Article

Kinetics of Odorant Recognition with a Graphene-Based Olfactory Receptor Mimicry

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Abstract: Malaria vector mosquito species rely on a handful of specific pheromones for mating; one of them, sulcatone (6-methyl-5-hepten-2-one), is also found in human exudation. Therefore, a complete understanding of the insect’s olfaction, and rapid real-time methods for odorant detection, are required. Here, we mimic the odorant recognition of the nerve cells of an insect’s antenna with a synthetic graphene-based bio-electro-interfacial odorant receptor. By this means, we obtain the kinetics of the genuine odorant recognition reaction and compare them to electro-antennogram data that represent the more complex scenario of a living insect. The odorant-binding proteins OBP 9A and 9B only associate with their ligands weakly, showing KDs of between 2.1 mM and 3 mM, while the binding kinetics of OBP proteins depend on the structural feature of a cystine knot and are modulated by the local milieu within a protein-aided enhancement zone.

Keywords: biosensor; graphene; field effect transistor; insect; pest; odorant-binding protein; olfaction; malaria; sulcatone; 6-methyl-5-hepten-2-one

1. Introduction

The small-molecule pheromone 6-methyl-5-hepten-2-one (sulcatone) merits attention for its two-fold role in one of the critical safety issues of our time, being linked to climate change [1,2]. It is associated with the spread of malaria, being one of the five main aggregation pheromones involved in swarming initiation and also mating success in malaria vector mosquito species [3–5]. The pheromone attracts the mosquito species, Anopheles arabiensis and An. gambiae, and increases mating for another five malaria vectors; moreover, it is a degradation product of terpenes and, thus, indicates the breakdown of plant defense [6]. In comparison, the structurally similar 3-octanol is a degradation product of lipids and indicates the activity of fungi [7] and, thus, the potential presence of mycotoxins [8]. For both C-8 marker compounds, rapid, real-time monitoring is highly desirable.

Here we used a graphene-based field-effect transistor (FET), which we linked communicatively to the odor binding proteins, TcasOBP9A and TcasOBP9B, of the beetle Tribolium castaneum to mimic the nerve cell receptor (Figure 1). The nerve cell receptor TcOr1 is highly conserved (ortholog of Drosophila Or83b) and prevails in most insects [9–12]. Beyond detecting two critical pheromone-volatiles in real time, this approach allows us to compare the reaction kinetics of odorant recognition in the insect’s antenna of our synthetic receptor, and so discover previously unknown regulatory pathways. We had previously established our bio-transistor, which detected the binding of various small molecules to Apis mellifera OBP14 [13], and revealed that the binding kinetics of OBP proteins depend on their cysteine bridging pattern [14]. While OBP14 contains 2 S-S-bonds, with cysteines linked according to the pattern 1–3, 4–5 (with no cystine knot, 2 is an additional free cysteine), OBP9A/B has 3 S-S-bonds that form a cystine knot with the (rotaxane-) pattern 1–3, 2–5,
4–6 [15]. A comparison of wild-type OBP14 and Amel SS-OBP14, a Gln44Cys, His 97Cys S-S-OBP14-double-mutant (containing an additional disulfide bond between helices α3 and α6, to achieve a 1–3, 2–5, 4–6 pattern) showed that the restriction of molecular dynamics and the stability increase in the rotaxane substructure, which results from cystine knot formation [16], while leaving the crystal structure largely congruent to the non-rotaxane ones, accelerates the on-rate in small-molecule binding and, ultimately, leads to tighter binding [14]. Therefore, it seems a logical step to link such an OBP to our sensor platform that shows a double cystine knot crosslinking pattern, especially considering future field sensor applications where stable proteins are required. We chose *Tribolium castaneum* OBP9A and OBP9B, according to their high expression levels in the antenna, which suggested an important role in olfactory processes; moreover, RNA-interference experiments in which both proteins were knocked out demonstrated a massive loss of sensitivity of the insect antennae [17].

On the dendrites of the living insect’s sensillum, pheromone volatiles are absorbed by a lymphatic liquid that can be viewed as a protein-aided enhancement zone, in which OBPs bind, protect, enrich and transport the pheromone [18] (Figure 1). However, the stability/dynamics-dependent ligand-binding kinetics leave room for speculation that smell-sensing may be controlled by a specific local milieu in the vicinity of the nerve cell membrane that would modulate the OBPs’ stability/dynamics. There are plenty of reports of nerve cells signaling via the release of ions or small molecules [19], thus changing, very locally, the milieu of the surrounding medium. Usually, such experiments are conducted with advanced microscopic techniques and the results are viewed in the light of cell-cell communication [20,21]; however, here, a case of cell-OBP communication becomes a matter for investigation. It is beyond the scope of this paper to investigate the local milieu at the smell receptor’s surface; however, we show a comparison between our synthetic receptor (i.e., a FET-transistor, linked to OBP9A/B) and the electroantennograms (EAG) of living *Tribolium castaneum* antenna. The binding of sulcatone and 3-octanol appears to be energetically equal (with comparable $K_D$) but the binding kinetics are strongly accelerated in the living antenna.

