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A biomimetic bitter receptor-based biosensor with high efficiency immobilization and purification using self-assembled aptamers

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It is of substantial interest to mimic mechanisms of biological sensing systems for the development of novel biosensors. This paper presents a novel biomimetic bitter receptor-based biosensor for the detection of specific bitter substances, in which bitter receptors were used as sensitive elements for the first time. A simple and practical self-assembled aptamer-based strategy was proposed for functional immobilization and purification of bitter receptors. A human bitter receptor, T2R4, was expressed on the plasma membrane of HEK-293 cells and fused with a His_6-tag on its C-terminal. The membrane fractions containing the expressed T2R4 were extracted and immobilized on the gold surface of a quartz crystal microbalance (QCM) pretreated with a monolayer of self-assembled aptamers that can specifically recognize and capture biomolecules labeled with His_6-tags. The QCM device was used to monitor the responses of T2R4 to various bitter stimuli. The results indicate that this biosensor can detect denatonium with high sensitivity and specificity, which is the specific target of T2R4. In addition, this biosensor shows dose-dependent responses to a certain concentration range of denatonium. The sensitivity of bitter receptor-based biosensors prepared by an aptamer-based method is 1.21 kHz mM^{-1}, which is 2 times higher than that prepared by a SAM-based method. The major advances on bitter receptor immobilization and purification presented in this work could substantially be very useful for developing other membrane receptor-based biosensors and molecular sensor arrays. This bitter receptor-based biosensor has great potential to be used as a valuable tool for bitter detection as well as for the research of taste signal transduction.

1 Introduction

Membrane receptors are a major class of molecular sensors located on the cell membrane. Ubiquitous in nature, membrane receptors are present in all living cells and are known to play a pivotal role in the detection of extracellular biochemical or biophysical signals. Especially, in the biological olfactory and taste systems, olfactory receptors and taste receptors are membrane molecular sensors for the detection of environmental chemical signals presented by odorants or tastants, which can provide important information on safety, food, and communications.[1–3] In the recent decade, the advances in the research of olfactory and taste transduction mechanisms have made a significant impact on the development of biomimetic receptor-based biosensors for chemical sensing.[4,5] Various functional membrane receptors have been used as sensitive elements for the development of novel biomimetic biosensors for the detection of chemical substances, involving the employment of olfactory receptors and taste receptors.[6–10] Within this context, high efficiency immobilization and purification of membrane receptors on the solid supports are still the main challenges to develop novel biomimetic biosensors for chemical sensing.

In view of their key importance in biomimetic receptor-based biosensors, there is a strong need to develop highly efficient and convenient methods for the immobilization and purification of membrane receptors to satisfy the need for biosensing applications. To this end, a large number of methods for sensor surface modification have been developed to meet the specific requirements in handling functional membrane receptors.[11,12] One of the commonly used methods is physical absorption, which usually use membrane receptors coupled directly to the sensor surface without any modification.[13,14] Although the physical adsorption methods are simple and convenient, these methods usually suffer from the reduction in the stability and repeatability of the biosensors due to the difficulties in controlling the chemical and physical properties of adsorbed sensitive molecules. Alternatively, various methods based on surface chemical treatment, for example covalent polymer

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coating, have been demonstrated with high stability and repeatability.[15–17] These methods can improve the surface robustness to thermal, mechanical, and solvolytic instabilities. However, these methods are typically laborious, costly, and time-consuming. Moreover, these physical and chemical methods are not able to provide sufficient specificity for the efficient purification of membrane receptors. Therefore, it is necessary and favorable to develop novel methods for sensor surface modification that could not only be simple, convenient, and robust to environmental factors, but also provide sufficient specificity for the purification of membrane receptors.

