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Smelling Sulfur: Copper and Silver Regulate the Response of Human Odorant Receptor OR2T11 to Low-Molecular-Weight Thiols

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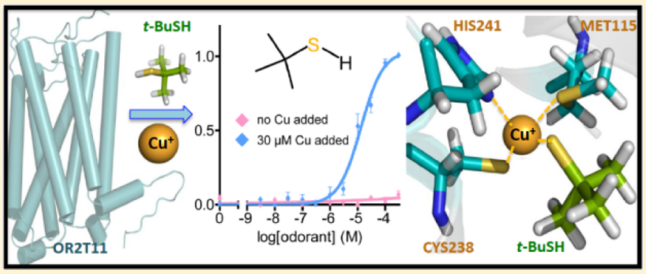
Supporting Information

ABSTRACT: Mammalian survival depends on ultrasensitive olfactory detection of volatile sulfur compounds, since these compounds can signal the presence of rancid food, O_2 depleted atmospheres, and predators (through carnivore excretions). Skunks exploit this sensitivity with their noxious spray. In commerce, natural and liquefied gases are odorized with *t*-BuSH and EtSH, respectively, as warnings. The 100-million-fold difference in olfactory perception between structurally similar EtSH and EtOH has long puzzled those studying olfaction. Mammals detect thiols and other odorants using odorant receptors (ORs), members of the family of seven transmembrane G-protein-coupled receptors (GPCRs). Understanding the regulator cofactors and response of ORs is particularly challenging due to the lack of X-ray structural models. Here, we combine computational modeling and site-directed mutagenesis with saturation transfer difference (STD) NMR spectroscopy and measurements of the receptor response profiles. We find that human thiol receptor OR2T11 responds specifically to gas odorants *t*-BuSH and EtSH requiring ionic copper for its robust activation and that this role of copper is mimicked by ionic and nanoparticulate silver. While copper is both an essential nutrient for life and, in excess, a hallmark of various pathologies and neurodegenerative diseases, its involvement in human olfaction has not been previously demonstrated. When screened against a series of alcohols, thiols, sulfides, and metal-coordinating ligands, OR2T11 responds with enhancement by copper to the mouse semiochemical CH_3SCH_2SH and derivatives, to four-membered cyclic sulfide thietane and to one- to four-carbon straight- and branched-chain and five-carbon branched-chain thiols but not to longer chain thiols, suggesting compact receptor dimensions. Alcohols are unreactive.



INTRODUCTION

One- to four-carbon thiols have long been known for their unpleasant odors and very low odor threshold levels. There is presumed to be an evolutionary basis for the exquisite olfactory sensitivity of humans and other mammals to volatile sulfur compounds, since these compounds can signal the presence of rancid food, oxygen depleted toxic atmospheres, and predators (through organosulfur compounds found in carnivore excretions). As early as 1887, Emil Fischer wrote that concentrations of ethanethiol as low as 0.05 parts per billion (ppb) are "clearly perceptible to the sense of smell".[1] A monograph on the senses notes that ethanol "is only perceptible in air in a concentration of 0.4% w/w, whilst ethyl mercaptan [ethanethiol] is perceptible at 0.3×10^{-8}% w/w; our perception of it is one hundred million times more delicate".[2] Other mammalian species also show high sensitivity to one- to four-carbon thiols. For example, spider monkeys (*Ateles geoffroyi* L.) show extremely low threshold detection values for ethanethiol, 0.001 ppb.[3] Thiols with very low odor thresholds are also present in skunk scent as (*E*)-2-butene-1-thiol[4] and in garlic breath as 2-propenethiol,[5,6] odorants featured in the 2004 Nobel Prize Lecture of Linda Buck.[7] These odor properties are exploited in the common use of 2-methyl-2-propanethiol (*t*-butyl mercaptan; TBM), which has an odor threshold of 0.029 ppb,[8−10] as a natural gas odorant and

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ethanethiol as a liquefied petroleum gas (LPG, propane) odorant.[11,12] Due to their very low odor thresholds, certain thiols also have an important sensory impact as trace aroma components in wine,[13] beer,[14,15] cheese,[16] onions,[17] grapefruit,[18] durian,[19] roasted coffee,[20] and sesame seeds,[21] among other foodstuffs. In addition to methanethiol, found in breath and foot odor and flatus,[22] other low-molecular-weight thiols have also been implicated in unpleasant body odors, e.g., chiral 3-methyl-3-sulfanylhexan-1-ol identified in armpit odor can be perceived at levels as low as 0.000001 ppb.[23]

In 1977, Robert Crabtree made the insightful proposal that thiols "bind chemically to a nasal receptor, or group of receptors, containing a transition metal at the active site" and that "copper(I), particularly when coordinated to a "soft" anionic centre such as I or SR, ... seems to be the most likely candidate for a metallo-receptor site in olfaction".[24] The choice of copper is notable, given that it is an essential element for human health,[25,26] is widely distributed, e.g., in saliva and plasma, and has been implicated in smell function[27] (as has zinc),[28] although the connection to human olfaction has not been firmly established.

Humans can distinguish a very large number of odorants[29] using less than 400 different olfactory receptors (ORs),[7,30,31] members of the family of seven transmembrane G-protein-coupled receptors (GPCRs). Understanding the regulator cofactors and response of ORs is particularly challenging due to the lack of X-ray structural models. Thus, the molecular basis for the remarkable olfactory sensitivity of humans to thiols compared to structurally analogous alcohols remains unknown.

