud cell were coupled to the bias voltage, which can generate a corresponding fluctuation in photocurrent. Therefore, it can record the membrane potential changes of a single taste bud cell by recording the fluctuation in photocurrent. For this, a bias voltage was applied to LAPS chip by the working electrode and the reference electrode to get the LAPS *I-V* curve on the illuminated spot. The *I-V* curve is a typical “S” shaped curve, which is utilized to choose the proper working point for long-term recording of photocurrent fluctuation when LAPS works in the continuous mode. It is, therefore, able to record electrical cellular responses from single taste bud cell by extracellular recording of cell membrane potential changes with LAPS chip.

For extracellular electrical recording, a representative bitter substance, denatonium, was utilized to stimulate taste bud cells cultured on LAPS surface. Besides, to confirm the origination of extracellular electrical recordings, a hemichannel blocker, CBX, which can dramatically inhibit the membrane current and ATP release of taste bud cells, was applied to taste bud cells. Fig. 3(a) shows a typical extracellular electrical recording from single taste bud cell with and without application of CBX. As shown in the upperpart of Fig. 3(a), that is No CBX applied, it can be observed the application of denatonium leads to significant increase in the rate of firing spikes. On the contrary, as shown in the lower part of Fig. 3(a),

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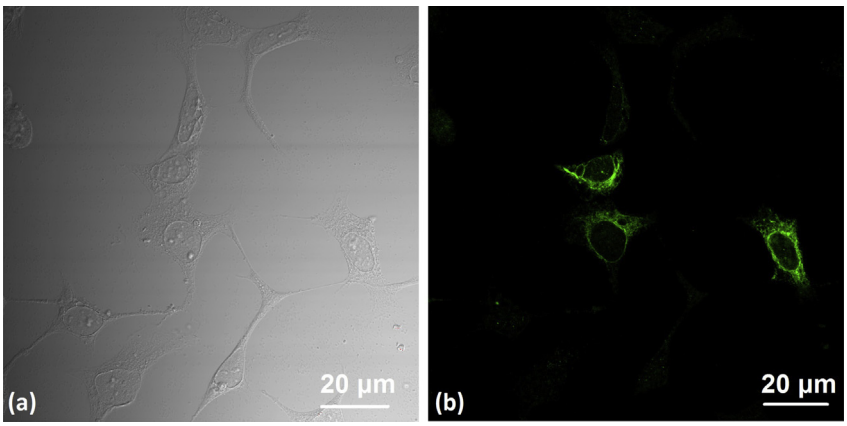

Fig. 1. Fluorescent imaging of taste bud cells staining by mouse monoclonal anti-IP_3R3 and FITC conjugated to goat antimouse IgG antibodies. (a) Optical field and (b) fluorescent field.