In the recent decade, self-assembled monolayers (SAMs) have been applied in the sensor surface modification in order to provide a simple and efficient approach to improve the efficiency and stability of coupling between sensitive receptors and transducers.[18–21] SAMs are prepared by spontaneous tethering of molecules with active chemical moieties onto reactive solid surfaces, which holds the advantages of easy preparation, low cost, and versatility. On the other hand, aptamers, which are nucleic acid ligands, have also been applied in biosensors as sensitive elements due to their high sensitivity and specificity to their molecular targets. The binding affinity and specificity of aptamers are comparable to those of antibodies, which have been widely viewed as promising complementary molecules to antibodies.[22,23] The combination of SAMs and aptamers may provide novel solutions for the high efficiency coupling of sensitive receptors with transducers due to the combination of high stability of SAMs with high specificity of aptamers.

With the new insights into the mechanisms of biological taste sensation, more and more efforts have been devoted to the development of novel biomimetic taste biosensors in recent years. Many types of biomimetic taste biosensors have been developed by the combination of various transducers with taste functional components such as taste buds,[24] taste bud cells,[9,25,26] and taste receptors.[10] Due to the employment of biological taste components, these biosensors are characterized with high sensitivity, rapid response and excellent selectivity, which have great potential to be applied in many fields including biomedicine, environmental protection, and food safety. However, there is no report on the use of bitter receptors for the development of biomimetic receptor-based biosensors for the detection of bitter substances.

In this study, we developed a biomimetic bitter receptor-based biosensor for specific bitter substance detection, in which bitter receptors were used as sensitive elements for the first time. In addition, a self-assembled aptamer-based strategy was proposed to generate highly efficient bitter receptor immobilization and purification on a sensor surface. A human bitter receptor, T2R4, was functionally expressed on the plasma membrane of HEK-293 cells and characterized by immunofluorescent staining. This bitter receptor-based biosensor was developed on the basis of a quartz crystal microbalance (QCM), which is a commonly used mass sensitive transducer for the research of molecular biosensors. The immobilization of T2R4 was characterized by electrochemical measurements. The performances of this bitter receptor-based biosensor were tested by recording the resonant frequency shifts of a QCM in response to various bitter stimuli. The specificity and sensitivity of this biosensor were tested and compared with the SAM-based method. In addition, an olfactory receptor, ODR-10, was used as a control receptor to investigate the cross-selectivity of bitter receptor-based biosensors.

2 Experimental

2.1 Bitter receptor preparation

A human bitter receptor, T2R4, was prepared by extracting the membrane fraction of cells expressed with T2R4. Firstly, the expression plasmid *pCEP4/rho-T2R4-His$_6$* was constructed, which contained the full length cDNA of T2R4 and the His_6-tag. After confirmation by DNA sequencing, the plasmid was used to transfect human embryonic kidney cells (HEK-293) using Lipofectamine™ 2000 (Invitrogen). The expression of T2R4 was validated by immunofluorescent staining using anti-His_6-tag rabbit IgG as the primary antibody and FITC conjugated anti-rabbit IgG as the secondary antibody. The nucleus of HEK-293 cells was stained using 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA), which can pass through an intact cell membrane and bind strongly to A–T rich regions in DNA. Images were taken using a fluorescent microscope (IX81-FV1000, Olympus, Center Valley, PA, USA). Membrane fractions containing expressed His_6-tagged T2R4 were extracted from transfected HEK-293 cells using the membrane protein extraction kit (Sangon Biotech, China). The resulting solution was the mixture of total plasma membrane proteins containing T2R4 and was stored at −70 °C for further experiments. Membrane fractions were also extracted from HEK-293 cells without any transfection to be used as the negative control. An olfactory receptor, ODR-10, with a His_6-tag was prepared as described in our previous reports and employed to investigate the cross-selectivity of bitter receptor-based biosensors.[27,28]

2.2 Bitter receptors coupled to QCM devices

QCM devices of 5 MHz AT-cut quartz crystals with polished gold electrodes were used as the transducer and fixed in a chamber allowing only the exposure of one gold electrode surface to the solution in the chamber. Thiol-modified anti-His_6-tag aptamer ($5'$-HS-$(CH_2)_6$-GCTATGGGTGGTCTGGTTGGGATTGGCCCCGGG AGCTGGC-$3'$) was self-assembled on the gold surface of QCM. Then 11-mercaptoundecanoic acid (11-MUA) (Sigma-Aldrich, USA) was used to block the free sites of the electrode surface and provide suitable steric spaces for the aptamers. Membrane fractions containing expressed T2R4 were dissolved in phosphate buffer solution (PBS) containing 0.5% Triton X-100 and added to the gold surface of QCM for 3 h incubation at 37 °C. After washing with distilled water thoroughly, the biosensors were ready for further experiments. The same protocol was also applied for the preparation of olfactory receptor-based biosensors, in which His_6-tagged ODR-10 was used as a sensitive element. The olfactory receptor-based biosensors were used to investigate the cross-selectivity of bitter receptor-based biosensors.

