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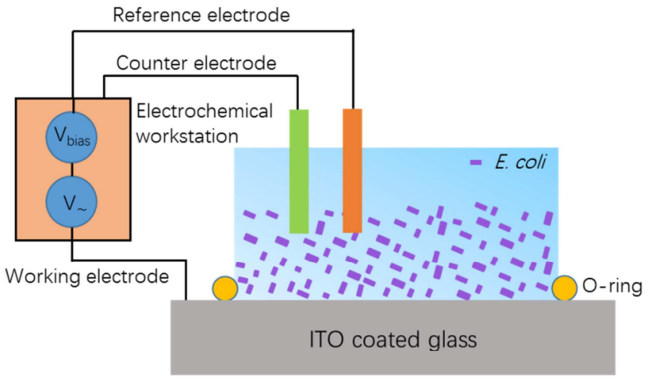

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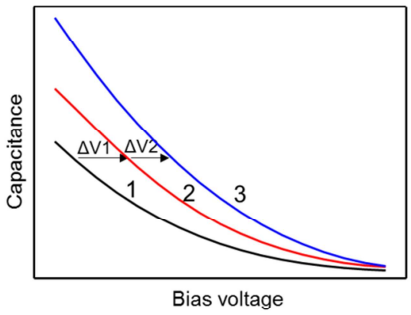

(b)

A bioelectronic taste sensor based on bioengineered *Escherichia coli* cells combined with ITO-constructed electrochemical sensors

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Abstract: In this study, we developed a novel bioelectronic taste sensor for the detection of specific bitter substances. A human bitter taste receptor, hT2R4, was efficiently expressed in *Escherichia coli* (*E. coli*), which was used as the primary recognition element. A simple and low-cost electrochemical device based on ITO-based electrolyte-semiconductor (ES) structure was innovatively employed as the transducer to assess bacterial metabolic consequences of receptor activation in real time. An apparent increase in extracellular acidification rate was observed, which was resulted from the triggering of hT2R4 receptors by their target ligand of denatonium. The sensor showed dose-dependent responses to denatonuim ranging from 50 nM to 500 nM, while non-bioengineered bacteria without hT2R4 receptors exhibited negligible responses to the same stimulus. In addition, the specificity of the proposed taste biosensor was verified using other typical bitter substances such as quinine and alpha-naphthylthiourea (ANTU). This research provides a simple and inexpensive approach for the construction of bioelectronic taste sensors.

Key words: taste receptor, cell acidification, ITO-based ES sensor, *E. coli* cells

1. Introduction

As one of five basic sensations, the taste has gained tremendous attentions for its potential applications in many fields such as food safety, pharmaceutical industry and environment monitoring. Recently, bioelectronic devices based on taste cells and receptors have been widely developed for highly selective detection of target chemicals [1]. The recognition element can be taste cells [2, 3] and taste buds of animals containing native taste receptors [4-6]. The preparation of primary cells need killing animals each time, and the types of the obtained taste receptor can be hardly controlled. Mammalian cell lines or bacterial cells that are heterologously expressed with functional taste receptors could solve these problems [7-9]. Compared to mammalian cell-based expression systems, bacteria-based receptor expression systems are more efficient and robust for the mass production of recombinant proteins [10, 11]. In most cases, the expressed receptors are collected and purified from the lysed cells (e.g. *Escherichia coli* (*E. coli*)), and then coupled with the secondary transducer for specific sensing purposes. The complex process may suffer from problems such as loss of biological activity or unsuccessful binding of receptor proteins. Using bioengineered bacteria directly as the primary recognition element would help to preserve the receptor activity and simplify the construction of taste sensors.

To develop taste biosensors, various types of secondary signal transducers have been used, such as field-effect transistors (FETs) [9-12], microelectrode arrays (MEAs) [5, 6],

light-addressable potentiometric sensors (LAPSs) [2-4] and quartz crystal microbalance (QCM) devices [13]. For receptor-based bioelectronic tongues, responsive signals resulted from interactions between ligands and receptors immobilized on sensor surface were detected [9-13]. On the other hand, cell- or tissue-based taste sensors mainly measured cell membrane potential changes induced by the activation of receptors [3-6]. These methods mentioned above require either refined manufacturing processes, or expensive sensor materials or instrumentations.