We previously reported evidence for the central role of copper in discrimination by mouse OR MOR244-3[32,33] of the social signaling compound (methylthio)methanethiol (MTMT; **1**).[34] The binding of MTMT at the active site of this receptor was rationalized with a QM/MM model involving chelation of copper by **1**, validated by mutagenesis.[35] Here we describe identification of a human OR, OR2T11, highly responsive to short-chain thiols, especially 2-methyl-2-propanethiol, also showing a strong copper effect, and surprisingly, an effect also seen with ionic and nanoparticulate silver. We compare the relative activities of human OR OR2T11 with two mouse ORs, MOR244-3 and MOR244-2, toward metals alone as well as toward short- and longer-chain thiols, H_2S, metal-coordinating ligands, as well as alcohols, in the presence of metals as well as a potent metal-sequestering agent. Our study combines computational modeling, site-directed mutagenesis, and measurements of the receptor response profiles with saturation transfer difference (STD) NMR spectroscopy, a powerful spectroscopic method for detecting weakly binding small-molecule interactions with large proteins. We find that OR2T11 is surprisingly selective toward low-molecular-weight thiols and is unresponsive to longer chain thiols as well as alcohols, suggesting compact dimensions for the metallo-receptor predicted by Crabtree.[24]

RESULTS

OR2T11 Is a Human Thiol Receptor with a Copper Effect. To explore whether the metal enhancement effect seen with mouse OR MOR244-3 is common to other ORs and whether humans also use the same strategy for sensitive thiol detection, we employed a two-pronged approach. First, we conducted a screening for known receptor−ligand pairs for thiols, carboxylic acids, and amines, compounds known to coordinate metals,[28] using three representative metal salts: $CuCl_2$, $NiCl_2$, and $ZnSO_4$. Both nickel and zinc ions are similar to copper ions in terms of coordination properties. This screening involved 35 mouse receptors and 7 human receptors as well as 30 odorous ligands, of which 20 are carboxylic acids and 10 are amines, corresponding to a total of 99 mouse and 18 human receptor−ligand pairs. We found no metal effect in any of the pairs with the ligand and metal concentrations tested (100 and 10 μM of each ligand and 30 μM of $CuCl_2$, $NiCl_2$, or $ZnSO_4$, Figure S1). However, we cannot exclude the possibility of the existence of a metal effect in other, orphan ORs or other classes of volatile compounds with unknown ORs.

Second, with the above limitation in mind, we extended the search for human ORs with a metal effect by targeting specific thiols with high human sensitivity, such as TBM. Of the 330 human ORs screened, a few ORs emerged from the screen (Figure 1). Follow-up confirmation experiments with an extended concentration range showed two receptors with strong responses to TBM, including OR2W1 (Figure S2) and OR2T11. A third human receptor, OR2C1, also emerged from screens for thiol receptors and was responsive to longer straight-chain monothiols (Figure S2), consistent with previous reports.[36,37] Of these ORs, OR2T11 was the one and only human receptor for TBM with a strong copper effect that could be effectively counteracted with the addition of the copper chelator tetraethylenepentamine (TEPA) (Figure 2A). Human OR2T11 and mouse MOR244-3 are not orthologous, and the ORs closest to MOR244-3 in the human genome, OR4E2 and OR4E1, do not respond to MTMT.

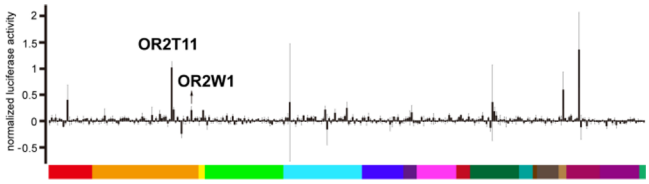

Figure 1. Screening for human ORs for TBM. 330 unique human ORs were screened against TBM using luciferase assay (N = 3). Colored blocks along the x-axis indicate different human OR families. All receptors with a response over 0.2 were separately verified and false-positive results were subsequently excluded.

We next assessed the receptor specificity of OR2T11 using other thiols in both the luciferase reporter gene system and the real-time GloSensor system. We found that upon $CuCl_2$ addition, OR2T11 was only capable of responding to small monothiols, including methanethiol, ethanethiol, 1-propanethiol, 2-propanethiol, 1-butanethiol, all branched-chain four-carbon thiols (2-methyl-2-propanethiol, 2-methyl-1-propanethiol, and 2-butanethiol), and selected branched-chain or cyclic five-carbon thiols (3-methyl-2-butanethiol, 2-pentanethiol, and cyclopentanethiol) and short, straight-chain dithiols (Figures 2A, S3, and S4; Tables S1 and S2).

OR2T11 also responded to MTMT, representative of small α-mercaptothioethers, and selected disulfide derivatives, including 2,3,5-trithiahexane and bis(methylthiomethyl) disulfide, which may be reduced to α-mercaptothioethers, (ethylthio)-methanethiol, and 1-(methylthio)ethanethiol, which are analogous to 1-butanethiol and 2-butanethiol, respectively, and cyclopentanethiol analogue 2-thiolanethiol (Figures S3−S5). The unsaturated monothiol prop-2-ene-1-thiol, commonly known as allyl mercaptan, also activated OR2T11 (Figures 2A, S3, and S4), as did the small cyclic sulfide thietane, to which

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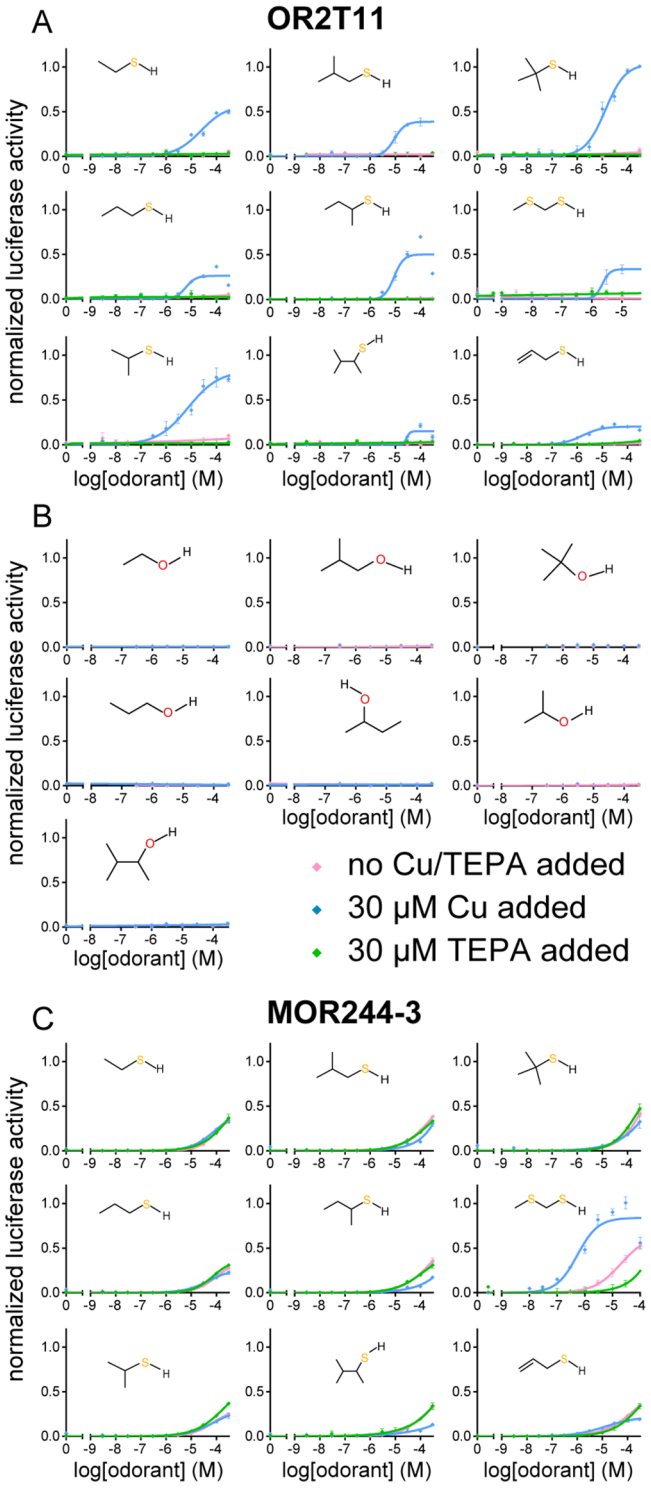