![Figure 1. Nerve cell receptor in the insect's antenna, mimicked by our synthetic receptor. Left side: nerve cells in the insect's sensillum are equipped with the ion-channel receptor TcOr1, which binds OBPs (which, in turn, bind odorants) and responds with ion signaling [22]. TcOr1 faces into a liquid lymphatic solution that constitutes a protein-aided enhancement (or enrichment) zone [11,12]. Right side: synthetic receptor. Instead of a nerve cell membrane containing ion channels, the signal is generated by a highly surface charge-sensitive graphene semiconductor surface. OBPs remain communicatively linked to the graphene, which is sustained by a Si/SiO substrate. In both cases, the response is read out electrically, with electrodes placed at the antenna (for electroantennograms) or on the graphene surface (for rGO-FET measurements). The structures of both odorants are depicted at the bottom.](image-url)
2. Materials and Methods

Protein production. OBP9A and OBP9B were expressed in E.coli as described previously [13,14] and purified with standard chromatographical methods, including a final-size exclusion step with a Sepharose 200 column. The chromatograms showed a sharp, symmetric peak with no shoulders and a reducing SDS-Page of different fractions within this peak confirmed one sharp band. The non-reducing SDS-Page showed the same molecular weight; thus, the S-S-bonds have formed a cystine-knot pattern.

The amino acid sequences of OBP9A and OBP9B are the following:

- TcasOBP9A: AMSEAQLKAAVKLVRNMCQPKSKATNEDIEKMHHGDWNIDRTAMCYMHCAILNSKLITKENV
- TcasOBP9B: AMSEQMAALKLVRNVQPKKTATNEQIEAMHTGNWDLDKNGKCYMCILNMYKLGKDNC
- TcasOBP9A: FNRDYITLAENPLTALKTASIAANLCKDSAKTLDDKCAAYEISKCYMYLDNPEKYFLP
- TcasOBP9B: FDWEAGIATLKAQAPESVRPDAISVPNCKDKTTSKDEAEAYEIAHHCMYLDNPEKYFLP

Preparation of large graphene oxide (GO) sheets. Graphene oxide (GO) was prepared by a modified Hummers/Offeman method, as follows [23]. First, 2 g of 125–250 µm-sized graphite flakes (NGS Naturgraphit GmbH, Leinburg, Germany) were stirred in 12 mL of concentrated sulfuric acid (98%) for 4 h at 80 °C. After cooling down to room temperature, the suspension was sonicated for 30 min with 500 mL deionized H₂O, then the flakes were filtrated at 0.2 µm pore size and dried in an exicator overnight. The graphite flakes were again suspended in concentrated sulfuric acid (120 mL) and stirred for 30 min at −20 °C. Subsequently, 15 g of potassium permanganate was interspersed over a period of 2 h while the exothermal reaction was kept at −20 °C, and further stirred at RT for another 2 h. For oxidative exfoliation, the suspension was diluted stepwise into 1 L of deionized H₂O and a 30% solution of H₂O₂ was added. Subsequently, the graphene oxide was skimmed from the water surface and stirred in fresh deionized H₂O.

Preparation of rGO-FETs. The employed protocol followed the procedure described elsewhere in the literature [13,14]. Briefly, silicon substrates with a 300 nm oxide layer were chosen as a basal layer for the FETs. The SiO₂ substrates were cleaned with a standard RCA cleaning procedure. To obtain an adhesive self-assembled monolayer, the substrates were submerged in a 2% aminopropyltriethoxysilane (APTES) solution in ethanol for 1 h. After rinsing with ethanol, the substrates were heated to 120 °C for two hours and afterward cooled to room temperature. The graphene oxide flakes were applied to the APTES-modified Si-Wafer via drop-casting of the graphene-oxide suspension. The deposited graphene oxide was reduced in hydrazine vapor using hermetically sealed glass Petri dishes that contain the substrates and a droplet of hydrazine at 70 °C overnight. Flake distribution was first checked with an optical microscope and selected devices, then characterized using SEM (see Supplementary Materials S1). T-shaped source and drain electrodes were applied by the evaporation of gold onto the graphene surface, as shown in the sketch in Figure 1 and further described by the authors of [24].

