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Chapter 1

Biomimetic Olfactory Biosensors and Bioelectronic Noses

Marielle El Kazzy, Charlotte Hurot, Jonathan S. Weerakkody, Arnaud Buhot and Yanxia Hou

1.1. Introduction

The environment in which we live is rich in odorants emitted from various natural and unnatural sources (plants, bacteria, industrial activities and other human activities). Odors are mainly composed of hydrophobic volatile organic compounds (VOCs) with molecular weights of less than 300 Da. They cover many chemical classes including organic acids, alcohols, aldehydes, ethers, esters, amides, amines, hydrocarbons, halogenated hydrocarbons, ketones, nitriles, aromatics, phenols, other nitrogen-containing compounds, and sulfur-containing compounds. Nowadays, the analysis of VOCs is of great interest to a diverse variety of fields, such as environmental monitoring, public safety and security, the food and beverage industry, the cosmetics and perfume industry, medical diagnostics and health monitoring, etc.

Traditional analytical methods, such as gas chromatography coupled to mass spectroscopy (GC-MS), are very accurate, reliable and able to identify different substances in a sample. Moreover, when combined with olfactometry, they can provide not only detailed chemical information but also sensory information. Nevertheless, these techniques require expensive equipment as well as expertise in

Yanxia Hou
Univ. Grenoble Alpes, CEA, CNRS, IRIG, SyMMES, UMR5819, F-38000 Grenoble, France

operating them. They are often time-consuming and laborious to perform. On the other hand, the human nose is often used to analyze odorants and assess the quality of products in the food, beverage, cosmetics, and perfume industries. However, its use is limited by the fact that panelists are expensive to train and employ, and sometimes they can give biases in results highlighting the subjectiveness of the human sense of smell. Consequently, these approaches are not practical for use in a large scale. In such a context, there is an increasing demand for a device that could mimic the human nose and provide an objective, quantitative, rapid, and reliable analysis of odorants.

An interesting alternative could be electronic noses (eNs), which consist of an array of cross-reactive chemical sensors combined with an appropriate transduction system, and use advanced mathematical procedures based on pattern recognition and/or multivariate statistics for signal processing. Over the last three decades, eNs have demonstrated their great potential and efficiency as analytical tools for the analysis of VOCs in many fields [1-4]. However, for most existing eNs the main drawback is the lack of good selectivity. To overcome it, in the last decade, an increasing number of research activities have been concentrated on the development of olfactory biosensors and bioelectronic noses by incorporating biological material from the olfactory system (i.e. proteins, derivatives of cells and tissues, etc.) as sensing material in the device [5-9].

To clarify the terms, in this chapter, an olfactory biosensor refers to a single sensor system, while a bioelectronic nose refers to a multiplexed system with an array of olfactory biosensors.

Thanks to the current knowledge about the biological olfaction and recent achievements in genetic engineering, biotechnology and nanotechnology, such bioelectronic noses can better mimic the biological olfactory system with improved performances such as high sensitivity and selectivity. However, the integration of these sensing biomaterials to a bioelectronic nose device remains challenging due to the complex and fragile structure that governs their proper functionality.

After introduction, in the second part of this chapter, for better understanding the different strategies and technical solutions employed for the development of biomimetic olfactory biosensors and electronic noses, we will present the source of inspiration: the olfactory system.

We will introduce the mechanism of smell and odor discrimination principles. Then we will describe the main components of the olfactory system that are currently used as raw sensing materials for the design of biosensors namely the olfactory receptors (ORs) and some other proteins such as odorant binding proteins (OBPs) and enzymes involved in biological olfaction. In the third part, we will give a review on the development of biomimetic olfactory biosensors and bioelectronic noses based on olfactory tissues and cells, olfactory receptors, and odorant binding proteins. For each system, we will highlight the immobilization strategy employed to conserve the biological properties of the sensing material as well as its performances for the analysis of odorants. Finally, we will make a general conclusion and give some perspectives.

1.2. The Olfactory System A Source of Inspiration

1.2.1. How Do We Smell ?

Among the human senses, the mechanisms of sight, hearing, and touch that are based on physical stimulus are well understood. Today, we are able to fabricate devices that can record and reproduce the signals to mimic these senses, for example, camera for sight, microphone for hearing, and tactile sensor in smart phones and tablet PCs for touch. In contrast, the mechanism by which the biological nose can detect and identify odorant molecules based on chemical stimulus has long remained unknown. In fact, in 1970, when the organic chemist Robert Luft was asked, how olfactory perception is achieved, he replied "If you answer to this question, the Nobel Price is yours" [10]. And indeed, in 2004, Linda B. Buck and Richard Axel received the Nobel Prize in Physiology or Medicine [11] for their groundbreaking work on the molecular basis of odor recognition in 1991 [12]. In their famous study, they identified, for the first time, a multigene family that encodes the core proteins of the olfactory system: the olfactory receptors. They isolated and cloned 18 different ORs that bound odorants. For which, they also determined their structural features and other properties that will be detailed later.