As a comparison, bitter receptor-based biosensors were also prepared by the SAM-based method. A self-assembled

monolayer of 16-mercaptohexadecanoic acid (MHDA) (10 mmol L^{-1}) was formed on the gold surface of the QCM electrode by 24 h treatment. After activation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 75 mmol L^{-1}) and *N*-hydroxysuccinimide (NHS, 15 mmol L^{-1}) for 1 h, membrane fractions with expressed T2R4 were added and immobilized on the SAMs. Distilled water was used to remove the excessive membrane fractions and the nonspecific adsorption.

2.3 Characterization of bitter receptor immobilization

The stepwise process of bitter receptor immobilization was characterized by electrochemical cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The electrochemical measurements were performed using the electrochemical workstation (Zahner, Germany). All the measurements were performed in a Faraday cage with PBS (pH 7.0) with 5 mM $K_4[Fe(CN)_6]$ and 5 mM $K_3[Fe(CN)_6]$. The gold electrode of the QCM served as the working electrode. Pt wire and an Ag/AgCl (3 M KCl) electrode were used as a counter electrode and a reference electrode, respectively. CV was performed in the potential range from −0.3 V to +0.7 V at a rate of 50 mV s^{-1}. EIS was performed at an alternating potential with an amplitude of 5 mV in the frequency range from 1 Hz to 100 kHz.

immunofluorescent staining was employed to quantitatively characterize the immobilization of bitter and olfactory receptors on the gold surface of QCM. Anti-His_6-tag rabbit IgG was used as a primary antibody and FITC conjugated anti-rabbit IgG was used as the secondary antibody. The fluorescence intensity of bitter and olfactory receptors immobilized on the gold surface of the QCM by the self-assembled aptamer-based method and the SAM-based method was measured and calculated by fluorescent microscopy and the self-developed software (IX81-FV1000, Olympus, Center Valley, PA, USA).

2.4 Performance testing of bitter receptor-based biosensors

To test the performances of bitter receptor-based biosensors prepared by different immobilization strategies, the specificity and sensitivity were measured using a QCM200 system (Stanford Research Systems, Inc., USA) by using the following protocol. Various bitter substances including 30 mM $MgSO_4$, 1 mM denatonium, 1 mM D-(−)-salicin, and 1 mM quinine were used to test the specificity. Different concentrations of denatonium were applied to measure the sensitivity. All the bitter substances were purchased from Sigma-Aldrich (USA) and dissolved in distilled water at desired concentrations to be used as bitter stimuli. The bitter stimuli were introduced into the detection chamber of membrane receptor-based biosensors after the frequency baseline of the QCM was stabilized. The introduction of bitter stimuli will result in the shift of the QCM resonant frequency due to the mass changes on the QCM surface. The frequency shifts of the QCM were recorded and calculated as the responses of bitter receptor-based biosensors to bitter stimuli. All the measurements were carried out under the same environmental conditions with humidity at 10% and temperature at 25 °C to minimize the influences of environmental factors on the measurements.

3 Results and discussion

3.1 Expression of bitter receptors

Membrane receptors can respond to the specific stimuli with extremely high sensitivity and specificity. This makes membrane receptors ideal candidates to be used as sensitive elements in biosensors. However, membrane receptors usually contain trans-membrane domains. The hydrophobic environment of a cell membrane is crucial to the natural structure and function of membrane receptors. In order to use membrane receptors as sensitive elements, it is necessary to express membrane receptors in the plasma membrane of a heterologous cell system. In this study, a human bitter receptor, T2R4, was used as the sensitive element for bitter detection and was expressed on the plasma surface of human embryonic kidney cells (HEK-293).[23,29] The schematic structure of the constructed T2R4 expression vector *pCEP4/rho-T2R4-*His_6 is shown in Fig. 1a. A *rho-tag* import sequence was inserted into the N-terminal of T2R4 to facilitate the cell plasma membrane expression. A His_6-tag that is six histamine acids was inserted into the C-terminal of T2R4 for the purpose of functional immobilization as well as purification on the sensor surface.

