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Nanovesicle-based bioelectronic nose platform mimicking human olfactory signal transduction

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ABSTRACT

We developed a nanovesicle-based bioelectronic nose (NBN) that could recognize a specific odorant and mimic the receptor-mediated signal transmission of human olfactory systems. To build an NBN, we combined a single-walled carbon nanotube-based field effect transistor with cell-derived nanovesicles containing human olfactory receptors and calcium ion signal pathways. Importantly, the NBN took advantages of cell signal pathways for sensing signal amplification, enabling ~100 times better sensitivity than that of previous bioelectronic noses based on only olfactory receptor protein and carbon nanotube transistors. The NBN sensors exhibited a human-like selectivity with single-carbon-atomic resolution and a high sensitivity of 1 fM detection limit. Moreover, this sensor platform could mimic a receptor-meditated cellular signal transmission in live cells. This sensor platform can be utilized for the study of molecular recognition and biological processes occurring at cell membranes and also for various practical applications such as food screening and medical diagnostics.

1. Introduction

Human olfactory systems recognize diverse odorants with a remarkable sensitivity and selectivity through olfactory signal transduction which is initiated by the binding of odorant to human olfactory receptors (hORs) followed by the cascade of enzymatic reactions and is terminated by the signal transmission to a brain (Firestein, 2001; Krautwurst et al., 1998; Shepherd, 1994; Zhao et al., 1998). Recently, bioelectronic artificial noses with human-like responses have been developed by coating hORs on field effect transistor (FET) devices (Lee and Park, 2010; Kim et al., 2009a; Yoon et al., 2009; Goldsmith et al., 2011). In this case, the binding of odorant molecules onto the hORs on the FETs was directly measured by monitoring the charge state change of the hORs *via* the FET without relying on olfactory signal transduction pathways. On the other hand, signal pathways related with cell responses to external stimuli have been studied using various techniques such as a fluorescence assay, surface plasmon resonance, patch-clamps, and FET-based sensing methods (Lee et al., 2009; Wicher et al., 2008; Patolsky et al., 2006; Voelker and Fromherz, 2005; Merz and Fromherz, 2005). For example, the responses of hOR-expressed cells have been studied to understand olfactory cell signaling, which enabled the development of a sensor platform for odorant detection. However, the approach based on such cell-based platforms often requires expensive, time-consuming, and labor-intensive procedures. As an alternative strategy, nanovesicles with functional components for receptor-dependent signal transduction have been produced from mammalian cells (Pick et al., 2005), while they have not been utilized for either biosensor applications or olfactory signal transduction study.

Herein, we developed a nanovesicle-based bioelectronic nose (NBN) platform that mimicked the signal pathways of human olfactory systems and thus recognized specific odorant with a high sensitivity and a human-like selectivity. In this work, we expressed human OR 2AG1 (hOR2AG1) protein in human embryonic kidney (HEK)-293 cells, and nanovesicles produced from the cells were immobilized on single-walled carbon nanotube-based FETs (swCNT-FETs). The activity of the nanovesicles containing the hOR protein and related signal pathways could be monitored electrically

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using the underlying swCNT-FETs, leading to an operation as a bioelectronic nose. Significantly, our NBN device took advantages of cell signal pathways for sensor signal amplification, enabling ~100 times better sensitivity than that of previous bioelectronic noses based on only olfactory receptor protein and swCNT-FETs (Kim et al., 2009a). The NBN device could detect odorant at 1 fM concentration and exhibited a high selectivity with single-carbon-atomic resolution. Furthermore, we performed extensive control experiments to show that the NBN device could mimic the receptor-mediated signal pathway of human olfactory systems. NBNs can be a powerful platform for a fundamental study regarding diverse G protein-coupled receptors (GPCRs) and related cellular signal pathways, and they also can be utilized for various practical applications such as food screening and medical diagnostics.

2. Materials and methods

2.1. Cloning of human olfactory receptor

We used an hOR2AG1 gene as a model olfactory receptor gene. The full-length hOR2AG1 coding sequence (GenBank accession No. NC000011) was amplified by polymerase chain reaction (PCR) from human genomic DNA mixture (Novagen, Germany). The PCR was performed with a mixture of *Tag* polymerase (Takara, Japan), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM of each primer, and 100 ng human genomic DNA using the following temperature cycle: 35 cycles of 94 °C for 5 min, 55 °C for 30 s, and 72 °C for 1 min. Here, we used following primers; hOR2AG1-N: TGGAATTCATGGAGCTCTGGAACTTCA, hOR2AG1-C: AGCTCGAGCTAGAGCGTGGAGTGTG. Afterwards, the amplified hOR2AG1 gene was inserted into in-frame between the BamHI and XhoI restriction sites of an expression vector, pcDNA3 (Invitrogen, USA) using DNA ligation kit (Takara, Japan). Then, a flag-tag (amino acid sequences; N-DYKDDDDK-C) and a rho-tag import sequence (the N-terminal 20 amino acids of rhodopsin; N-MNGTEGPNFYVPFSNKTGVV-C) were inserted into BamHI-EcoRI restriction sites located in the N-terminus of olfactory receptor gene for Western blot analysis and targeting receptor protein onto a cell membrane surface, respectively.