As shown in Fig. 1.1, ORs are incorporated into the plasma membrane of the dendritic extrusions of olfactory neurons (ONs) called cilia. Each olfactory neuron expresses about a million copies of only one type of

receptor. To enable the detection of odorant molecules, those non motile cilia are projected into the mucus that covers the nasal epithelium. On the other extremity, the unmyelinated axons of all the ONs carrying the same type of ORs penetrate the ethmoid bone and reach the olfactory bulb where they converge into clusters called glomerulus. The glomeruli are then connected to the olfactory cortex via mitral and tufted cells that constitute second order neurons needed for processing the incoming signals [13, 14].

When the respiratory air enters the nasal cavity, the carried odorant molecules are dissolved in the mucus or transported by some other proteins to the vicinity of the ORs. Depending on their affinity, the ligands bind more or less strongly to the receptor and trigger a signaling cascade in the ONs. This generates an electrical impulse that is transmitted to the olfactory cortex where the signal is processed and the olfactory message is decoded.

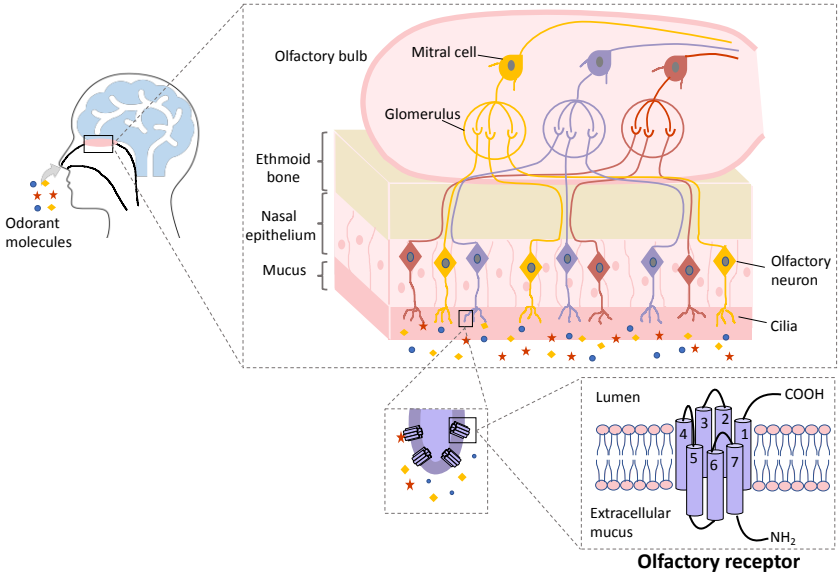


Fig. 1.1. Scheme of the structure of the olfactory system.

1.2.2. The Mystery of Odor Discrimination (Odor Coding)

Often underrated and overlooked compared to the other senses, our noses actually hold many hidden "super powers". Indeed, with roughly 400 different types of ORs, our odor detector can discriminate more than ten thousand different odors and can even distinguish between two odorant enantiomers. For example, the (S)-(+)-2-methyl butanoic acid is perceived as tender and fruity while its enantiomer (R)-(-)-2-methyl butanoic acid smells like cheese or sweat [15]. But the most striking fact is that our olfactory system can detect some odorants such as 2-isobutyl-3-metoxypyrazine (IBMP – bell pepper odor) at sub nanomolar concentrations (0.01 nM in aqueous solution) [16]. This is equivalent to detect a few milligrams in an Olympic size swimming pool! Furthermore, it was recently reported that humans can discriminate more than 1 trillion olfactory stimuli [17].

To break the olfactory code, scientists have suggested many theories and proposed different models such as the "lock-key" model presented by Amoore [18] and the vibrational theory [19] by Malcolm Dyson. Despite all these models, the mystery behind the outstanding resolution and diversity of odor perception remained unsolved until the identification of the multigene family by Buck and Axel [12]. This discovery has opened new doors to study and investigate interaction modes between the ORs and odorant molecules.

With this intention, thanks to electrophysiological measurements, Firestein *et al.* [20] concluded that olfactory receptors are broadly tuned and can interact with a large spectrum of distinct odorant molecules. Similarly, using calcium imaging Krautwurst *et al.* [21], Malnic *et al.* [22] and Touhara [23] have confirmed that most ORs are indeed selective but not specific. In fact, one receptor can interact with more than one odorant molecule and each odorant molecule can be recognized by multiple olfactory receptors. This observation has led to the conclusion that the olfactory system uses a combinatorial code to discriminate and identify odors. This result can finally explain why odorant molecules having similar structures (such as enantiomers) could elicit a completely different physiological response.