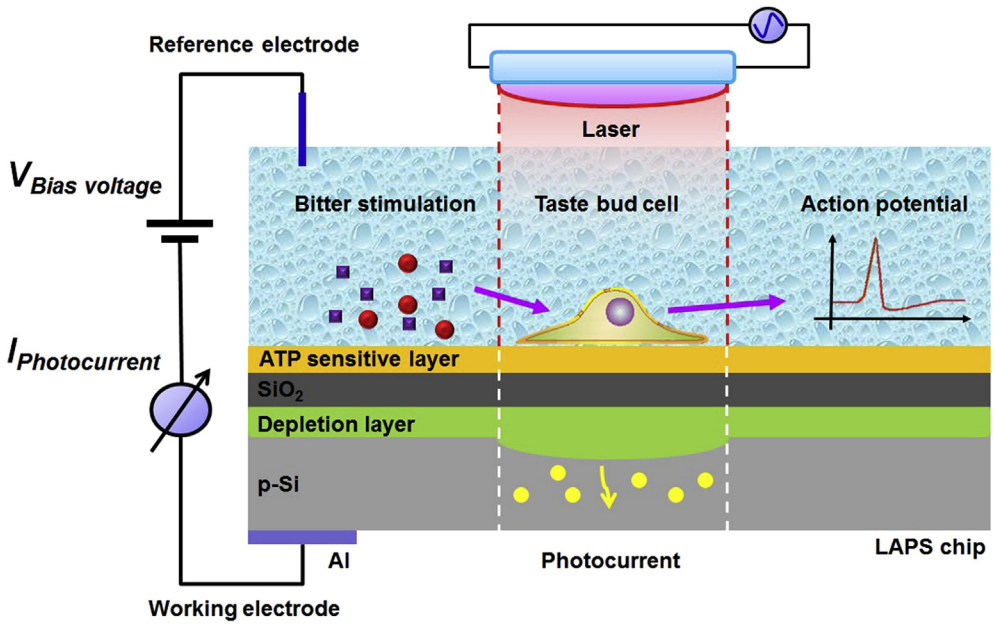

Fig. 2. Schematic diagram showing mechanisms of LAPS chip for extracellular recording of cell membrane potential changes from single taste bud cell under bitter stimulation.

that is With CBX applied, no significant change in the rate of firing spikes can be observed before and after the application of denatonium. This is due to the block effect of CBX to the hemichannel, which can inhibit the generation of firing spikes on taste bud cells during the application of denatonium. Fig. 3(b) shows the statistical results of electrical extracellular recording from taste bud cells. In case of no CBX applied, it is indicated the rate of firing spikes in response to denatonium (14.1 ± 0.8 spikes/10s) is significantly higher than that of without denatonium stimulation (2.7 ± 0.9 spikes/10s, $p = 7.69 \times 10^{-9}$, $n = 8$). In case of with CBX applied, there is no significant difference in the rate of firing spikes before and after the application of denatonium stimulation. It is indicated the rate of firing spikes with CBX application after denatonium stimulation (2.9 ± 0.6 spikes/10s) is significantly lower than that of without CBX application after denatonium stimulation (14.1 ± 0.8 spikes/10s, $p = 4.51 \times 10^{-4}$, $n = 6$). All the results proved that this biosensor successfully recorded the enhancive effects of denatonium and inhibitory effects of CBX on the rate of firing spikes, which suggest it can perform extracellular electrical recording at the single taste bud cell level.

3.3. Recording of ATP release from single taste bud cell

Fig. 4 shows the mechanism of LAPS for extracellular recording of local ATP concentration from a single taste bud cell. LAPS chip can measure the changes in surface charge resulting from redistribution of charged molecules occurred directly at the gate surface of LAPS chip or within the Debye length from the surface. As shown in Fig. 4(a), for detecting ATP released by taste bud cell, LAPS chip was functionalized with ATP-sensitive aptamers that can bind with ATP molecules, which will lead to the surface charge changes because of the leave of ATP aptamers from LAPS surface. Charge changes associated with ATP exposure will result in the modulation of the flat-band potential. In this case, if a bias voltage is applied to the LAPS to make LAPS works in the discrete mode and a focused laser is illuminated on the measurement spot, the charge changes before and after ATP exposure on the illumination spot can be measured by recording the changes in LAPS photocurrent, which can be read out by the shift of the LAPS *I-V* curves as shown in Fig. 4(b). Therefore, LAPS chip functionalized with ATP-sensitive aptamers can record the local ATP concentration changes