It is well known that metabolic perturbations of living cells play a critical role in the assessment of cellular activities including receptor stimulation [14-16]. The activation of receptors can modulate the rate of extracellular acidification by causing metabolic changes that affect the demand for ATP. Therefore, by measuring extracellular acidification, functional ligand-receptor interactions in cells can be assessed indirectly. LAPS is the most commonly used technique for metabolic recording [7, 17-21]. It is featured with an electrolyte-insulator-semiconductor (EIS) structure and is illuminated with a modulated light source to generate an alternating photocurrent as the sensor signal. Traditional LAPS has a pH sensitivity of about 57 mV/pH when consists of a sensing layer of Si_3N_4 [19]. Recently, a new concept based on electrolyte-semiconductor (ES) structure has been suggested in LAPS system for biological sensing and imaging purposes [22-25]. Especially, the introduction of indium tin oxide (ITO) as the semiconductor [23, 24], enables the ES structure-based sensor to be a simple, low-cost and sensitive assay in chemical and biological sensing applications.

Inspired by the previous work, herein, we report for the first time, the use of ES structure

as a (bio-)chemically sensitive capacitor to monitor bacterial metabolism and metabolic consequences of receptor activation in real time. To functionalize bacterial cells, a human bitter receptor-hT2R4, which belongs to the large family of G-protein coupled receptor, was expressed in *E. coli* as a study model. A direct current (dc) polarization via reference electrode is applied to the bare ITO electrode, superimposed to a small alternating current (ac) voltage to measure the capacitance through an impedance analyser. The capacitance is modulated both by the bias voltage and interface chemical state (e.g. solution pH). This sensor requires no additional steps arising from protein extraction, cell/protein immobilization, or sensor chip modification and encapsulation. Benefitted from the simple processing and testing procedure, the proposed sensor system shows great potentials in areas of pharmaceutical industry and food screening. For example, it could be used to detect the bitter taste in Chinese herbal medicine to determine whether it is acceptable for patients or not, or to detect the bitter compounds in contaminated food which are usually regarded as a sign of toxin presence. Moreover, by expressing with different types of taste receptors, the sensor system could assess other tastes including sweet, sour, salty and umami, thus providing essential information on the state, quality and nutrition of food.

2. Experimental section

2.1. The preparation of bioengineered *E. coli* cells

Construction of the plasmid. The target DNA sequence of *t2r4* was amplified by PCR to obtain gene with a his_6-tag on the 3' terminal. Both the PCR product and *pMAL-c5E* vector

(donated by Shannxi Institute of Microbiology) were digested with *BamH* I and *Nco* I. Then the digested *t2r4* was cloned into *pMAL-c5E* using T4 DNA Ligase (Takara, Japan) to obtain the recombinant plasmid *pMAL-c5E-t2r4/his*$_6$, which was identified by the double digestion and DNA sequencing. The upstream and downstream primers for *t2r4* amplification are *5'- cgcat gccat ggcta tgctt cggtt attct at-3' and 5'- cggga tccct aatga tgatg gtgat gatgt ttttt gaaac aaaga atc-3', respectively.*

Transformation of recombinant plasmid. The recombinant plasmid *pMAL-c5E-t2r4* was transferred into the *E. coli* DH5α (purchased from TIANGEN) by the heat shock method. For the first, the recombinant plasmid *pMAL-c5E-t2r4* was incubated with *E. coli* DH5α cells for 30 min on ice. Then, the mixture was kept in 42 ℃ water bath for 90 s and on ice for 5 min, respectively. Following selection of transformed cells on solid lysogeny broth (LB) medium supplemented with ampicillin (50 μg/mL), the recombinant plasmid *pMAL-c5E-t2r4* was recovered from a subset of colonies for sequencing.