In fact, it is this cross-reactive principle that has inspired the design of electronic noses . Such devices rely on an array of wide-spectrum sensing materials (the "ORs") coupled to a transducer (the "neurons") and associated with a pattern-recognition system (the "brain") to

identify the odor. One sensing material must be able to bind different odorant molecules, and reversely, one odorant molecule can bind different sensing materials.

1.2.3. Structure and Signaling Process of Olfactory Receptors

For better understanding the olfaction mechanism in vertebrate at a molecular level, in this part, the structure and signaling process of vertebrate ORs will be presented. The work of Buck and Axel suggested the existence of a superfamily of odorant receptors characterized by seven transmembrane domains and exhibiting variability in regions that potentially represent the binding site of odorant molecules. They have also supported the idea that an intracellular G protein could be involved in the transduction mechanism. Since this breakthrough, many studies have been performed to identify, characterize and deorphanize the members of this large receptor family in both vertebrate and invertebrate [20, 21, 24-27]. Encoded by a multigene family [12, 28], ORs are heptahelical transmembrane G-protein-coupled receptors (GPCRs) that bind odorants. They belong to the class A, like rhodopsin or adrenergic receptors and constitute the largest family of GPCRs identified in the mammalian genome (~1000 genes) [13, 26, 29, 30]. Today, we know that the human genome harbors 396 functional OR genes [31].

The transduction mechanism of ORs in vertebrates and especially in humans has been extensively studied and described [30, 13, 32-36]. It is schematically illustrated in Fig. 1.2. Like all the members of GPCRs family, ORs transduce the binding event via the activation of a molecular switch: the guanine nucleotide-binding protein also called G-protein. Olfactory G-proteins (G_{olf}) are heterotrimeric proteins that consist of three subunits: alpha ($G\alpha_{olf}$), beta (β) and gamma (γ) [37, 38]. The last two subunits often form a stable dimer: The $G\beta\gamma$ complex. The binding of an odorant molecule induces a conformational change in the receptor that will activate its associated G-protein. When the G-protein is activated, the guanosine diphosphate (GDP) initially bound to the alpha subunit is replaced by a guanosine triphosphate (GTP). As a consequence, the α subunit dissociates from the G-protein complex and activates an enzyme: the adenylyl cyclase (AC). AC catalyzes the conversion of adenosine triphosphate (ATP) into the cyclic-3′, 5′-Adenosyl monophosphate (cAMP) neurotransmitter. The increasing concentration of cAMP triggers the opening of the cAMP-dependent

membrane cation channels and thus enabling the entry of sodium and calcium ions (Na^+ and Ca^{2+}) into the ciliary lumen. Moreover, the influx of Ca^{2+} induces the opening of the Ca^{2+}-dependent Cl^- channels, which allows the exit of chloride ions Cl^- and a further depolarization of the cell. If the resulting membrane potential is large enough, an electrical signal is generated and propagates, via the axons, to the olfactory cortex in the brain.

It should be noted here that the change in conformation that occurs upon an odorant binding event is a very important fact that allows the use of ORs for bioelectronic nose development. Indeed, the conformational change elicits a change in the electrical properties of the protein [39]. Thus, using different transduction strategies, it is possible to obtain measurable and detectable signals.

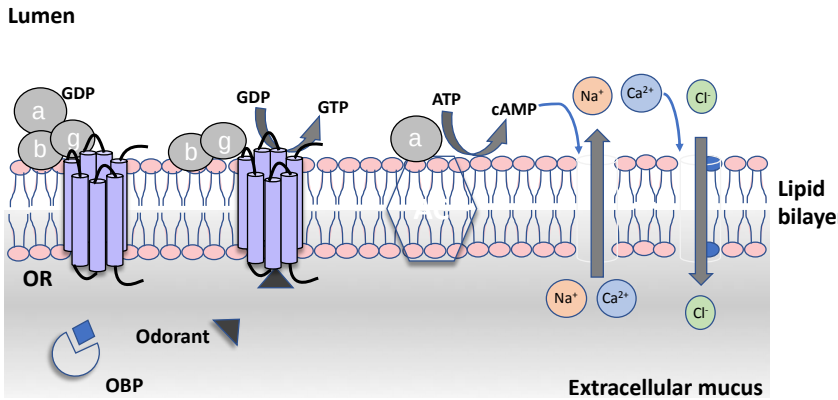


Fig. 1.2. Transduction mechanism of olfactory receptors.

1.2.4. Important Implication of Other Proteins

Some other proteins such as odorant binding proteins and odorant degrading enzymes also play a very important role in the olfaction mechanism. Their structure and functions are presented below.