The expression of T2R4 was validated by immunofluorescent staining. The nucleus of HEK-293 cells was labeled using DAPI. As shown in Fig. 1b and c, the results demonstrate the successful expression of T2R4 in HEK-293 cells. Furthermore, as indicated by the nucleus staining of HEK-293 cells, the expressed T2R4 are mainly distributed on the plasma membrane of HEK-293 cells. This could facilitate the activity maintenance of expressed bitter receptors and makes them suitable to be used as sensitive elements in biosensors for bitter detection.

3.2 Coupling of bitter receptors to transducers

Membrane fractions containing expressed His_6-tagged T2R4 were extracted from transfected HEK-293 cells and coupled

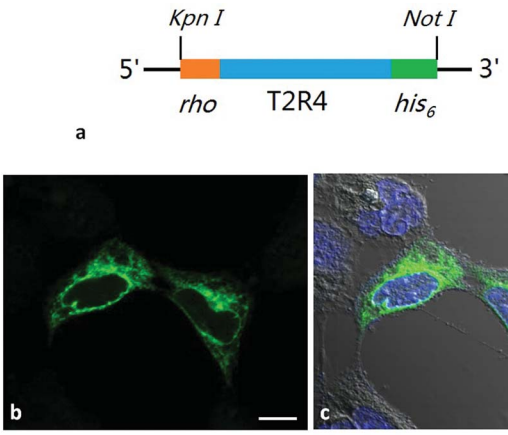

Fig. 1 (a) Schematic diagram of the constructed T2R4 expression vector *pCEP4/rho-T2R4-*His_6. (b) Fluorescent image shows the expression of a human bitter receptor, T2R4, in HEK-293 cells. (c) Overlap image of the fluorescent field and optical field in the same region as shown in Fig. 1b. The blue fluorescence indicates the nucleus of HEK-293 cells. (Scale bar: 10 μm.)

to the gold surface of QCM devices by a self-assembled aptamer-based method. As schematically illustrated in Fig. 2, thiol-modified anti-His_6-tag forms a monolayer through a self-assembly process *via* Au–thiol binding on the gold surface of QCM devices.

In contrast to the SAM-based method, we exploited a new use of a thiol-modified anti-His_6-tag aptamers for the modification of a gold sensor surface. This unconventional use of self-assembled aptamers significantly simplifies the preparation process of bitter receptor-based biosensors. In addition to the immobilization of bitter receptors, self-assembled aptamers also functioned for affinity purification of bitter receptors because other biomolecules without His_6-tags will be washed out from the sensor surface. By this method, only the bitter receptors labeled with His_6-tags can be selectively immobilized on the sensor surface. Moreover, this method allowed the bitter receptors to remain embedded in the cell membrane, which can facilitate the maintenance of the natural structure and function of bitter receptors. We hypothesize that this method could significantly improve the surface molecular immobilization and purification efficiency. As a result, the performances of bitter receptor-based biosensors based on self-assembled aptamers could be greatly enhanced due to the higher stability and specificity of aptamers as well as the higher density distribution of bitter receptors on the sensor surface. Furthermore, this method could also potentially eliminate the involvement of complicated and multiple processing steps for biosensor preparation, for example, the centrifugation and purification of bitter receptor proteins.