Expression and characterization of hT2R4 receptors. The *E. coli* Rosetta (DE3) cells (purchased from TIANGEN) were transferred with the *pMAL-c5E-t2r4* vector and incubated in LB medium containing ampicillin. Gene expression was induced with 0.06 mM of isopropyl b-D-thiogalactoside (IPTG) followed by incubation for 6 h at 30 ℃. Finally, cells were harvested by centrifugation (5000 rpm, 10 min) and stored at -20 ℃ for further experiments. The SDS-PAGE analysis and Western blot analysis were performed to test the expression of hT2R4. To obtain the hT2R4 proteins, bioengineered *E. coli* cells were lysed by sonication. Insoluble fractions were collected by centrifugation (12000 rpm, 15 min) and then re-suspended in PBS. Standard Coomassie blue stained SDS-PAGE was

carried out and the resulting gel was transferred to a PVDF membrane. After blocking, the membrane was incubated with mouse anti-his_6 monoclonal antibody followed by HRP-conjugated goat anti-mouse IgG. The expression of hT2R4 was visualized using EZ-ECL Chemiluminescence Detection Kit for HRP. All the Western blot related reagents were purchased from Beyotime (China).

Bacterial activity assessment. The effect of the receptor expression on bacterial activity was determined by measuring the optical density of *E. coli* suspension at 600 nm (OD600) with a spectrophotometry (UV2600, UNIC, China). The absorbance of the native and bioengineered bacteria cultured in LB medium at 37 ℃ was continuously monitored in 1 h intervals.

2.2. ES sensor preparation and electrochemical measurements

Indium tin oxide (ITO)-coated glass (50 Ω/cm^2) was purchased from Diamond Coatings Limited, UK. The glass was cut into 1 cm × 1 cm pieces and cleaned in an ultrasonic bath with acetone, isopropyl alcohol, and ultrapure water before use.

All the electrochemical measurements were carried out on an electrochemical workstation (Zennium, Zahner Elektrik, Germany) and used the Pt wire and Ag/AgCl (in saturated KCl) electrode as a counter electrode and a reference electrode, respectively. The ITO substrate was mounted into a homemade measuring chamber as the working electrode (see Figure 1). Capacitance-voltage (*C*-*V*) and constant-capacitance (ConCap) measurements were carried out at a frequency of 10 Hz with an ac voltage of 10 mV. In the ConCap mode, the capacitance of the ES sensor is kept constant by using a

feedback-control circuit which enables direct monitoring of potential changes at the sensor surface. For pH sensitivity measurements, a range of 10 mM phosphate buffer solutions (pH 4-9) with 100 mM NaCl were used. For *E. coli* acidification measurements, 0.8 mM pH 7.2 phosphate buffer solution with 170 mM NaCl was adopted. Glucose and bitter chemicals including denatonium, quinine and alpha-naphthylthiourea (ANTU) were purchased from Sigma-Aldrich. All solutions were prepared using ultrapure water (18.2 MΩ·cm).