Figure 2. Human OR2T11 responds to selected monothiols and α-mercaptothioethers in the luciferase assay as shown by dose−response curves to various (A) thiols and (B) alcohols. (C) MOR244-3 selectively employs copper in response to thiol ligands.[32] *y*-axis indicates normalized response ± SEM (N = 3). All responses are normalized to the highest thiol response of each OR.

MOR244-3 also had a response and strong copper effect (Figure S7). Notably, thietane is a mouse alarm pheromone and predator scent analog.[38] OR2T11 did not respond to hydrogen sulfide (in the form of NaSH at pH 6, Figures S3 and S5).

Among the odorants with no effect on OR2T11 are 5- to 10-carbon straight-chain monothiols (Figures S3 and S4). In assessing OR2T11 receptor selectivity, the real-time GloSensor system was found to be more sensitive than the luciferase reporter gene system, given that a small number of the above odorants were only active in the former but not in the latter system. In addition, the alcohol counterparts to the responsive monothiols are also nonresponsive (Figure 2B).

Because of these newly discovered monothiol odorants for the human receptor OR2T11 with a copper effect, we sought to test these compounds on MOR244-3, the mouse OR previously shown to have a copper effect. Surprisingly, we found that while MOR244-3 responded with a strong copper effect to α-mercaptothioethers[32] it also responded to most of the monothiols but without a copper effect (Figure 2C). Finally, using CuCl as a source of copper ion gave results similar to those of $CuCl_2$ in the case of both OR2T11 and MOR244-3 (Figure S6). This is not unexpected, e.g., in view of the reducing environment found in cells as well as redox processes involving Cu(II) and thiols previously discussed by us,[32] processes occurring in the model wine[39] and processes suggested to occur in the regulation of the sense of taste by copper.[40]

OR2T11 and MOR244-2 Are Odorant Receptors That Are Activated by Metal Alone. In addition to copper ion, we also tested the effect of other metal ions on OR2T11. We found that among 12 other types of metals tested, silver (including AgAc, $AgNO_3$, and colloidal silver) could also support elevated response of OR2T11 toward TBM (Figure 3A). Interestingly,

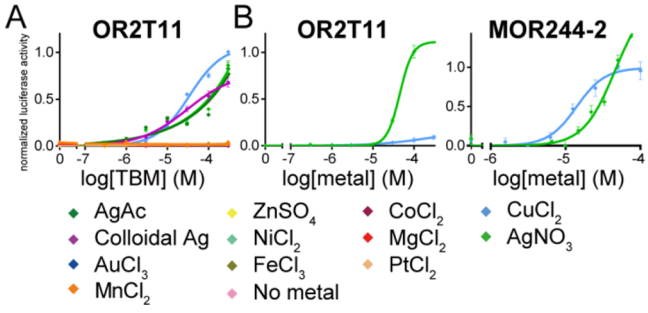

Figure 3. Both OR2T11 and MOR244-2 are activated by metals. (A) Dose−response curves of OR2T11 against increasing concentrations of TBM with the concentration of different metals held constant at 30 μM. (B) OR2T11 (left) responds to $AgNO_3$, while MOR244-2 (right) responds to both $CuCl_2$ and $AgNO_3$. *y*-axis indicates normalized response ± SEM (N = 3). The responses in B are normalized to the highest metal response of each OR.

we found that OR2T11 had a dose-dependent activity toward silver ion alone (Figure 3B, left). Another member of the mouse MOR244 family, MOR244-2, shared similar metal-responsive properties with OR2T11 in that it responded to both $CuCl_2$ and $AgNO_3$ (Figure 3B, right). To search for an odorous ligand for MOR244-2, we screened MOR244-2 against a panel of odorant mixtures and selected individual ligands with different structural features. We found that unlike MOR244-3, MOR244-2 did not respond to MTMT or any other thiols tested or to any ligand mixtures tested (Figure S8 and Table S3)

Observation of Odorant/Receptor Interactions with Metal Effect by STD NMR Spectroscopy. STD NMR spectroscopy is being exploited in many areas of bioscience

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including drug discovery.[41] STD NMR is a method for detecting the interactions of small ligands with large proteins. The detection is ligand-based, meaning that there is no mass limit on the target protein and expensive protein labeling is not required.[41,42] This technique relies on the fact that a bound ligand receives saturation transfer from a protein through spin diffusion through the nuclear Overhauser effect and works best with weak binding ligands with dissociation constants of K_d = 10^{-8}–10^{-3} mol L^{-1}. The resulting spectrum is the result of subtracting a spectrum in which the protein is selectively saturated (on-resonance) and a spectrum recorded without protein saturation (off-resonance).[42] STD NMR and the slightly modified saturation transfer double difference (STDD) NMR allow the observation of small-molecule interactions with live cells and have been used to study GPCR's with a 24 000-fold excess of ligand to protein using only 512 scans.[41,43−45]