Odorant binding proteins were detected for the first time by Pelosi *et al.* [40] in 1982 while searching for olfactory receptors using radioactively labeled odorant 2-isobutyl-3-metoxypyrazine (IBMP). Since then, the initially called pyrazine binding proteins were widely studied both in vertebrates (cow/bovine [41, 42], rat [43], rabbit [44], mouse [45, 46],

humans [47], etc.) and insects (honeybee [48], drosophila [49], mosquitos [50], moth [51], etc.); and their amino acid sequence, structure and ligand affinities were extensively investigated. Additionally, many interesting reviews that summarize and discuss the work reported in the literature have been published [16, 52-58].

Highly concentrated in the nasal mucus of vertebrates [47] and in the sensory lymph of insects [55, 57], OBPs are small soluble proteins with low molecular weight (around 20 kDa) [16]. They are able to bind different classes of odorants in a reversible manner with a micromolar range dissociation constant [16, 52]. Vertebrate OBPs belong to the lipocalin family which also includes proteins such as bilin-binding protein (BBP) and retinol-binding protein (RBP) [59]. Despite presenting a great divergence in their amino acid sequence vertebrates OBPs share a common three-dimensional structure. This special architecture consists of a β-barrel formed by eight antiparallel β-sheets and a short α-helical domain at the C-terminus [60, 61]. On the other hand, insect OBPs are characterized by a structure that involves a hydrophobic cavity composed of six α-helical domains stabilized by three disulfide bonds [55-57].

Despite their well-defined structure, the physiological functions of OBPs in olfaction and chemoreception are not yet fully understood. Nevertheless, based on their characteristics (solubility, cross reactivity, molecular weight…) and their homology to carrier proteins such as alpha 2-microglobulin, some hypotheses have been proposed. Pevsner *et al.* [42, 62] first suggested that, like hemoglobin transports oxygen, OBPs may play a role in odorant transport and facilitate their diffusion across the aqueous mucus since odorant molecules are mainly hydrophobic. In addition, this could help in preserving these chemical messengers from enzymatic degradation [55]. In insects, a special class of binding proteins known as PBPs (pheromone binding proteins) was identified. Just like OBPs, PBPs transport pheromones through the sensillar lymph to reach their receptors and protect them from degrading enzymes [63, 57]. Finally, OBPs are also suspected to act as scavengers for odorants [33, 54]. They are thought to participate in olfactory signal termination by removing the odorant molecules from the mucus.

Indeed, another important part in the olfaction process is the signal termination. In fact, one of the remarkable features of the biological nose is its ability to rapidly recover after exposure to odorant

molecules. This desensitization task also involves another protein family including protein kinase (such as protein kinase A or protein kinase C) and specialized G-protein receptor kinase (such as rhodopsin kinase)
[53, 57, 64]. Those odorant degrading enzymes act directly on the ORs by phosphorylation, which stops their biological activity. It is important to note that phosphorylation of the receptors can also be induced by some odorants.

In order to ensure a real time detection and avoid the saturation of the olfactory receptors, the olfactory system must be constantly regenerated from odorant stimuli. Additionally, odorant molecules and pheromones are xenobiotic compounds thus, they must be eliminated from the system. In vertebrate, this task is assured by cytochrome P-450 enzymes which are a subset of monooxygenases [53, 64]. First identified in the liver, those enzymes have been found to be very active in the olfactory epithelium in the vicinity of the ORs [65]. Moreover, in many insects, enzymes such as esterase and oxidase that degrade sex pheromones were detected in the antenna and sensillar lymph [53, 57, 63].

To summarize, the remarkable performances in term of sensitivity and selectivity make the biological nose an extraordinary analytical system. Indeed, with millions of years of evolution, it has excellent performance for odor detection, discrimination, and recognition. Therefore, it presents a great source of inspiration for the design of highly accurate odor analysis tools. In the following part of this chapter, we will present the progress on the development of biomimetic olfactory biosensors and bioelectronic noses.

1.3. Biomimetic Olfactory Biosensors and Bioelectronic Noses

Since the mechanism of olfaction was unveiled, numerous teams from around the world have started to develop a new generation of odor analysis tools by mimicking the human nose. For this, they incorporated sensitive biomaterials from the olfactory system including ORs, OBPs, enzymes, and olfactory tissues into electronic devices. Indeed, so far, the sensitivity and selectivity of those bio-components for specific ligands can hardly be matched by any artificial material. In the last few decades, increasing research activities have concentrated on the exploitation of these biomaterials for the development of novel

olfactory biosensors and bioelectronic noses. These systems have improved performances compared to classical eNs, which often employ chemical materials such as metal oxides, polymers, etc. However, their development is quite challenging due to several issues, namely the protein production in large quantities, the poor stability of biomaterials, the short lifetime and the poor reproducibility of biosensors. Fortunately, thanks to recent important advances in biotechnologies, nanotechnologies, and data processing, great progress has been made on their development, as summarized by several reviews [5, 27, 66-72]. In this part, we will present and discuss the ingenious strategies proposed by different teams to tackle these technical challenges for building robust, sensitive and selective olfactory biosensors and bioelectronic noses.