The stepwise process of bitter receptor immobilization was characterized by electrochemical cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). As shown in Fig. 3a, CV scans for the bare QCM gold electrode reveal reversible cyclic voltammograms, which indicate a well prepared clean gold surface. For the step of aptamer immobilization, a dramatic decrease in faradic current indicates the formation of a self-assembled aptamer monolayer on the gold electrode surface due to the insulating property of the aptamer monolayer. A significant increase of the charge transfer resistance (R_{ct}) can be observed as shown in Fig. 3b, which are Nyquist plots of impedance spectra of the same preparation process. After the step of blocking, faradic current further decreased due to the blocking of non-specific adsorption spots as well as the formation of more condensed mixed SAMs on the gold electrode surface. The step of bitter receptor immobilization resulted in the slight increase of current. Fig. 3b further indicates a decrease in polarization resistance in this step. The reason is assumed to be due to the conformational changes and structural re-arrangement of molecules immobilized on the gold electrode surface originating from the interactions between bitter receptors and self-assembled aptamers. These results demonstrated that the bitter receptors with His_6-tags were efficiently immobilized on the surface of the QCM gold electrode.

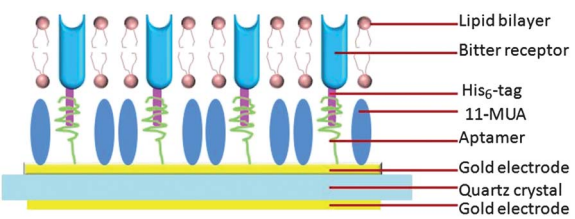

Fig. 2 Schematic diagram shows the surface structure of bitter receptor-based biosensors prepared by the self-assembled aptamer-based method.

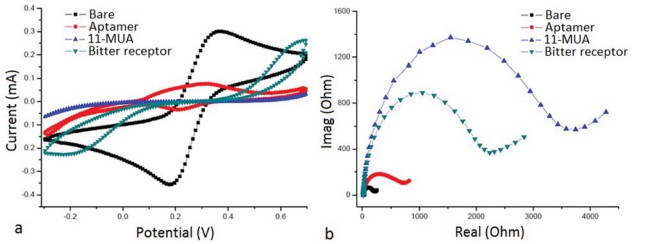

Fig. 3 Bitter receptor immobilization on the gold surface of the QCM device with the self-assembled aptamer-based method characterized by (a) CV and (b) EIS.

3.3 Performance of bitter receptor-based biosensors

According to the Sauerbrey equation, the mass change on the QCM surface originating from the interactions between bitter receptors and bitter stimuli can be monitored by recording the frequency shift of the QCM. The more mass changes will result in the higher frequency shifts, which indicate the higher responses of bitter receptor-based biosensors to the specific bitter stimuli. In this study, the responses of biosensors were calculated by the differences in resonance frequency shifts of the QCM before and after bitter stimuli introduction to the detection chamber.

The specificity of bitter receptor-based biosensors was tested using various bitter substances including $MgSO_4$, denatonium, D-(−)-salicin, and quinine. As shown in Fig. 4, the results showed that biosensors with T2R4 have significantly higher and specific responses to denatonium, which is the natural ligand of T2R4. While the negative control biosensors (membrane fractions without T2R4) show no significant response to the bitter stimuli tested. The much smaller responses of negative control biosensors could originate from the non-specific adsorption of bitter substances onto the sensor surface. In addition, an olfactory receptor, ODR-10, with a His_6-tag was employed to further investigate the specificity of bitter receptor-based biosensors for bitter detection. It is indicated that biosensors using ODR-10 as the sensitive element prepared by the same protocol as that of bitter receptors show no significant response to bitter stimuli. All the results demonstrate the high specificity of bitter receptor-based biosensors for bitter detection.

In order to investigate the purification effect on the performance of bitter receptor-based biosensors, we compared the sensitivity of the bitter receptor-based biosensors prepared by the self-assembled aptamer-based method and the SAM-based method. The sensitivity of these biosensors was tested using different concentrations of denatonium. The results shown in Fig. 5 indicate that bitter receptor-based biosensors prepared by