In addition to the experiments discussed above, STD NMR was used as a complementary method for studying the binding of TBM to OR2T11 and MOR244-3. This method confirms the metal effects detected by the luciferase and GloSensor assays. The results of our STD NMR experiments are presented in Figure 4. Figure 4A shows the NMR spectrum for TBM dissolved in acetone-d_6. Protons corresponding to TBM appear at 1.4 ppm. The remaining spectra in Figure 4 are difference spectra ($I_O - I_{SAT}$). Figure 4B,C shows the STD spectra of cells transfected to express OR2T11 in the presence of TBM after and before the addition of $CuCl_2$, respectively. One peak (1.4 ppm) corresponding to the methyl protons of TBM is clearly visible. This peak indicates the binding of TBM to OR2T11. There is a significant increase in the area of the peak in the presence of $CuCl_2$ indicating an increase in binding in the presence of $CuCl_2$. Figure 4D,E shows the STD spectra of cells transfected to express OR2T11 in the presence of TBM after and before the addition of $AgNO_3$, respectively. Again, one peak (1.4 ppm) corresponding to the protons of TBM is clearly visible and is an indicator of binding. While not as dramatic as the increase in binding seen upon the addition of $CuCl_2$, the addition of $AgNO_3$ to the cell suspension increases the binding of TBM to OR2T11. Figure 4F,G shows the STD spectra of cells transfected to express MOR244-3 in the presence of TBM after and before the addition of $CuCl_2$, respectively. However, there is no significant increase in the area of the peak upon addition of $CuCl_2$. This indicates that the binding of TBM to MOR244-3 was not increased with the addition of $CuCl_2$. Figure 4H,I shows the STD spectra of cells transfected to express MOR244-3 in the presence of TBM and after and before the addition of $AgNO_3$, respectively. There is no significant increase in the area of the peak, indicating no change in the binding of TBM to MOR244-3 with $AgNO_3$ addition.

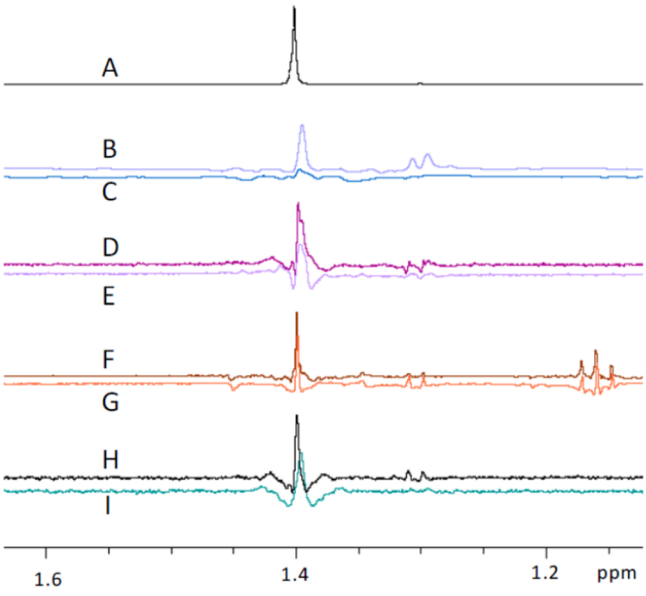

Figure 4. (A) NMR spectrum of TBM in acetone-d_6; STD spectrum of cells transfected to express OR2T11 in HBSS/D_2O with TBM and (B) with $CuCl_2$ or (C) prior to $CuCl_2$ addition. STD spectrum of cells transfected to express OR2T11 in HBSS/D_2O with TBM and (D) with $AgNO_3$ or (E) prior to $AgNO_3$ addition. STD spectrum of cells transfected to express MOR244-3 in HBSS/D_2O with TBM and (F) with $CuCl_2$ or (G) prior to $CuCl_2$ addition. STD spectrum of cells transfected to express MOR244-3 in HBSS/D_2O with TBM and (H) with $AgNO_3$ or (I) prior to $AgNO_3$ addition.

It should be noted that the reference spectra (I_O) of all the cell suspensions (not shown) contained multiple peaks located between 3.0 and 4.2 ppm due to glucose and Hana3A metabolites; the doublet located at 1.3 ppm was due to lactate. In addition, two sets of controls were performed. First, the STD spectra of transfected cells without TBM or metals were acquired. The absence of the peak (1.4 ppm) seen throughout Figure 4 verifies that the binding was not due to the cells interacting with the buffer solution. In addition, this peak was also not observed in STD spectra of nontransfected Hana3A cells with the addition of TBM and metal salts. These results clearly indicated that the binding seen in Figure 4 is a result of the cells being transfected to express the OR and not a general response of the Hana3A cells to the TBM/metal salts.

The amplification factor (A_{STD}) is obtained by multiplying the relative STD effect of a given signal (the intensity of a signal in the difference spectrum divided by its intensity in the reference spectrum, I_{STD}/I_0) with the molar ratio of ligand relative to the protein, [odorant]/[OR].[46−48] The concentration of OR was taken to be 13 nmol/L, and the concentration of odorant was taken to be the average of the ERETIC (electronic reference to access in vivo concentrations) values[49] for the samples with and without the metal salts. Previously,[50] we have shown that for the three independent experiments with TBM, $CuCl_2$, and MOR244-3, I_{STD}/I_O was equal to 0.002 with a relative standard deviation of 13%. A_{STD} values were 280, 460, and 630 and increased with TBM concentration. This could reflect binding kinetics, but since the proteins are most likely saturated, the A_{STD} values should be equivalent. In this scenario, the relative standard deviation is 39%. Four sources of uncertainty were expected to impact the peak integrals in our experiments: slight spectral deviations between the on-resonance and off-resonance spectra that result in slight imperfections in subtraction; repeatability in the concentration of OR due to variation in transfection rate and cell density; baseline drift; and peak overlap. Although odorant concentrations determined by the ERETIC method should be accurate to less than 0.1 mmol/L, it should be noted that the ERETIC and STD reference spectra had different resolutions, which led to an increase in the uncertainty for this value.