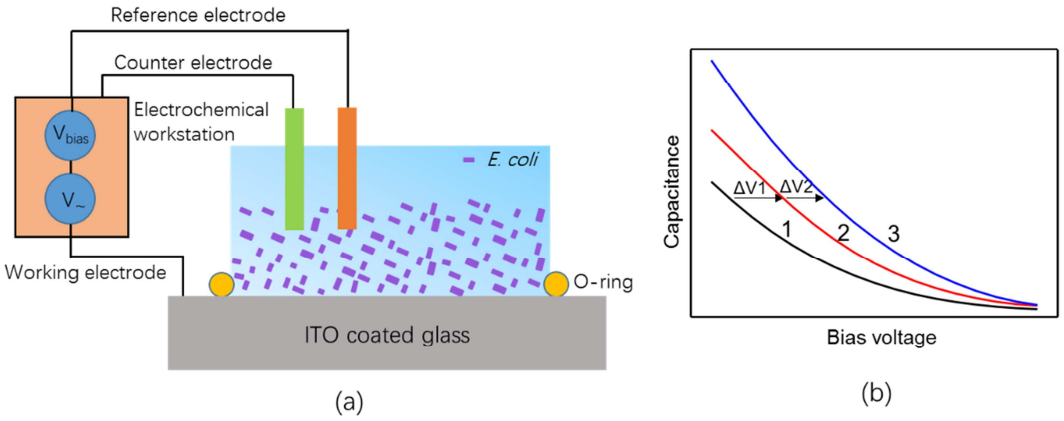
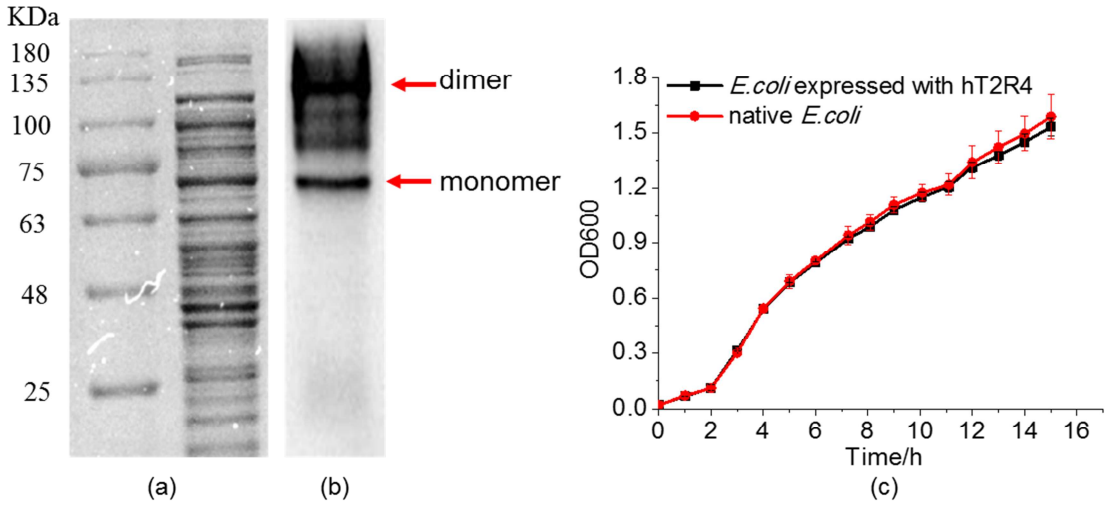


Figure 1. (a) Schematic diagram for ITO-based electrochemical measurements and (b) expected shifts of capacitance-voltage (*C-V*) curves due to the extracellular acidification process of *E. coli* cells. Lines 1, 2 and 3 represent results measured without glucose, with glucose and with target bitter stimulus, respectively. *ΔV1* and *ΔV2* represent the voltage shifts at a constant capacitance induced by extracellular acidification and acidification enhanced by bitter stimulation, respectively.

3. Results and discussion

3.1 Expression of bitter receptor in *E. coli*

The previous report has shown that extracellular acidification measurements on human embryonic kidney (HEK)-293 cells expressed with bitter receptors can reflect the function of receptor stimulation [7]. It is therefore suggested that extracellular acidification can be considered as a specific cellular response indicating specific interactions between expressed bitter receptors and their ligands. However, the expression of bitter receptors in mammalian cells usually suffers from low expression efficiency, toxicity, and stringent growth conditions to maintain cell activities. These make it difficult to obtain convenient and reliable biosensors for practical applications, especially the application related to in-field measurements. Bioengineered bacteria expressed with bitter receptors provides a possible approach to solve this problem, which is easy to culture in-field and shows decisive advantages in high expression efficiency and robustness in mass production. As a G-protein coupled receptor, hT2R4 is usually expressed on the plasma membrane of mammalian cells for cellular signal transduction. The triggering by its ligand mediates a diverse array of cellular functions such as cell metabolism [7]. As a result, this study explores the possibility of using hT2R4 receptor expressed *E. coli* cells as the sensitive element for bitter sensation.



163

164 Figure 2. (a) SDS-PAGE analysis and (b) Western blot analysis of hT2R4 produced from

165 *E. coli*. The dark bands marked by the red arrows indicate the expression of human taste

166 receptor hT2R4 protein. (c) The growth curves of native *E. coli* and *E. coli* expressed with

167 hT2R4 by analyzing the optical density at 600 nm.

168 The expression of hT2R4 in *E. coli* was demonstrated by the SDS-PAGE analysis and

169 Western blot analysis, which both show a band of 70 kDa in the pictures (Figure 2). The

170 133 kDa band represented the dimeric forms of the receptor protein, which was

171 sometimes obtained in the Western blot analysis [26]. Furthermore, the effect of receptor

172 expression on bacterial proliferation was evaluated by measuring the optical density of *E.*

173 *coli* suspension. As shown in Figure 3c, the growth rates for native and hT2R4 expressed

174 *E. coli* cells were almost the same, indicating no obvious effects on the growth of bacteria.