1.3.1. Olfactory Tissues and Cells-based Olfactory Biosensors and Bioelectronic Noses

One biomimetic option that has been investigated for developing olfactory biosensors and bioelectronic noses consist in using olfactory tissues and cells as sensing materials [5, 73-79].

P. Wang's group designed a biomimetic cell-based olfactory biosensor by combining different olfactory tissues with an electronic transduction system. In the first example [77] they investigated the use of olfactory neurons and olfactory bulb cells as sensing material. Light-addressable potentiometric sensor (LAPS), a surface potential detector, was used as the transducer. This technique enables the detection of odorant binding by monitoring the extracellular potential of the cells. Olfactory neurons and bulb cells were collected from rat pups and cultivated on the silicon surface of LAPS chip (Fig. 1.3a), which was coated with a mixture of poly-l-ornithine and laminin to improve its biocompatibility and thus promote cell adhesion. The performances of this olfactory biosensor were tested upon the injection of different concentrations of acetic acid. As a result, the system showed a linear dose-response. In addition, the sensitivity of the device to glutamic acid (Glu) (an important neurotransmitter in the olfactory bulb) was also tested. The device was capable to detect Glu at a concentration of 25 μM.

In a second study [78], they coupled olfactory mucosa tissue to a LAPS device (Fig. 1.3b). The olfactory tissue was extracted from Sprague–Dawley rats and the isolated mucosa was deposited on the

sensor surface with cilia receptors side up. To improve the attachment of the tissues, the surface was initially coated with dissolved cellulose nitrate and dried in air. Butanedione and acetic acid solutions were used to stimulate the system. It was shown that the olfactory mucosa tissues conserved their function and the obtained system was sensitive and responded to odorant stimulus. Moreover, when freshly isolated tissue was used, the biosensor could respond to odors for up to at least 2 h on LAPS.

In another example, for developing a cell-based olfactory biosensor, T. H. Park's group used artificial olfactory cells [79]. They expressed the rat olfactory receptor OR I7, combined with a rho-tag import sequence at the N-terminus, on the surface of HEK-293 cells. These cells were attached to the surface of the sensor chip covered with poly-D-lysine. Various odorants including heptanal, octanal, nonanal, decanal, and helional were analyzed. These odorant molecules, initially dissolved in DMSO, were diluted in a running buffer containing Ca^{2+}. To evaluate the performances of the obtained olfactory biosensor, surface plasmon resonance (SPR) was adopted as transduction technique. In this system, the signal detected by the transducer was not linked to the conformational change of the olfactory receptor but was rather due to the influx of calcium induced by the odorant binding event. Since the olfactory receptors are expressed on the surface of cells they are, thus, located several micrometers away from the sensor surface, which is much greater than the detectable SPR evanescent field of ~200 nm (c.f. Fig. 1.4). Therefore, the system cannot detect the conformational change generated by the odorant binding. However, this binding event still triggered the intracellular signal transduction involving the Ca^{2+} ion influx, which caused changes in the intracellular composition. Such changes may generate a variation in the local refractive index leading to an SPR signal. As expected, the system presented a higher affinity to octanal (the specific ligand of OR I7) with a linear response in the range of 10^{-1} to 10^{-4} M. This study showed that it is possible to use SPR for cell-based olfactory biosensors. Most importantly, the authors have underlined and confirmed the role of calcium ions in the signaling process of olfactory receptor. A result that they proved in a previous study [80], where they investigated the cAMP and IP_3 pathways triggered by odorants binding to ORs expressed in heterologous cells (HEK-239).

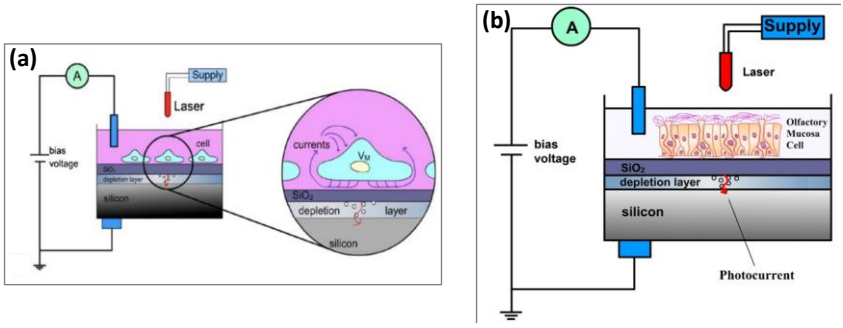


Fig. 1.3. Olfactory cells and tissues-based biosensors (a) [77], (b) [78].