Of particular interest is the ratio of A_{STD} in the presence of TBM with and without the addition of metal salts. This ratio allows us to examine the metal effect with the I_{STD}/I_O of the samples since the molar ratio of ligand relative to the protein does not change significantly upon addition of metal salts.

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Using the relative standard deviation reported previously (13%) gives a propagated uncertainty of 18% for these ratios. The ratio of the A_{STD} values of the samples transfected to express OR2T11 containing TBM with and without $CuCl_2$ is 4.0. This can be compared to the analogous A_{STD} values with and without $AgNO_3$, which is 1.4. If the same method is applied to the samples transfected to express MOR244-3, then the ratios are 0.9 and 1.1 for the spectra comparing binding with and without $CuCl_2$ and $AgNO_3$, respectively. These results indicate a metal enhancement effect for OR2T11 but not for MOR244-3 when binding TBM.

Homology modeling and QM/MM studies. Figure 5A,B shows the QM/MM structural models of OR2T11 and MOR244-2, respectively, obtained by using the X-ray crystal structure of the human M2 muscarinic receptor[51] as a template, as recently reported for the mouse OR MOR244-3.[35] The models provide valuable insights on the odorant binding sites and share common features, including a highly conserved disulfide S−S bond thought to be critical for structural stability. Two binding sites for Cu(I) were identified in OR2T11. Both sites are supported by site directed mutagenesis and activation profiles, showing a lack of response to thiols when mutating the key amino residues responsible for Cu binding (Figure S9C,D). While it is possible that there are other explanations for the loss of function on mutagenesis, mutagenesis of multiple other sites failed to alter the response and copper effect (Figure S10).

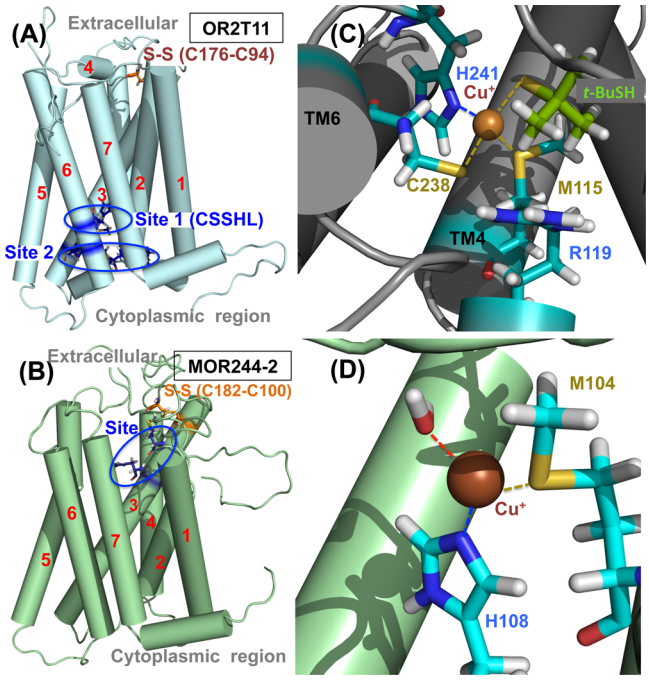

Figure 5. QM/MM modeling reveals metal-binding sites in OR2T11 and MOR244-2. (A) OR2T11 homology model. Two binding sites are shown as blue solid and dotted circles. The disulfide (S−S) bond is also shown on the top of the model. (B) MOR244-2 homology model. The disulfide (S−S) bond is shown as an orange stick. The blue solid circle represents the binding site. (C) QM/MM optimized model of OR2T11 with TBM. The distance between Cu^+ and the S_{ligand}, S_{M115}, S_{C238}, and N_{H241} are 2.24, 2.61, 2.24, and 2.09 Å, respectively. (D) QM/MM optimized metal binding site in MOR244-2. The distance between Cu^+ and the S_{M104}, N_{H108}, and the water molecule are 2.22, 1.99, and 2.05 Å, respectively.

Site 1 involves M115 of TM3 and residues C238 and H241 from TM6 near the end of the TM6 and TM3, while site 2 consists of M56 of TM2 and M133, R135, and C138 of TM4 (Figure S9B). Site 1 has a CSSHL (Figure 5A) motif close to the cytoplasmic region, similar to other candidate pentapeptides (e.g., CGSHL) previously proposed for metal binding sites in the cytoplasmic end of TM6.[52] In addition, site 1 shares similarities with the Cu binding site suggested for MOR244-2, with Cu(I) bound to M104 and H108 of TM3 (Figure 5B), although the Cu binding site in MOR244-2 is close to the extracellular domain. In contrast, site 1 is near the cytoplasmic region with Cu(I) binding in a trigonal planar configuration by coordination to the heteroatoms N_{H241}, S_{C238}, and S_{M115} with distances Cu−N_{H241}, Cu−S_{C238}, and Cu−S_{M115} of 2.01, 2.15, and 2.33 Å, respectively (Figure S9A). Interestingly, the Cu−S_{M115} distance increases to 2.5−3.0 Å upon ligand binding, forming a distorted tetrahedral configuration with distances Cu−S_{ligand}, Cu−N_{H241}, and Cu−S_{C238} of around 2.20 Å and Cu−S_{M115} of about 2.2−2.5 Å for thiols containing a single S atom and 2.5−3.5 Å for thiols containing a second S atom (Figure S11). It is, therefore, clear that the active site undergoes coordination rearrangements upon ligand binding. We note that amino acid residue R119 provides critical H-bonding interactions that stabilize the underlying structural rearrangements at the active site. Relative binding energies for a series of alkanethiols are predicted to range between 12−37 kcal mol^{-1} (Table S4). There is no correlation between relative binding energies for these alkanethiols and computed ligand−copper bond distances.

Site 2 binds Cu(I) with a tetrahedral coordination to S_{M56}, S_{M133}, S_{C138}, and N_{R135} with distances of 2.40, 2.73, 2.17, and 1.97 Å, respectively. Upon ligand binding, these coordination bond lengths increase to 3.21, 4.08, 2.27, and 1.99 Å, respectively. Analogous to the increase in the Cu−S_{M115} distance in site 1, the Cu−S_{M133} and Cu−S_{M56} distances are significantly elongated to coordination bond lengths larger than 3.00 Å upon thiol ligand binding (Figure S12A,B).