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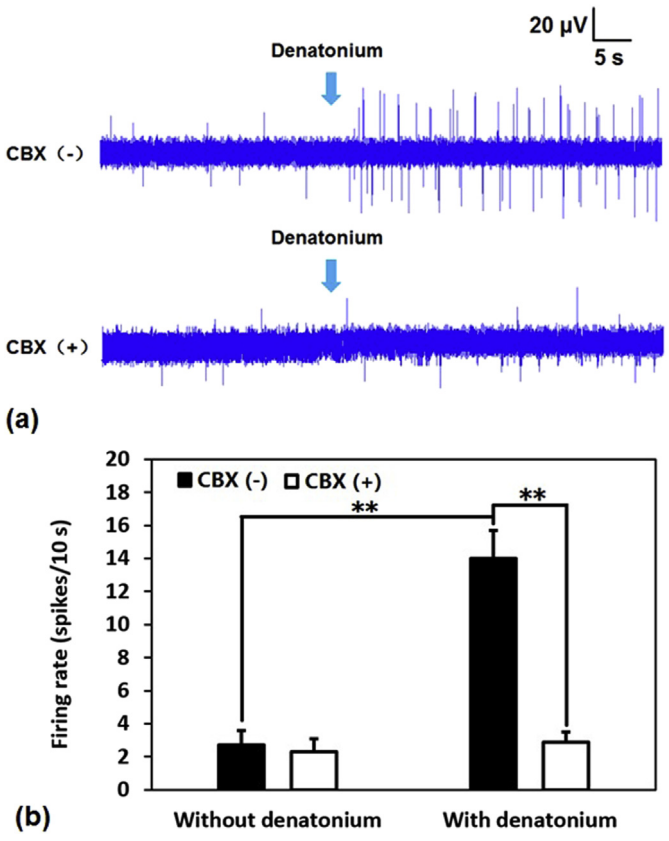

Fig. 3. (A) Typical extracellular membrane potential recording from a taste bud cell using LAPS chip under denatonium stimulation (1 mM) with and without application of CBX. CBX (−), CBX free; CBX (+), CBX application (10 μM). (b) Enhancive effects of denatonium (1 mM) and inhibitory effects of CBX on firing spikes of taste bud cells. All data are represented by the mean ± standard error of the mean (SEM). ** $p < 0.01$, Student's *t*-test. The mean and SEM of 8 experiments are indicated.

resulting from ATP release by a single taste bud cell. As a consequence, the generation of photocurrent can be separated into two parts with time resolution to separate the signals resulting from cell membrane potential changes and ATP release. The influences between two types of signals can also be avoided by the time-resolved measurement. In addition, signals resulting from cell membrane potential changes are in the high frequency domain compared to the signals resulting from ATP release, which can also avoid the interactions between two types of signals. While the influences of ATP release from neighboring taste bud cells were controlled by reducing the surface distribution of taste bud cells, which is as low as 1×10^4 cm^2. This makes distances (around 100 μm) between neighboring cells enough to perform the ATP measurement at the

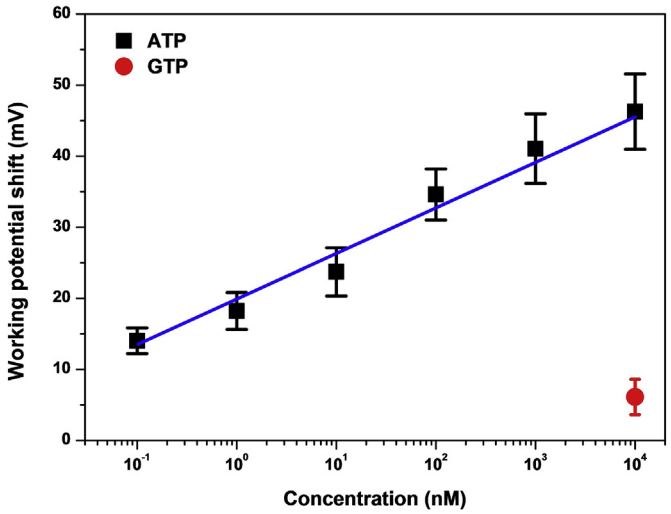

Fig. 5. Performance test of aptamer-based LAPS biosensor for detecting ATP. The mean and SEM of 3 experiments are indicated.