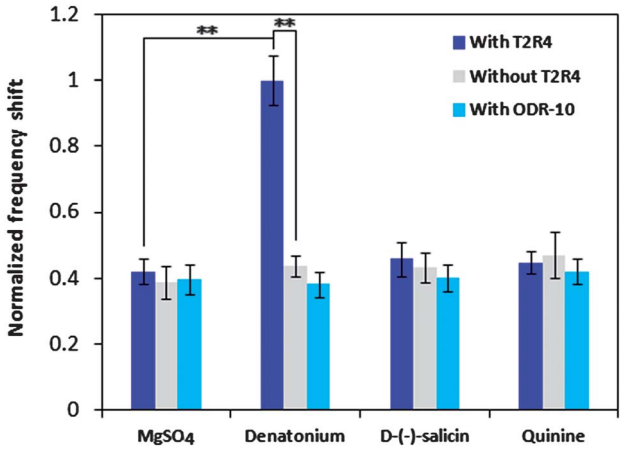

Fig. 4 Responses of biosensors with different sensitive elements (with T2R4, without T2R4, and with ODR-10) to various bitter stimuli. The responses of various bitter stimuli were normalized to that of denatonium. All the data are represented by mean ± SD. $^{**}p < 0.05$, Student's *t*-test. The mean and SD of 6 experiments are shown.

both methods show dose-dependent linear responses to denatonium in the concentration range from 0.01 mM to 0.3 mM. The limit of detection (LOD) is 1.58×10^{-6} M for aptamer-based biosensors and 2.31×10^{-5} M for SAM-based biosensors, respectively, which are calculated by the intersection point of the extrapolated linear region of the calibration curve with abscissa. In contrast, the biosensors using ODR-10 as a sensitive element prepared by the self-assembled aptamer-based method show no significant responses to denatonium at the different concentrations tested. The sensitivity (S) of bitter receptor-based biosensors was calculated by dividing the resonant frequency shifts (Δf in kHz) of the QCM by the concentration of denatonium (ΔC in mM), that is $S = \Delta f/\Delta C$. The sensitivities of bitter receptor-based biosensors prepared by the self-assembled aptamer-based method and the SAM-based method were 1.21 kHz mM^{-1} and 0.636 kHz mM^{-1}, respectively. The biosensors prepared by the self-assembled aptamer-based method show more than 2 times improvement in sensitivity as compared with the biosensors prepared by the SAM-based method.

As compared with the SAM-based method, biosensors prepared by the self-assembled aptamer-based method have much higher performances. We anticipated that this is mainly due to the higher density distribution of bitter receptors on the gold surface of the QCM prepared by the self-assembled aptamer-based method. Because, for biosensors prepared by the SAM-based method, many other kinds of membrane proteins could also be immobilized on the sensor surface due to the lack of specific binding between taste receptors and SAMs. In contrast, the specific aptamers can selectively capture bitter receptors labeled with His_6-tags, which will finally result in a higher distribution density of bitter receptors on the sensor surface.

In order to further verify the coupling efficiency, immunofluorescent staining was employed to specifically characterize the density of bitter receptors (T2R4 with His_6-tag) immobilized on the gold surface of the QCM by the self-assembled aptamer-based method and the SAM-based method. The density of bitter receptors on the sensor surface was calculated by the fluorescent density within a certain area. The results of immunofluorescent staining are shown in Fig. 6. The fluorescent intensity of biosensors prepared by the self-assembled aptamer-based method was significantly higher than that prepared by the SAM-based method, which indicates that more bitter receptors were immobilized on the gold surface of the QCM within a certain area. The results indicate that the self-assembled aptamer-based method can immobilize more bitter receptors on the sensor surface with higher density than that of the SAM-based method. This could contribute greatly to the higher sensitivity of the biosensors prepared by the self-assembled aptamer-based