2.2. Formation of nanovesicles from HEK-293 cells

Adherent HEK-293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. Then, the cells were seeded (10^5 cells/ml) into 6-well plates, transiently transfected with pcDNA3-flag-rho-hOR2AG1 using lipofectamine (Invitrogen, USA), and cultivated again for 48 h. Then, we produced nanovesicles containing hOR2AG1 from the cells *via* 20 min incubation at 37 °C with 300 rpm agitation in serum-free DMEM containing cytochalasin B (20 μg/ml). After that, we separated the remaining cell bodies and the nanovesicles through differential centrifugation. Here, the cells were first separated from the mixture of the cells and the nanovesicles through centrifugation at 1000 × *g* for 30 min. The nanovesicles were then retrieved through centrifugation at 15,000 × *g* for 30 min and resuspended in PBS (pH 7.4) containing protease inhibitor cocktail (Sigma–Aldrich, USA). The produced nanovesicles were either used immediately. Or, they could be stored at −80 °C for several weeks and used later. For the preparation of ion channel blocked nanovesicles, the produced nanovesicles were incubated with PBS containing 10 mM of $MgCl_2$ for 30 min. The protein concentration of the nanovesicle was determined using a BCA reagent (Pierce, USA) with bovine serum albumin as a standard.

2.3. Expression of human olfactory receptors

The expression of hOR2AG1 was first confirmed by Western blot analysis. The insoluble fractions of parent cells and nanovesicles were electrophoresed on polyacrylamide gels and transferred to a polyvinylidenefluoride membrane (PVDF, Bio-Rad, USA). Then, the membrane was blocked with 5 wt% skim milk in PBST (PBS containing 0.1 vol% Tween-20, pH 7.4). The membrane was incubated with an anti-flag antibody (1:1000 dilution, Cell Signaling, USA) and horseradish peroxidase-conjugated secondary antibody (1:2000, GE healthcare, UK). Blots were observed using an enhanced chemiluminescence detection kit (ECL, Amersham-Pharmacia Biotech, UK).

2.4. Immunofluorescence analysis

Cell surface expression of the hOR2AG1 in HEK-293 cells was verified by immunofluorescence analysis. Cells were fixed in 4% paraformaldehyde for 20 min and incubated at 37 °C for 1 h in PBS containing the primary antibody (1:1000, anti-flag rabbit antibody, Cell signaling, USA). After washing three times with PBS, cells were incubated with the 1:1000 dilution of polyclonal anti-rabbit Alexa594-linked antibody (Invitrogen, USA) at 37 °C for 1 h. To visualize hOR2AG1 protein, pictures of fluorescent cells were taken with a Nikon Eclipse TE 300 microscope (Nikon Corporation, Japan) with excitation and emission set to 590 nm and 617 nm, respectively.

2.5. FE-SEM imaging of nanovesicles

Cell-derived nanovesicles were doped on a slide glass surface and then lyophilized by freeze-drying. The lyophilized nanovesicles were then coated with platinum (10 nm) by sputtering. The morphology of the nanovesicles was visualized using a FE-SEM (JEOL JSM-6700F, Japan).

2.6. Confocal fluorescence imaging of nanovesicles

Nanovesicles were stained by 10 min of incubation with Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, λ_{ex} = 550 nm, λ_{em} = 570 nm, 1.25 μg/ml, Molecular Probes, USA) as a hydrophobic membrane-associated probe and then placed onto a glass substrate precoated with 0.1 mg/ml PDL. The fluorescence observation of the nanovesicles in aqueous solutions was performed using an upright confocal fluorescence microscope (Nikon C1, Japan) equipped with a fluorescence system and a digital camera.

2.7. Calcium signaling in nanovesicle

Cells were loaded with 5 μM Fura2-AM (Molecular probes, USA) in PBS containing 2 mM $CaCl_2$ at 37 °C for 30 min. The cells were then washed with PBS and incubated in PBS containing 2 mM $CaCl_2$ for 1 h to allow the complete hydrolysis of Fura 2-AM by intracellular esterase. Subsequently, nanovesicles were produced as described above. The produced nanovesicles containing hOR2AG1 and Fura 2-AM were incubated for 2 h and immobilized on 96-well plates precoated with 0.1 mg/ml PDL. Then, a Ca^{2+} dependent fluorescent signal upon the addition of specific ligand (1 mM of AB) was measured at 510 nm through dual excitation at 340 nm and 380 nm using a spectrofluorophotometer (Tecan, Switzerland). Using this ratio, the level of intracellular Ca^{2+} was determined.