To summarize, the cell-based olfactory biosensors are very interesting. In particular, they allow studying the process of odorant recognition at a single cell level. However, their practical on-field applications are quite limited by the short lifetime of these sensing materials and the strict and complex conditions required for cell culture.

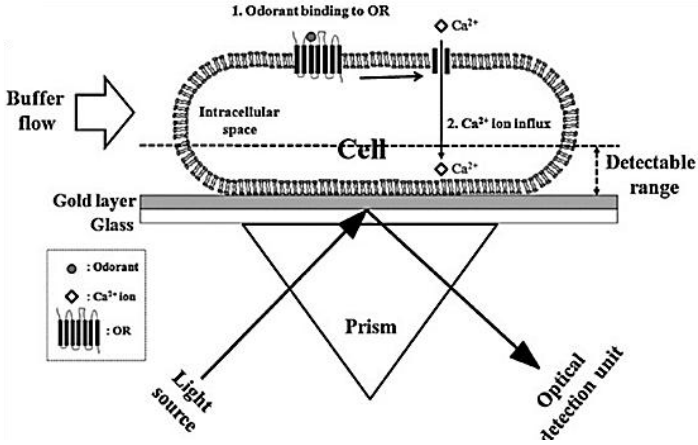


Fig. 1.4. Schematic illustration of a cell-based olfactory biosensor using SPR as transduction system [79].

1.3.2. Olfactory Receptor Based Olfactory Biosensors and Bioelectronic Noses

ORs are quite suitable for the development of olfactory biosensors and bioelectronic noses for several reasons. Firstly, they have high sensitivity and selectivity for odorant molecules. Secondly, their conformational change induced by odorant binding elicits a change in the electrical properties of the protein [39]. Therefore, it is possible to couple them with an appropriate transduction system to obtain a measurable and detectable signal. Thirdly, genetic engineering of proteins enables the addition of tags or other specific sequences to facilitate their purification as well as their immobilization on the sensors. Nevertheless, the exploration and exploitation of ORs for the development of bioelectronic noses is still very challenging from a technical point of view. This is mainly related to their production in large quantities and their stability in suboptimal environmental conditions after being incorporated into bioelectronic noses.

Indeed, the use of OR-based bioelectronic nose for industrial applications requires an efficient and high throughput protein production. During the first studies, the expression of ORs in heterologous cells was found to be very challenging, mainly due to their hydrophobic nature. Gimelbrant *et al.*[81] reported that when expressed in commonly used eukaryotic cell lines the ORs remained trapped in the endoplasmic reticulum or in the Golgi apparatus and could not be translocated into the plasma membrane. Moreover, a study on *C. elegans* [82] showed the importance of membrane associated proteins in targeting the olfactory receptors to the plasma membrane of olfactory neurons cilia. However, in order to investigate the binding affinities and transduction mechanisms of olfactory receptors, several teams succeeded to express those membrane proteins in non-neuronal cells. For this, different strategies were explored. For instance, using the baculovirus expression vector system (BEVS), Ramming *et al*. [26] succeeded to express ORs in surrogate Sf9 insect cells. Krautwurst [21] empirically found that a rhodopsin N-terminal extension can facilitate the traffic of the receptors, to the plasma membrane of human embryonic kidney (HEK-293) cells. Wetzel *et al.* [83] functionally expressed human OR17-40 fused with the membrane import sequence of
5- hydroxytriptamine receptor ($5\text{-}HT_3$) in HEK-293 cells and *Xenopus laevis* oocytes. Moreover, a receptor-transporting protein 1S (RTP1S) that is known to promote the translocation of OR in mammalian cells

has been used to assist and increase the expression of the receptors [84]. Since then, great progress has been made. Today, several strategies exist for the expression of ORs [85], including cell-based expression [86-89], extracts from tissue or cells, cell-free expression and chemical synthesis [7, 90, 91]. Finally, prokaryotic systems such as *Escherichia coli* and yeast cells can also be used.

For the elaboration of olfactory biosensors and bioelectronic nose based on ORs, clearly, the incorporation of ORs into the sensor is another crucial yet tricky task. It can highly affect the sensitivity, stability and reproducibility of the corresponding device. ORs are membrane proteins and as such, depend on a lipid bilayer environment to maintain their structural stability and functionality. Here, we will present state of the art on the development of olfactory biosensors and bioelectronic noses based on ORs. In particular, we will focus on different innovative strategies reported in the literature to maintain their biological activities when immobilized on a sensor.