Electrical characterization of synthetic receptors. The resulting FET chips were sorted, based on conductivity, and only chips that showed a resistance smaller than ~800 Ohms were used. A silver-silver chloride reference electrode (Flex Ref, World Precision Instruments, Sarasota, USA) was used to operate the FET device in a liquid gate configuration. Source-drain-currents (I_{SD}) were plotted against the different gate voltages (V_G) to optimize the measuring range (Figure 2A). A V_G of −0.6 V was finally chosen for all subsequent measurements. The source-drain bias V_{SD} = 0.05 V.
were monitored in real time, using a Keithley 4200 semiconductor characterization system. (Figure 2A) revealed that the synthetic receptor can be tuned and has a preferred operating (which, in the functional receptor, would link an OBP to the graphene surface). The linker 

\[ \text{µ} \]

Therefore, 20\( \mu \)L of a 5 \( \mu \)M PBSE solution in THF was placed onto the detection area of the rGO-FET, followed by 10 \( \mu \)L of a 10 \( \mu \)M protein solution in PBS buffer (10 mM; pH 8.0); the chip was incubated for 2 h at 4 \( ^\circ \)C. The same buffer was also used to equilibrate the system until baseline stability was obtained, and to prepare odorant solutions of sulcatone and 3-octanol (Sigma-Aldrich) at concentrations of between 300 \( \mu \)M and 5 mM.

The synthetic receptors were contacted in a polymer flow cell and the source-drain currents were monitored in real time, using a Keithley 4200 semiconductor characterization system.

3. Results

**Proteins.** Expression and purification of the proteins OBP9A and OBP9B were controlled by SDS-Page and resulted in sharp single bands, both at the same MW. The presence of the disulfide bonds was checked by comparing reducing and non-reducing gels.

**Plain synthetic transistor.** The plain rGO-FETs were only loaded with the PBSE-linker (which, in the functional receptor, would link an OBP to the graphene surface). The linker also serves as a passivation layer, thus preventing all non-protein-covered surfaces from generating non-specific signals [13]. The transistor/receptor setup is depicted in Figure 1 and in panels G and H in Figure 2.

Feeding the odorants sulcatone, 3-octanol, and 2-octanone, at concentrations in the range of the maximum used in the following experiments (up to 5 mM), into these plain receptors resulted in no significant response, as shown in Figure 2B.

**Synthetic ligand-binding receptor.** Further controls were performed with the full functional receptor, consisting of an rGO-FET coupled to an OBP9B. A gate voltage swipe (Figure 2A) revealed that the synthetic receptor can be tuned and has a preferred operating range of between \(-0.6\) and \(0\) V. Repeated exposure of the receptor to sulcatone stimuli of 0.5 mM or 1 mM proved it to have excellent baseline stability and reproducible signal amplification (Figure 2D). Detailed analysis of the single concentration steps yielded a high
signal-to-noise ratio. The green dash-dotted and the blue dotted lines in Figure 2C are the simulated boundaries of a 20% error interval of the observed reaction rate. Fitting with established models [13], shown as a red line, yields an R² of 0.99.

Synthetic odorant receptors were set up with OBP9A and OBP9B and stimulated with the odorants sulcatone and 3-octanol at stepwise-increasing concentrations from 300 µM to 6 mM as the source-drain current, ∆I_SD, was monitored on-line in real time (shown in Figure 3A–D). The subsequent rinsing step with pure buffer re-set the system to the pristine baseline level, demonstrating the full reversibility of the affinity reaction between the receptor and the odorant. These raw data were subjected to a global analysis that proved the internal consistency of each data set (each OBP–odorant combination) as described previously by the authors of [13]. Dissociation constants (K_D) were obtained in two ways. For the kinetic approach, see the k/c odorant plots in panels E–H, in which the intercept and slopes indicate k_off and k_on, thus, a kinetic K_D is given by their quotient. For thermodynamic K_D, which more directly represent the equilibrium state, the relative response was plotted against the stimulus concentration (Figure 3, panels I–L), which also yielded the receptor occupation. Fitting in a Langmuir isotherm yielded the K_D summarized in Table 1. The kinetic K_Ds corresponded to the thermodynamic ones within an error margin of 20%; thus, proving the internal consistency of the data.