3.2 pH sensitivity of ITO-based ES sensor

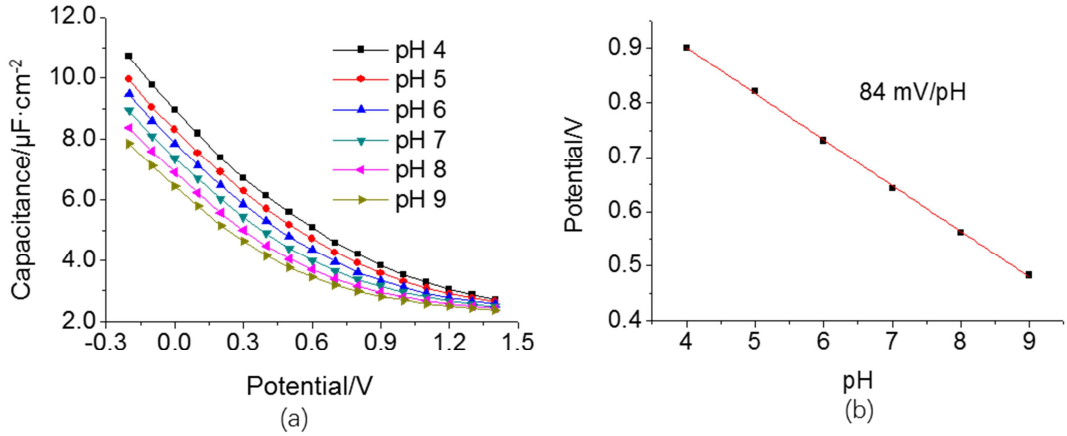


Figure 3. (a) *C-V* curves of ITO-coated glass at different pH values and (b) the pH sensitivity of the ITO-coated glass (R^2 = 0.998).

In an electrolyte-semiconductor (ES) structure, there are two capacitors in series: the interfacial (electrode/electrolyte) double layer capacitance and the space charge capacitance. Since the space charge capacitance is much smaller than double layer capacitance (2-3 orders of magnitude), the capacitance value measured from this model is assumed to be mainly contributed by the space charge capacitance. Figure 3a depicts capacitance (*C*) measured for ITO substrate as a function of applied voltage in a series of pH phosphate buffer solutions (10 mM, 0.1 M NaCl, pH 4-9). As an n-type semiconductor, there is an apparent decrease in capacitance response to higher applied voltages, which is resulted from the decrease of space charge capacitance. Furthermore, the capacitance shifts towards lower potentials as pH increases due to the change of the surface charge state. The sensor showed a high pH sensitivity of 84 mV/pH at a constant capacitance of 5 $\mu F \cdot cm^{-2}$ (Figure 3b), which was higher than that of traditional LAPS, making this a

promising pH sensor for biochemical analysis. After measuring *C-V* curves in buffer solutions from pH 4 to 9 in sequence, the *C-V* curve returned to its initial position when the pH was changed back to 7 (data not shown), showing good reversibility and stability of ITO-coated glass for pH sensing.