1.3.2.1. Membrane Fractions

In order to maintain the three-dimensional structure of ORs, the general idea is to keep them in a lipidic environment. The first strategy consists in expressing the ORs in a heterologous system and then breaking the plasma membrane into fractions carrying ORs, also called nanosomes in the literature. In this way, ORs are embedded in natural membranes. Thanks to genetic engineering, it is possible to make expression of ORs with special tags fused at their terminus. Certain tags such as polyhistidine and c-Myc tags may help in immobilizing and/or orientating ORs on the biosensor surface in an appropriate way to make their binding sites accessible for odorant molecules. Alternatively, if a specific antibody against the OR of interest exists, it can also be used for efficient OR immobilization. In the past two decades, various olfactory biosensors based on membrane fractions were developed using different ORs including I7 rat olfactory receptors (ORI7) [92], human olfactory receptor OR1740 [93-96] ODR-10 of *C. elegans* [97-100] and human olfactory receptor 2AG1 (hOR2AG1) [101, 102]. Here we will present some of them as examples.

In close collaboration, N. Jaffrezic's group and E. Pajot-Augy's group [92] succeeded to express rat ORI7 in yeast *Saccharomyces cerevisiae.* They had previously optimized the experimental conditions. When

inducting receptor expression under low temperature at 15 °C, a low expression level was obtained but the expressed ORs were properly addressed to the plasma membrane, as confirmed by Western blotting and immunolocalization methods [103]. Furthermore, they proved that ORI7 conserved their biological activities well in the membrane fractions. Thus, they incorporated such membrane fractions to develop an olfactory biosensor based on electrochemical impedance spectroscopy (EIS). To ensure the appropriate orientation of ORs after immobilization, they first anchored antibodies specific to the OR on the biosensor through self-assembled multilayers. This immobilization strategy was first validated using another GPCR: rhodopsin [104]. In practice, as presented in Fig. 1.5a, first the gold electrode was functionalized with mixed self-assembled monolayers (SAMs) composed of 16-mercaptohexadecanoic acid (MHDA) and 1,2-dioleoyl- *sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) sodium salt (biotinyl-PE). The free space on the surface was blocked with goat antibodies. In the following step, neutravidin was introduced and bound to the biotin moieties on the mixed SAMs. Thanks to its four identical binding sites for biotin, biotinylated polyclonal antibody (Biot-Ab) specific to OR I7 was then immobilized on the gold surface. Finally, the membrane fractions carrying ORs were incorporated into the gold electrode by binding to the specific antibody with controlled orientation. EIS was used to evaluate the sensitivity and selectivity of the obtained olfactory biosensor towards odorants (specific heptanal and octanal, non-specific helional for negative control) in aqueous solution. The hydrophobic odorants were initially dissolved in dimethyl sulfoxide (DMSO) in order to facilitate their dissolution in aqueous solution. The authors have proven that ORs are active after their incorporation into the biosensor thanks to the immobilization strategy. The obtained system showed good sensitivity and selectivity for aldehydes with a linear response for concentrations between 10^{-13} and 10^{-4} M. It was stable during 7 days.

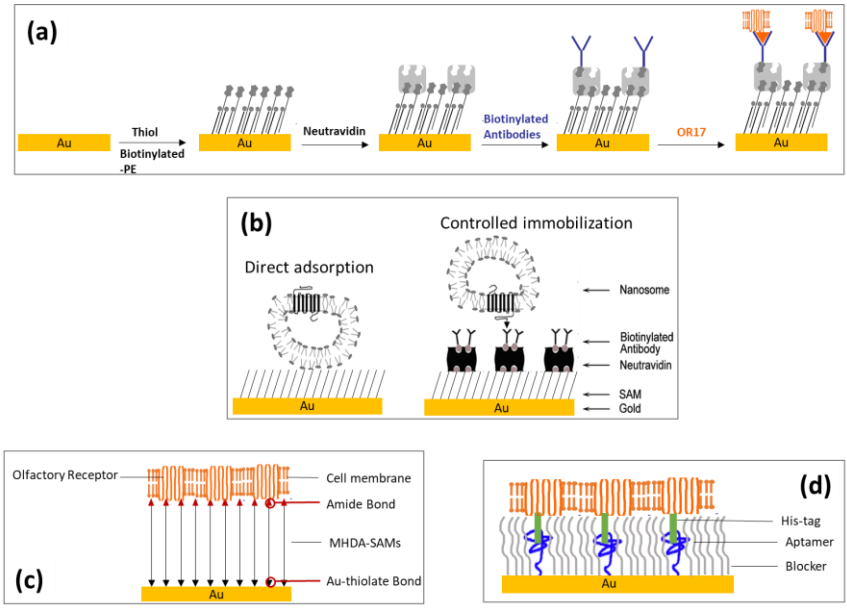


Fig. 1.5. Different strategies developed for the immobilization of membrane fractions carrying ORs: (a) antibody-directed specific immobilization [92], (b) comparison of physical adsorption and antibody-directed specific immobilization [96], (c) covalent bond [99], and (d) aptamer-assisted specific immobilization [100].