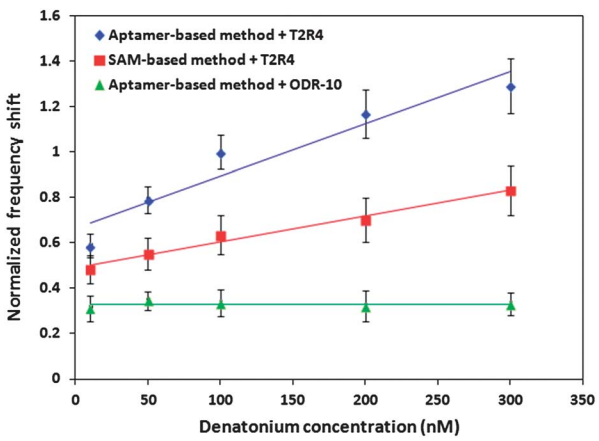

Fig. 5 The receptor-based biosensors prepared by the self-assembled aptamer-based method and the SAM-based method respond to different concentrations of denatonium. The responses of various concentrations of denatonium were normalized to that of denatonium at a concentration of 100 nM. All the data are represented by mean ± SD. The mean and SD of 8 experiments are shown.

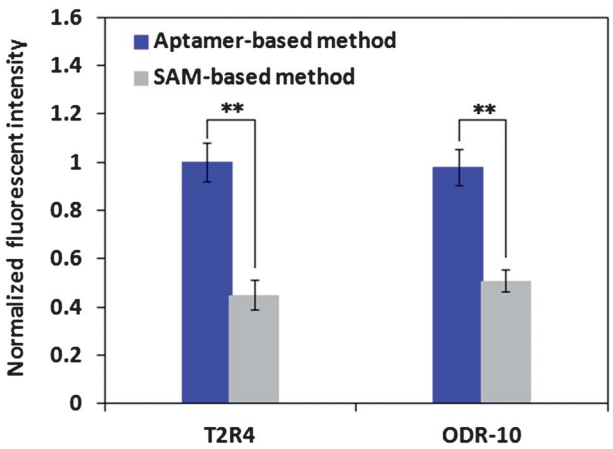

Fig. 6 The surface fluorescent intensity of bitter and olfactory receptors immobilized on the gold surface of the QCM by the self-assembled aptamer-based method and the SAM-based method. The fluorescent intensity was normalized to the mean fluorescence intensity of the bitter receptor immobilized by the self-assembled aptamer-based method. The data are represented by mean ± SD. $^{**}p < 0.05$, Student's *t*-test. The mean and SD of 4 experiments are shown.

method. In addition, olfactory receptors (ODR-10 with His_6-tag) were also investigated by the same protocol as bitter receptors to further demonstrate the enhanced effect on the coupling efficiency of receptors with transducers. As shown in Fig. 6, the results of immunofluorescent staining indicate the higher density of olfactory receptors immobilized by the self-assembled aptamer-based method than that of the SAM-based method. This suggests that the self-assembled aptamer-based method could also be applied for other types of receptors which have a similar structure to bitter receptors.

The stability and repeatability of the bitter receptor-based biosensors prepared by the self-assembled aptamer-based method were also investigated. The biosensors were kept at 4 °C when no measurements were taken. When the same bitter stimuli were applied (0.1 mM denatonium), the biosensors show stable and repeatable responses within 2 days (data not shown). The responses to the same bitter stimuli decreased significantly after 2 days. This may be due to the irreversible conformation changes of bitter receptors, which could cause a dramatic loss in their activity of binding target molecules. Compared with cell-based biosensors for bitter detection, this bitter receptor-based biosensor can detect the specific bitter substance with higher sensitivity and specificity in a long-term and stable manner. Cell based biosensors usually suffer from unstable status of cells used as sensitive elements for bitter detection, which leads to a dramatic decrease in the biosensor performances such as lower repeatability, unstable responses, and limited measurement time (usually within 2 h).[9,10,25] In addition, cell-based biosensors usually need a cell culture system, which complicate greatly the preparation of biosensors.

4 Conclusions

In summary, we developed a biomimetic bitter receptor-based biosensor for the detection of specific bitter substances. It is demonstrated that the use of the self-assembled aptamer-based method can simplify the preparation of bitter receptor-based biosensors and greatly improve their performances due to the higher efficiency of immobilization and purification on bitter receptors. The major advances on bitter receptor immobilization and purification efficiency presented in this work could substantially promote and accelerate the research and applications related to membrane receptor-based biosensors and molecular sensor arrays, which demand high efficiency and stability on coupling of molecular sensors to transducers in order to improve their performances in practical applications.

Acknowledgements

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