Figure 3. Synthetic receptor kinetics. (A–D) The real-time response of synthetic receptors, set up with OBP9A or OBP9B (as shown in panel T) when binding stepwise increasing concentrations of sulcatone or 3-octanol. (E–H) Observed rate constants (k) of each concentration step, plotted against the concentration. Intercepts and slopes of the linear regressions yield kinetic off-rates k_off and on-rates k_on, respectively. (I–L) Binding equilibria (thermodynamics) of each OBP/odorant pair above. Responses to each concentration at saturation are plotted against the concentration. Langmuir fits (red curves) yielded the dissociation constants K_D. Dotted and dashed-dotted lines indicate ± 20% simulated error ranges. The second (red) ordinate shows receptor occupation. All synthetic receptor responses were measured in triplicate.
Table 1. Odorant-binding kinetics of OBPs.

| Protein (Species) | Ligand                | $K_D/\mu M$ | $k_{on}/M^{-1} s^{-1}$ | $k_{off}/s^{-1}$ |
|------------------|-----------------------|-------------|------------------------|-----------------|
| TcasOBP9A        | 6-Methyl-5-hepten-2-one (Sulcatone) | 2100        | 2.9                    | 0.008           |
| (Tribolium castaneum) | 3-Octanol           | 2300        | 3.4                    | 0.006           |
| TcasOBP9B        | 6-Methyl-5-hepten-2-one (Sulcatone) | 3000        | 1.3                    | 0.004           |
| (Tribolium castaneum) | 3-Octanol           | 2900        | 2.4                    | 0.006           |
| AmelOBP14 [13]   | Homovanillic acid    | 4           | 1130                   | 0.008           |
| (Apis mellifera) | Methyl vanillate     | 20          | 230                    | 0.010           |
|                  | Eugenol              | 40          | 170                    | 0.006           |
|                  | Citral               | 800         | 9                      | 0.003           |
|                  | Methyl eugenol       | 1400        | 6                      | 0.006           |
|                  | Geraniol             | 3300        | 3                      | 0.003           |
|                  | 6-Methyl-5-hepten-2-one (Sulcatone) | 3500        | 5                      | 0.008           |
| Amel SS-OBP14 [13] (Apis mellifera) | Eugenol            | 4           | 750                    | 0.006           |
| BmorPBP1 [26]    | Bombykol (pH 8)      | 5           | 20                     | 0.000094        |
| (Bombyx mori)    | Bombykol (pH 4)      | -           | no binding             | no binding      |
| Antenna EAG [25] | 6-Methyl-5-hepten-2-one (Sulcatone) | 1300        | 73                     | 0.095           |
| (Tribolium castaneum) | 3-Octanol           | 2500        | 32                     | 0.08            |

Compared to OBP9B, OBP9A yielded slightly higher but still similar on-rates and affinities, while wild-type OBP14 also yielded similar affinities to sulcatone. The same holds true for the OBP9A-binding 3-octanol. Overall, both odorants are comparable OBP-binders (Table 1). However, a comparison between wtOBP14 and the more disulfide-crosslinked Gln44Cys, His 97Cys mutant in terms of binding eugenol reveals a 10-fold affinity gain (on-rate-driven). In contrast, BmorPBP1 binds bombykol with high affinity, despite a loose S-S-pattern similar to OBP14 at pH 8, and differs from the S-S-mutant OBP14- or OBP14-binding higher-affinity ligands by a 100-fold lowered exchange rate (lower $k_{on}$ compensated for by lower $k_{off}$). Binding shows a strong pH dependence, with no detectable binding at pH 4 [26].

The synthetic receptors with TcasOBP9A/B were closely comparable to the living insect’s antennae of the *T. castaneum* in terms of binding equilibria but differed in the exchange rate, which was 10 times higher in the insect.

The electroantennograms (EAG) in Figure 2E,F are courtesy of Karthi Balakrishnan [25]. The kinetic constants, $K_D$ and $k_{on}$, were converted from pressure units ($\mu$bar and $\mu$bar$^{-1}$) to molar units using Henry’s law constants, at 0.14 mol m$^{-3}$ pa$^{-1}$ for sulcatone [27] and 0.31 mol m$^{-3}$ pa$^{-1}$ for 3-octanol [28]. The results are shown in Table 1.