Our experimental and computational analysis shows evidence of Ag(I) binding to both OR2T11 and MOR244-2, similar to Cu(I). In OR2T11, the coordination of Ag(I) is slightly different since it forms a dicoordinated structure with R135 and C138, with Ag−N_{R135} and Ag−S_{C138} distances of 2.17 and 2.36 Å, respectively (Figure S12C), while the Ag−S_{M56} and Ag−S_{M133} distances are elongated to 4.31 and 4.62 Å, respectively. In MOR244-2, Cu(I) binds at the periphery of the TM region, near the extracellular domain, by coordination to N_{H108}, S_{M104}, and a water molecule with distances of 1.99, 2.22, and 2.0 Å, respectively (Figure 5D). Ag(I) binds similarly with corresponding distances of 2.20, 2.46, and 2.50 Å, respectively (Figure S12D). The predicted binding modes are supported by mutagenesis studies of the binding site (Figure S12E) and with the alignment of OR2T11 and MOR244-3 primary sequences (Figure S13).

DISCUSSION

ORs are central for the sensing of chemically and structurally diverse odorants, some of the most important of these being volatile sulfur compounds. Earlier studies hypothesized the participation of metal ions in olfaction based on the potential binding abilities and strong smells of several classes of metal-coordinating chemicals.[24,28] Using a combined *in vitro* and *in vivo* strategy, we previously identified the first mouse OR for MTMT and its related compounds, exhibiting a prominent

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copper enhancement effect. We subsequently modeled the binding site residues.[32,35] In this study, we identify a second mammalian and first human OR with a metal effect through screenings specifically targeting potentially metal-coordinating ligands. We find that OR2T11 is activated by TBM in the presence of copper and silver and that OR2T11 responds to silver alone. It is known that copper and silver coordination polymers with 2-methyl-2-propanethiol[53,54] can be solubilized with different ligands. Computational studies show two binding sites for OR2T11, which is confirmed by site-directed mutagenesis studies. The absence of a metal effect in an extensive screening using deorphaned ORs for ligands including alcohols, amines, sulfides, thiols, and carboxylic acids indicates that the metal effect although now present in both humans and mice may be a rather restricted phenomenon within an organism's olfactory system and may be specific to certain compounds where sensitivity is of utmost importance.

The remarkable observation that OR2T11 responds with a strong metal effect only to small thiols mainly containing one to five carbons suggests an unusually small receptor cavity. The relative order of activity of the thiols, namely, *t*-BuSH > *i*-PrSH > *n*-PrSH > AllylSH, is in accord with measured human thresholds, and the suggestion that "with increasing substitution at the mercapto-containing carbon atom, also the Lewis basicity of the SH group and, thus, the binding affinity to metal ions are increased. Therefore, tertiary thiols are able to link more strongly to metal ions in the active site center of a hypothetical OR than primary thiols, leading to a more sensitive detection resulting in lower odor thresholds."[55] QM/MM calculations show [negative] binding energy in the order *t*-BuSH > *i*-PrSH > *n*-PrSH > EtSH > MeSH > AllSH > H_2S ≫ 3-methylbutane-2-thiol, in agreement with the Lewis basicity model, given the electron-withdrawing, Lewis basicity diminishing effect of the sp^2−sp^3 bonding in AllSH, together with the enhanced steric demands of 3-methylbutane-2-thiol. The order of the calculated binding energy is also in accord with measurements of the binding of these sulfur compounds to gold nanoparticles supported on metal oxides.[56] Steric effects can also be invoked comparing *i*-PrSH and *sec*-BuSH, as well as MTMT and *i*-BuSH compared to that of *n*-PrSH (Table S4). Spider monkeys are especially sensitive to small alkanethiols, showing threshold detection values of 0.00096, 0.16, and 0.63 ppb, toward ethanethiol, butanethiol, and pentanethiol, respectively.[3] Since straight-chain thiols with five to seven carbons have human odor thresholds even lower than those with one to four carbons[18,55] and the ten-carbon chiral (*S*)-1-*p*-menthene-8-thiol (grapefruit thiol) has a record-holding odor threshold of 0.0000066 ng/L (parts per trillion) in air, other strongly responding human thiol receptors are required, such as OR2W1 and OR2C1, which respond to straight-chain thiols with five to eight carbons (Figure S2) without a copper effect. Interestingly, OR2W1 responds only weakly to branched and straight-chain thiols with one to four carbons. OR2T11 does not respond to H_2S, which is significantly weaker as a Lewis base than the alkanethiols. That the "grapefruit thiol" is described as having a potent but not unpleasant odor and that the perception of the odor of thiols from food and beverages varies with the concentration also points to the existence of multiple thiol ORs. Indeed, it has been argued that higher ligand concentrations "actively recruit more receptors, thus changing the quality of the receptor output."[57,58] Our observation that OR2T11 responds only to compact thiols of one to five carbon atoms and the four-membered heterocycle thietane requires that molecular size and shape as well as chemical properties, e.g., metal binding,[59] must be involved in detection of thiols by OR2T11. It has been suggested that *Drosophila* are sensitive to the molecular volume of odorants.[60]

The identification of two mammalian ORs with a metal effect allows us to scrutinize the metal effect requirements for different receptors and ligands. While MOR244-3 responds with a strong copper effect to α-mercaptothioethers,[32] it also responds to most of the monothiols but without a copper effect. This suggests that the mechanism of activation of the two receptors by the same monothiols may be dramatically different, one with copper and one without, as elegantly confirmed by results from STD NMR. Furthermore, the activation of MOR244-3 may involve both copper-dependent and -independent pathways. In addition to identifying receptors with a metal effect, we also identify OR2T11 and MOR244-2 as the first examples of ORs that respond to metals. It is notable, but perhaps not surprising, that OR2T11 can harbor a metal at two different binding sites so that it can both have a metal effect and be activated by metals. Cases such as MOR244-2, which is expressed in the ventral zone of the main olfactory epithelium and responds to metals only at a single binding site with its volatile ligands yet to be identified, are more perplexing from an evolutionary standpoint. Why has a receptor evolved metal responses, and why would metal perception be important for an animal's fitness? Nonvolatile molecules, such as major histocompatibility complex peptide ligands, are shown to activate olfactory neurons to facilitate social recognition.[61] It is possible that some animals use their olfactory system to detect metals. Notably, a third member of the MOR244 family, MOR244-1, is also widely expressed in the mouse olfactory system but has no known ligand. Future studies involving knockout animals will help to elucidate the possible roles of MOR244-1 and MOR244-2 in olfaction.