3.3 Extracellular acidification measurements of *E. coli*

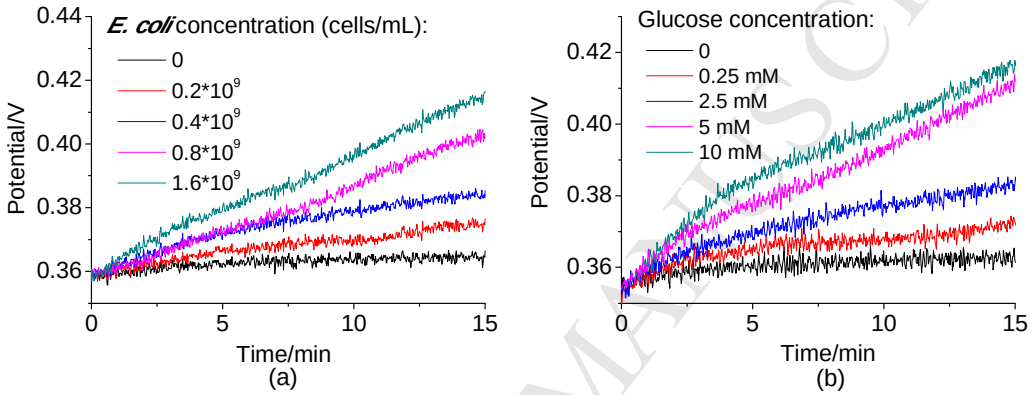


Figure 4. Potential changes of ConCap measurements (a) with different concentrations of *E. coli* (10 mM glucose) and (b) with different concentration of glucose ($1.6*10^9$ cells/mL). Working point of ConCap measurement was set to 5 $\mu F \cdot cm^{-2}$.

Cellular metabolism is the set of life-sustaining chemical transformations within the living cells. It begins with the uptake of the nutrition and oxygen, and produces acidic products such as acetate, lactate, succinate, etc., resulting in extracellular acidification. To validate the feasibility of the ITO-based ES sensor for metabolic activity measurements, extracellular acidification of native bacteria in suspension with different *E. coli* cell concentrations at a constant amount of glucose (Figure 4a), as well as with various glucose concentrations at a constant amount of cells (Figure 4b) were recorded using the

ConCap mode in real time. In contrast to *C-V* measurements, the ConCap mode permits a dynamic investigation of sensor behavior. During the testing period of 15 min, the potentials kept to increase at a constant capacitance of 5 μF·cm^{-2}, indicating more acidic values of the cell suspension solution. For example, the voltage increased by about 43 mV after 15 min's fermentation for the sample of 0.8 * 10^{9} cells/mL fed with 10 mM glucose, corresponding a pH decrease by 0.51. As expected, the potential increase is more significant when measured with a higher concentration of *E. coli* cells or glucose. The control sample without *E. coli* shows negligible changes in potential over the course of the experiment. It is worth to mention that some suspended cells would randomly land on the sensor surface during testing. As a result, it is desirable to investigate if the cell attachment affect the responsive sensor signals. The possible interference on sensor responses was excluded by the result shown in Figure 4b (the black curve), which showed no obvious potential perturbation for *E. coli* cells without glucose supplements.

3.4 Sensory responses of bioengineered bacteria to bitter substances

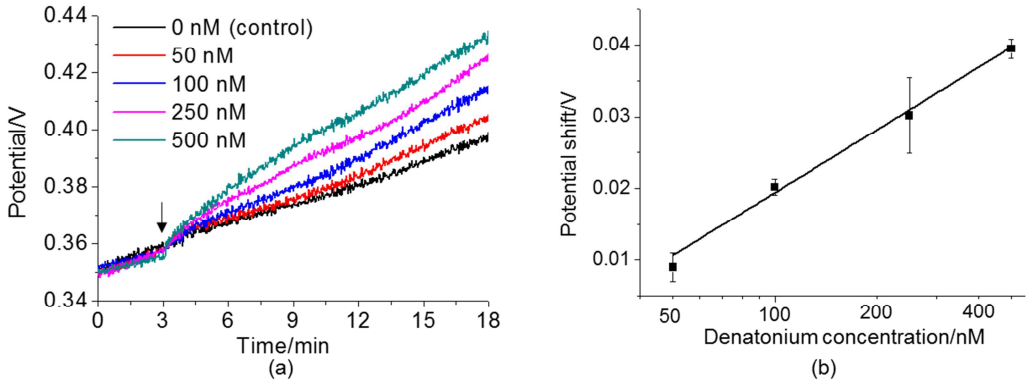


Figure 5. (a) Potential changes of ConCap recordings from bioengineered *E. coli* cells (1.6*10^{9} cells/mL, 10 mM glucose) in response to different concentrations of denatonium.

224 The black arrow marks the addition time of denatonium. Working point of ConCap
225 measurement was set to 5 $\mu F \cdot cm^{-2}$. (b) Corresponding statistical results of the taste
226 receptor-based biosensor in response to serial concentrations of denatonium (50 nM to
227 500 nM) evaluated 15 min after the addition of stimulus. The mean and stand error of the
228 mean (SEM) of three experiments are shown.