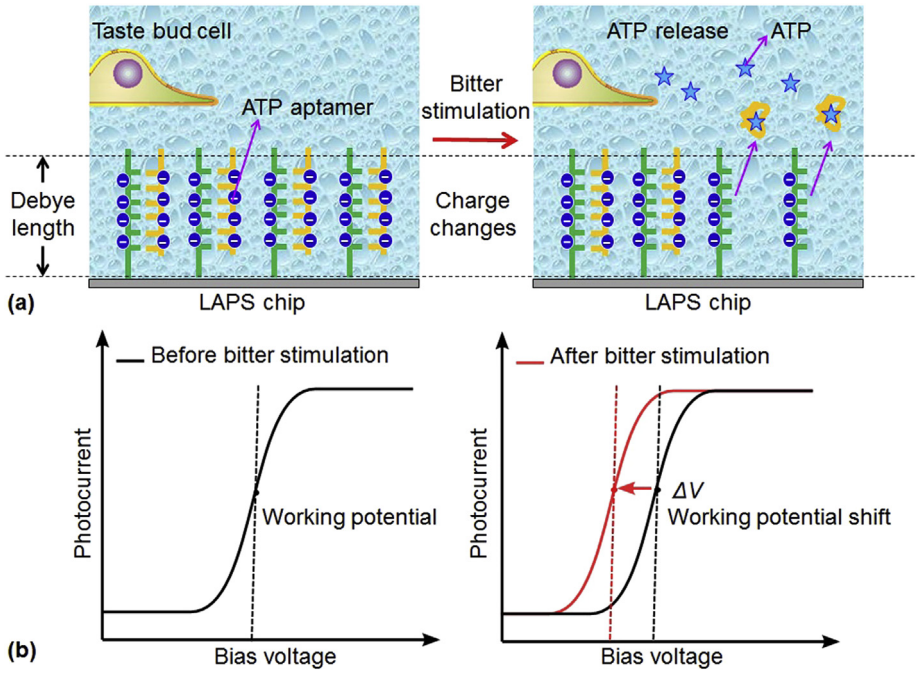

Fig. 4. Mechanism of LAPS chip for the extracellular recording ATP release from single taste bud cell under bitter stimulation. (a) Schematics of surface charge changes due to the ATP release from taste bud cell under bitter stimulation. (b) Working potential shift indicated by the LAPS *I-V* curves recorded before and after bitter stimulation.

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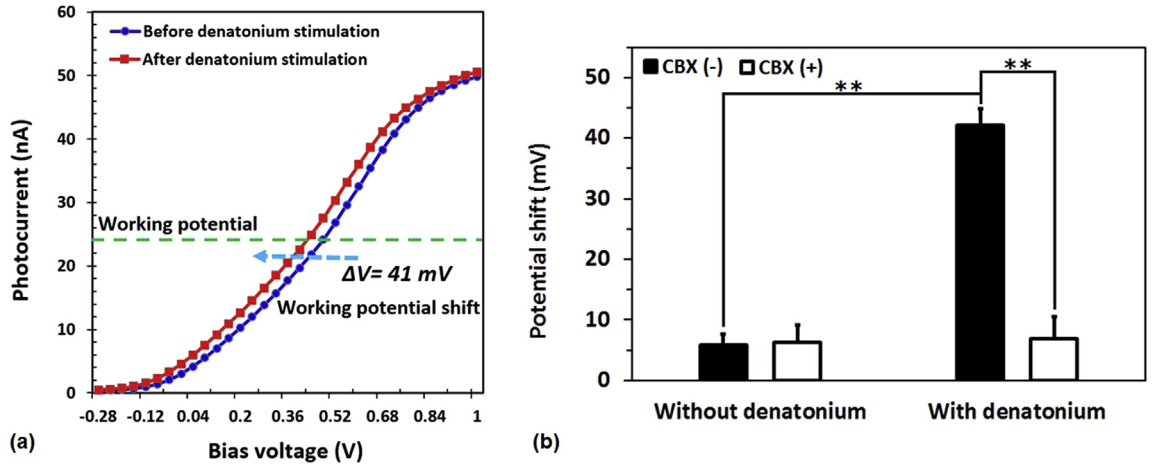

Fig. 6. (A) Typical LAPS *I-V* curves recorded before and after denatonium stimulation. (b) Enhancive effects of denatonium (1 mM) and inhibitory effects of CBX (10 μM) on ATP release of taste bud cells. All data are represented by the mean ± standard error of the mean (SEM). ** $p < 0.01$, Student's *t*-test. The mean and SEM of 6 experiments are indicated.

single-cell level.