4. Discussion

In the living insect’s antenna, an odorant is captured, accumulated, protected, and transported by an odorant-binding protein, which then hands the odorant over to the odorant receptor on the nerve cell membrane [18]. Speaking in technological terms, a membrane receptor can be described as a transistor. While a synthetic field-effect transistor transduces and enhances an electrical signal (electric field) that is applied to its gate into a source-drain current, initiating a “signal cascade” that ends with a graph on our screens, the receptor, as a biological transistor, transduces the biological signal (the presence of an odorant) into its own form of nerve cell signal cascade, which, in turn, ends up with the insect sensing a smell. Much like the synthetic FET, the signal cascade of the receptor is largely electrical, although the charge carriers are ions. The latest generation of synthetic FET transistors, which we introduced in a previous work [13] and which will be compared to a membrane receptor in the light of this new data, comes even closer to the principle of a biological transistor because it is biologically, not electrically, gated (triggered by an odorant). Briefly, the signal generation is based on the high sensitivity of graphene–SiO$_2$ sandwiches to local electric fields. At the nanometer scale, the semiconducting graphene layer is somewhat hilly in terms of topography and surface potential [29]; thus, it behaves like the 2-dimensional equivalent of a bulk semiconductor with p- and n-doped patches.
Applying a bias in a lateral direction between the source and drain electrodes yields an electrical circuit consisting of $x$ serial n-p-n-p junctions (a transistor array). The OBPs, which are communicatively close when linked to the graphene, exhibit a substantial change of dipole moment upon binding their ligands [30,31]; the signals displayed in the $I_D-V_G$ curves, as well as the $I_D$ vs time plots, prove that the transistor array reacts very sensitively to this field effect.

It has always been puzzling that the olfactory receptors in an insect’s antennae, as such, lack specificity and that the OBPs also fail to compensate for this missing selectivity, all of which still leaves the highly selective smell perception unexplained [31]. Therefore, it seems plausible that nerve cells may auto-regulate their sensing of a specific odorant by altering their local milieu (i.e., the milieu within their protein-aided enhancement zone), irrespective of the precise type of milieu regulation. We have shown previously that pH is part of the affinity modulating milieu [26]; furthermore, pH-dependent NMR structures of the pheromone-binding protein 1 [18] also point to a pH-modulated mechanism. However, it is premature to rule out other compounds of a modulating milieu at this stage. This milieu-regulation in insects’ antennae is likely to pose additional challenges to the development of bio-inspired sensing applications.

However, when used as a sensor, our system was capable of detecting even low-affinity OBP ligands and provided a certain level of progress in terms of selectivity and the ability to finely discriminate between small-molecule structures via the mutagenetic tailoring of the binding pocket [14,32,33]. Our synthetic smell receptor provides an opportunity to study smell-sensing in the absence of any presumed autoregulation (in a stable milieu, i.e., a buffer solution) and compare it to the living insect’s antennae. Remarkably, both systems—the FET-receptor and the living antenna—showed almost equal affinities to sulcatone and 3-octanol (this confirms that in both systems, the same binding reaction is predominant) but differed in the exchange rate, which is given by approx. 10-fold higher on- and off-rates in the antenna. This acceleration in the real antennae is crucial for the insect’s ultrafast smell-sensing, which allows for in-flight navigation.

A comparison between recombinant Apis mellifera OBP14 constructs, containing (mutant) or not containing (wt) an additional disulfide bond [14]; therefore, differing in a rotaxane-like intertwining of their polypeptide chains, reveals the importance of a stabilizing cystine knot for odorant binding. We hypothesize that the wild-type OBP14 (as in other similar OBPs that lack one of the 3 S-S bonds) exhibits enhanced conformational dynamics, which slows down odorant-binding due to a tweezer-like restrictive oscillation of the binding pocket. In fact, we found that OBPs were very stable already, with a vacant binding pocket, but this stability further increased when the binding pocket was occupied with an odorant, characterized by a melting-point increase from 57 °C to 64 °C [14]. This link between binding kinetics (especially the exchange rate) and the dynamics of the construct, indicates that any milieu that affected stability would result in differential on- and off-rates for odorants. In this context, the increased (but compensating for each other) on- and off-rates in living antennae, when compared to those we measured with our synthetic olfactory receptor, may result from a stabilizing milieu within the protein-aided enhancement zone.

Finally, our data show no significant differences between the OBP9 variants A and B. It has been reported that OBP9B is naturally expressed at high levels in the antenna, but if OBP9B is knocked out, OBP9A is ready as a substitute.

5. Conclusions

This work may open up a path for explaining how the insect’s olfactory system discriminates between odorants, despite the lack of specific receptors, and to a future understanding of ultrafast smell-sensing. It is only meant as a starting point and should encourage others to shed further light on the molecular dynamics of OBPs, as well as the local milieu (and milieu changes) in which OBPs work in the antenna. This FET-
transistor, sensitive to the malaria vectors of mosquito pheromones, is an opportunity for future developments in malaria risk monitoring; this work should also raise awareness of the subtle regulatory mechanisms that might make it more difficult to mimic insects for odor-recognition applications.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/chemosensors10060203/s1](https://www.mdpi.com/article/10.3390/chemosensors10060203/s1).

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