Finally, using STD NMR we have directly observed the binding of TBM to OR2T11 and MOR244-3 in live cells, including the effect that copper and silver salts have on this binding. Our results show that STD NMR methods can successfully be used to monitor specific metal effects. This approach has the advantage of not being an end-point assay, allowing various concentrations of odorants and metals to be added to the same live cell samples and changes in binding monitored. In addition, STD NMR methods work well in conjunction with the luciferase and GloSensor assays, providing valuable data for the identification and mapping of ligand binding sites and offering an instrumental tool for resolving molecular mechanisms responsible for ligand binding and resulting signaling. Such studies could lead to a deeper understanding of olfactory sensing and, ultimately, to the possibility of achieving olfaction on a chip.[62]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06983.

Experimental details; characterization of OR−ligand pairs, OR response to selected compounds, effect of copper on OR response; screening for OR ligands and ORs; predicted binding sites; sequence alignments; models of ligands bound to ORs; response of ORs to ligands; sulfur compounds utilized; EC_{50} data; mixture

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panel details; ONIOM energies; primer sequences; references (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Fischer, E.; Penzoldt, F. *Justus Liebigs Ann. Chem.* **1887**, *239*, 131−135.
(2) Moncrieff, R. W. *The Chemical Senses*, 3rd ed.; Leonard Hill: London, 1967.
(3) Laska, M.; Bautista, R. M.; Hofelmann, D.; Sterlemann, V.; Salazar, L. T. *J. Exp. Biol.* **2007**, *210*, 4169−4178.
(4) Andersen, K. K.; Bernstien, D. T.; Caret, R. L.; Romanczyk, L. J. *Tetrahedron* **1982**, *38*, 1965−1970.
(5) Cai, X. J.; Block, E.; Uden, P. C.; Quimby, B. D.; Sullivan, J. J. *J. Agric. Food Chem.* **1995**, *43*, 1751−1753.
(6) Block, E. *Garlic and Other Alliums: The Lore and the Science*; RSC Press: Cambridge, U.K., 2010.
(7) Buck, L. B. *Angew. Chem., Int. Ed.* **2005**, *44*, 6128−6140.
(8) Nagata, Y. In *Odor Measurement Review*; Japan Ministry of the Environment, Government of Japan: Tokyo, 2003; p 118−127.
(9) Nagata, Y.; Takeuchi, N. *Bull. Jpn. Environ. Sanit. Center* **1990**, *17*, 77−89.
(10) Devos, M.; Patte, F.; Rouault, J.; Lafort, P.; Van Gemert, L. J. *Standardized Human Olfactory Thresholds*; IRL Press at Oxford University Press: Oxford, U.K., 1990.
(11) Roberts, J. S. In *Kirk-Othmer Encyclopedia of Chemical Technology*; Wiley-VCH: Weinheim, Germany, 1997.
(12) Sela, L.; Sobel, N. *Exp. Brain Res.* **2010**, *205*, 13−29.
(13) Pavez, C.; Agosin, E.; Steinhaus, M. *J. Agric. Food Chem.* **2016**, *64*, 3417−3421.
(14) Gros, J.; Peeters, F.; Collin, S. *J. Agric. Food Chem.* **2012**, *60*, 7805−7816.
(15) Vermeulen, C.; Lejeune, I.; Tran, T. T.; Collin, S. *J. Agric. Food Chem.* **2006**, *54*, 5061−5068.
(16) Sourabie, A. M.; Spinnler, H. E.; Bonnarme, P.; Saint-Eve, A.; Landaud, S. *J. Agric. Food Chem.* **2008**, *56*, 4674−4680.
(17) Granvogl, M.; Christlbauer, M.; Schieberle, P. *J. Agric. Food Chem.* **2004**, *52*, 2797−2802.
(18) Schoenauer, S.; Schieberle, P. *J. Agric. Food Chem.* **2016**, *64*, 3849−3861.
(19) Li, J. X.; Schieberle, P.; Steinhaus, M. *J. Agric. Food Chem.* **2012**, *60*, 11253−11262.
(20) Dulsat-Serra, N.; Quintanilla-Casas, B.; Vichi, S. *Food Res. Int.* **2016**, DOI: 10.1016/j.foodres.2016.02.008
(21) Tamura, H.; Fujita, A.; Steinhaus, M.; Takahisa, E.; Watanabe, H.; Schieberle, P. *J. Agric. Food Chem.* **2011**, *59*, 10211−10218.
(22) Suarez, F. L.; Springfield, J.; Levitt, M. D. *Gut* **1998**, *43*, 100−104.
(23) Kraft, P.; Mannschreck, A. *J. Chem. Educ.* **2010**, *87*, 598−603.
(24) Crabtree, R. H. *J. Inorg. Nucl. Chem.* **1978**, *40*, 1453−1453.
(25) Lee, S.; Barin, G.; Ackerman, C. M.; Muchenditsi, A.; Xu, J.; Reimer, J. A.; Lutsenko, S.; Long, J. R.; Chang, C. J. *J. Am. Chem. Soc.* **2016**, *138*, 7603−7609.
(26) Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, CA, 1994.
(27) Henkin, R. I.; Potolicchio, S. J.; Levy, L. M.; Moharram, R.; Velicu, I.; Martin, B. M. *Am. J. Med. Sci.* **2010**, *339*, 249−257.
(28) Wang, J.; Luthey-Schulten, Z. A.; Suslick, K. S. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 3035−3039.
(29) Bushdid, C.; Magnasco, M. O.; Vosshall, L. B.; Keller, A. *Science* **2014**, *343*, 1370−1372.
(30) Axel, R. *Angew. Chem., Int. Ed.* **2005**, *44*, 6110−6127.
(31) Hong, S.; Corey, E. J. *J. Am. Chem. Soc.* **2006**, *128*, 1346−1352.
(32) Duan, X.; Block, E.; Li, Z.; Connelly, T.; Zhang, J.; Huang, Z.; Su, X.; Pan, Y.; Wu, L.; Chi, Q.; Thomas, S.; Zhang, S.; Ma, M.; Matsunami, H.; Chen, G. Q.; Zhuang, H. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 3492−3497.
(33) Block, E.; Zhuang, H. *ACS Symp. Ser.* **2013**, *1152*, 1−14.
(34) Lin, D. Y.; Zhang, S. Z.; Block, E.; Katz, L. C. *Nature* **2005**, *434*, 470−477.
(35) Sekharan, S.; Ertem, M. Z.; Zhuang, H.; Block, E.; Matsunami, H.; Zhang, R.; Wei, J. N.; Pan, Y.; Batista, V. S. *Biophys. J.* **2014**, *107*, L5−L8.
(36) Saito, H.; Chi, Q.; Zhuang, H.; Matsunami, H.; Mainland, J. D. *Sci. Signaling* **2009**, *2*, ra9.
(37) Mainland, J. D.; Keller, A.; Li, Y. R.; Zhou, T.; Trimmer, C.; Snyder, L. L.; Moberly, A. H.; Adipietro, K. A.; Liu, W. L.; Zhuang, H.; Zhan, S.; Lee, S. S.; Lin, A.; Matsunami, H. *Nat. Neurosci.* **2013**, *17*, 114−120.
(38) Brechbuhl, J.; Moine, F.; Klaey, M.; Nenniger-Tosato, M.; Hurni, N.; Sporkert, F.; Giroud, C.; Broillet, M. C. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 4762−4767.
(39) Kreitman, G. Y.; Danilewicz, J. C.; Jeffery, D. W.; Elias, R. J. *J. Agric. Food Chem.* **2016**, *64*, 4095−4104.
(40) Henkin, R. I.; Bradley, D. F. *Proc. Natl. Acad. Sci. U. S. A.* **1969**, *62*, 30−37.
(41) Wagstaff, J. L.; Taylor, S. L.; Howard, M. J. *Mol. BioSyst.* **2013**, *9*, 571−577.
(42) Viegas, A.; Manso, J.; Nobrega, F. L.; Cabrita, E. J. *J. Chem. Educ.* **2011**, *88*, 990−994.
(43) Pereira, A.; Pfeifer, T. A.; Grigliatti, T. A.; Andersen, R. J. *ACS Chem. Biol.* **2009**, *4*, 139−144.
(44) Claasen, B.; Axmann, M.; Meinecke, R.; Meyer, B. *J. Am. Chem. Soc.* **2005**, *127*, 916−919.
(45) Bergeron, S. J.; Henry, I. D.; Santini, R. E.; Aghdasi, A.; Raftery, D. *Magn. Reson. Chem.* **2008**, *46*, 925−929.
(46) Bhunia, A.; Bhattacharjya, S.; Chatterjee, S. *Drug Discovery Today* **2012**, *17*, 505−513.
(47) Krishnan, V. *Curr. Anal. Chem.* **2005**, *1*, 307−320.
(48) Meyer, B.; Klein, J.; Mayer, M.; Meinecke, R.; Möller, H.; Neffe, A.; Schuster, O.; Wülfken, J.; Ding, Y.; Knaie, O.; et al. *Ernst Schering Res. Found. Workshop* **2004**, *44*, 149−167.
(49) Akoka, S.; Barantin, L.; Trierweiler, M. *Anal. Chem.* **1999**, *71*, 2554−2557.