Before measurement on ATP release from taste bud cells, the responses of this aptamer-based ATP biosensor were tested by standard ATP solution at different concentrations. Nonspecific target guanosine triphosphate (GTP) was used to test the specificity of this ATP biosensor. The measurement results were shown in Fig. 5, which suggest this biosensor has good linear responses at the ATP concentration ranging from 0.1 nM to 10 μM. In addition, this ATP biosensor shows no significant response to GTP at the concentration of 10 μM, which suggest good specificity for ATP detection. Further, this biosensor shows no significant response to compounds presented in measurement solutions, cell culture medium, and bitter stimulation (data not shown), which suggests good stability for ATP detection under the circumstances of cell measurement.

To confirm the ability of this biosensor for recording the ATP release from single taste bud cell, the LAPS *I-V* curves were recorded before and after the application of denatonium (1 mM). Also, CBX (10 μM) was employed to prove the origination of ATP extracellular recording. Typical LAPS *I-V* curves recorded before and after denatonium stimulation were shown in Fig. 6(a), which show the local ATP concentration released by taste bud cells responding to denatonium stimulation. It is observed a working potential shift of 41 mV was generated indicated by the shift of the *I-V* curve to the left of bias voltage. This means fewer negatively charged DNA molecules on the LAPS surface because of the leave of ATP-sensitive aptamers from LAPS surface. Based on the linear relationship between working potential shifts and ATP concentrations as shown in Fig. 5, the working potential shift of 41 mV marks the local ATP concentration of 3.6 μM. This result is comparable to previously reported results [3]. Fig. 6(b) shows the enhancive effects of denatonium (1 mM) and inhibitory effects of CBX (10 μM) on ATP release of taste bud cells. It is indicated the potential shifts in response to denatonium (42.1 ± 2.6 mV) are significantly higher than that of without denatonium stimulation (5.8 ± 1.8 mV, $p = 6.32 \times 10^{-7}$, n = 6). In case of with CBX applied, there is no significant difference in potential shifts before and after the application of denatonium stimulation. It is indicated the potential shifts with CBX application after denatonium stimulation (6.7 ± 3.4 mV) are significantly lower than that of without CBX application after denatonium stimulation (42.1 ± 2.6 mV, $p = 8.46 \times 10^{-5}$, n = 6). It is proved this biosensor could perform extracellular recording of local ATP release from single taste bud cell.

All the results approved this biosensor could perform dual extracellular recording of single taste bud cells under bitter stimulation. The enhancive effect of denatonium and the inhibitory effect of CBX on bitter signal transduction can be recorded by both extracellular electrical recording and ATP extracellular recording, which shows similar trends on bitter signal transduction. Thus, this biosensor is suitable to be applied to investigating bitter signal transduction. In the future, much more work still needs to be carried out to make this biosensor work better for taste signal transduction. For instance, dual extracellular recording performance should be tested with more kinds of taste cells and under more bitter substances or other tastants.

4. Conclusion

To develop novel and powerful tools for the research of taste signal transduction, this study built the taste bud cell-based biosensors, modify the surface of LAPS chip with ATP-sensitive aptamers, and test the possibility of dual extracellular recording of taste bud cells under bitter stimulations for the first time. All the results suggest one conclusion: this dual extracellular recording biosensor can be used to detect changes in cell membrane potentials as well as ATP release of single taste bud cell. Our primary study has confirmed the performance of this hybrid biosensor for dual extracellular recording and the design possibility of taste bud cell-based biosensor used for bitter signal transduction is also approved. In the following study, we will further test the performance of this hybrid biosensor using various taste stimulations, especially the regeneration of sensor capability for ATP release by the introduction of new ATP aptamer. Our future work will focus on multiparameter extracellular recording to provide more accurate information on taste cell sensing to explore the mechanisms of taste signal transduction.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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