229 Since cellular metabolism acts as an important regulator for various cellular functions
230 including receptor activation, extracellular acidification process was recorded to
231 investigate the responses of bioengineered bacteria to target chemicals. Figure 5a depicts
232 the ConCap recordings of *E. coli* cells ($1.6*10^9$ cells/mL, 10 mM glucose) expressed with
233 functional hT2R4 in response to different concentrations of denatonium, which is the
234 natural ligand of the receptor. With the addition of denatonium at time t = 3 min (marked by
235 black arrow in Figure 5a), the cellular metabolism activity was apparently enhanced. The
236 statistical results evaluated in 15 min after the addition of stimulus show does-dependent
237 responses to denatonium (Figure 5b). With the concentration increasing from 0 to 500 nM,
238 more intense acidification was observed. On the other hand, the response of native *E. coli*
239 cells to 500 nM denatonium was negligible (Figure 6). The results demonstrated the
240 communication of hT2R4 with denatonium promotes cellular metabolism consequently.
241 The obtained sensitivity for denatonium detection is similar to the previous reported
242 mammalian cell-based [7] and receptor protein-based [13] sensors. Whereas, it provides
243 a much more inexpensive, robust and simpler approach for the construction of
244 bioelectronic tasters. In the real-word, denatonium is added to consumer products to
245 prevent accidental ingestion with a concentration in the order of μM [27, 28], that is higher

than the limit of detection of the proposed taster. The European Food Safety Authority (EFSA) reported the rat median lethal inhalation concentration (LC50) of denatonium is around 0.2 mg/L (~400 nM) [29], which is in the detection range of our sensor. Therefore, the proposed bitter taste sensor would be useful for certain analytical applications. Moreover, the responses of the bioengineered and native cells to other bitter substances (quinine and ANTU) were also examined. As shown in Figure 6, both cells with and without hT2R4 expression exhibited very low responses to quinine and ANTU, indicating a good specificity of the developed taste sensor. The negative responses appeared in some measurements could be attributed to the measuring error.

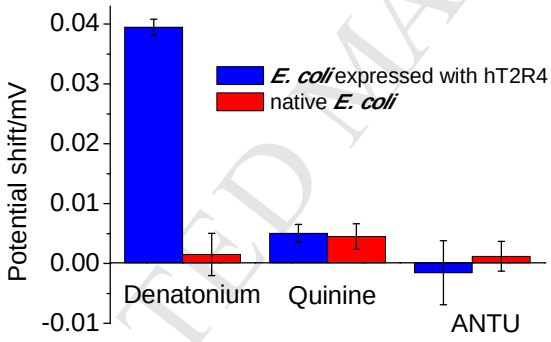


Figure 6. Responses of *E. coli* cells to various bitter stimuli at the concentration of 500 nM evaluated 15 min after the addition of bitter chemicals. The mean and stand error of the mean (SEM) of three experiments are shown.

4. Conclusions

In summary, we developed a label-free functional assay for detecting specific bitter substances on the basis of extracellular acidification measurements of bioengineered *E.*

coli cells. The human taste receptor hT2R4 was successfully expressed in *E. coli* and the cellular metabolism as well as the metabolic consequences of receptor activation were recorded using an ITO-based electrochemical ES sensor. The proposed biosensor showed does-dependent responses to the target ligand of denatonuim, while native *E. coli* did not response to the stimuli, indicating the communication of hT2R4 receptor with denatonium regulates cellular metabolism. To the best of our knowledge, it is for the first time that *E. coli* cells expressed with taste receptors are used as a sensitive element directly, thus no additional steps such as protein extraction and cell/protein immobilization are raised. In addition, the bioengineered cells in combination with ITO-based electrochemical ES system provide a considerably simple and low-cost strategy for the construction of bioelectronic tasters. For further application, other taste receptors could be expressed in bacteria as well thus rendering a better service in the fields of pharmaceutical industry and food screening.

Acknowledgments

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Highlights

- *E. coli* was successfully expressed with human taste receptor hT2R4 for specific detection of bitter substance of denatonium.

- A simple and low-cost ITO-based ES sensor was proposed to record the extracellular acidification process of bioengineered *E. coli*.

- The developed taste sensor shows does-dependent responses and high selectivity to denatonium due to the communication of hT2R4 expressed in cells with the bitter target.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors declare no conflict of interest.