DOI: 10.1021/jacs.6b06983
J. Am. Chem. Soc. 2016, 138, 13281−13288

(50) Burger, J. L.; Jeerage, K. M.; Bruno, T. J. *Anal. Biochem.* **2016**, *502*, 64−72.
(51) Haga, K.; Kruse, A. C.; Asada, H.; Yurugi-Kobayashi, T.; Shiroishi, M.; Zhang, C.; Weis, W. I.; Okada, T.; Kobilka, B. K.; Haga, T.; Kobayashi, T. *Nature* **2012**, *482*, 547−551.
(52) Turin, L. *Chem. Senses* **1996**, *21*, 773−791.
(53) Najafabadi, B. K.; Corrigan, J. F. *Dalton Trans.* **2014**, *43*, 2104−2111.
(54) Schneider, S.; Dzudza, A.; Raudaschl-Sieber, G.; Marks, T. J. *Chem. Mater.* **2007**, *19*, 2768−2779.
(55) Polster, J.; Schieberle, P. *J. Agric. Food Chem.* **2015**, *63*, 1419−1432.
(56) Sui, R.; Lesage, K. L.; Carefoot, S. K.; Furstenhaupt, T.; Rose, C. J.; Marriott, R. A. *Langmuir* **2016**, *32*, 9197−9205.
(57) Coureaud, G.; Langlois, D.; Sicard, G.; Schaal, B. *Chem. Senses* **2004**, *29*, 341−350.
(58) Wackermannová, M.; Pinc, L.; Jebavý, L. *Physiol. Res.* **2016**, *65*, 369−390.
(59) Block, E.; Jang, S.; Matsunami, H.; Sekharan, S.; Dethier, B.; Ertem, M. Z.; Gundala, S.; Pan, Y.; Li, S.; Li, Z.; Lodge, S. N.; Ozbil, M.; Jiang, H.; Penalba, S. F.; Batista, V. S.; Zhuang, H. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E2766−2774.
(60) Saberi, M.; Seyed-Allaei, H. *Sci. Rep.* **2016**, *6*, 25103.
(61) Spehr, M.; Kelliher, K. R.; Li, X. H.; Boehm, T.; Leinders-Zufall, T.; Zufall, F. *J. Neurosci.* **2006**, *26*, 1961−1970.
(62) Datta-Chaudhuri, T.; Araneda, R. C.; Abshire, P.; Smela, E. *Sens. Actuators, B* **2016**, *235*, 74−78.

DOI: 10.1021/jacs.6b06983
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