In another study [96], these two groups expressed OR1740 with a c-Myc-tag fused at its N-terminus using the same method. The membrane fractions carrying OR1740 had a uniform size with a diameter of ~50 nm. Moreover, in some control experiments, rat OR I7 and doubly tagged OR1740, with a c-Myc tag fused at its N-terminus and a HA-tag at its C-terminus, were also used. Here, a complete study was carried out in order to further optimize the immobilization method presented above. The main objective was to obtain an optimal immobilization procedure, which allowed for a good control over the density and especially the orientation of ORs, with a minimal non-specific interaction between odorant analytes and the underlayers. For this, three distinct SAMs carrying biotinyl groups at different densities were evaluated and compared. SPR was used for real time monitoring of step-by-step surface functionalization as well as for evaluating the performance of the obtained olfactory biosensor. Helional, an odorant

specific to OR1740, was used in solution at a concentration of 5 µM. In this study, the authors clearly showed the importance of immobilization to achieve proper orientation of ORs. They compared the performance of two olfactory biosensors, the first prepared by antibody-directed specific immobilization of ORs and the second by simple physical adsorption of ORs on SAMs (see Fig. 1.5b). The biosensor response was increased up to 50 % for the former. Moreover, OR1740 was functional only when immobilized via a tag attached to its C-terminus, but not via its N-terminus. Finally, the authors demonstrated that the best immobilization method selected in this study was also well adapted for micro- and nanosensor formats.

Other groups used another type of ORs for the development of olfactory biosensors: ODR-10 of *C. elegans* in membrane fractions. A first example is a study led by the group of T. H. Park [97], in which ODR-10 was successfully expressed in *Escherichia coli* but with a low expression level, as confirmed by SDS-PAGE and Western blot. The authors have insisted on the advantages of expressing the OR in bacteria compared to in the mammalian cell system such as human embryonic kidney (HEK-293) cells, which were used in their previous work [105]. Indeed, the former is much easier since it requires neither an import sequence for the expression of ORs on the cell membrane nor the construction of a stable cell line, which is often time consuming and expensive. For the immobilization of the membrane fractions carrying ORs on the biosensor surface, they considered a simple method based on physical adsorption, without precise control over the orientation of ORs on the biosensor surface. For this, they expressed ODR-10 fused with a GST-tag at the N-terminus and a 6×His-tag at the C-terminus. They lysed the bacterial cells by sonication and simply spread the obtained membrane fractions carrying ODR-10 on the gold plate of the quartz crystal. The ODR-10 of *C. elegans* is well characterized with a high affinity for diacetyl (butane-2,3-dione) [106]. In this study, quartz crystal microbalance (QCM) was used as transduction technique to evaluate the sensitivity and specificity of the obtained olfactory biosensor to various odorants, including diacetyl (specific analyte) and hexanal, heptanal, octanal and decanal (all for negative control). The odorant molecules initially dissolved in DMSO were diluted in sterilized water. Then, saturated vapors equilibrated with the odorant solutions were injected into the QCM system. The obtained olfactory biosensor showed good sensitivity and selectivity to the specific ligand (diacetyl) and exhibited a linear behavior in the range between 10^{-12}

and 10^{-5} M. Finally, it is noteworthy that the authors demonstrated that there were non-specific interactions between the odorants and the phospholipid membrane which was also observed by Wu [107].

Three other examples presented below were led by P. Wang's group. To show the importance of the immobilization technique for proper orientation of ORs and its impact on the sensitivity and stability of the biosensor, they employed three different immobilization strategies.

In their first study [98], they expressed the ODR-10 of *C. elegans* on the plasma membrane of human breast cancer MCF-7 cells. A *flag-tag* was fused on its N-terminal for the easy visualization of its expression on the plasma membrane and a *rho-tag* import sequence was inserted to improve the expression level of ODR-10. The efficient expression of the receptors in the MCF-7 cells at the mRNA level was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Besides, fluorescent staining experiments revealed that the ORs were effectively expressed in the cells at high levels and evenly distributed across the plasma membrane. After sonication, the membrane fractions containing ODR-10 were immobilized by physical adsorption on gold layer without precise control over the orientation of ORs. Surface acoustic wave (SAW) was used as transduction technique. The performance of the obtained olfactory biosensor was evaluated using various odorants, including specific diacetyl and non-specific ethanol, butanol, pentanedione, hexanal, and isoamyl acetate. The odorant samples were freshly prepared in Tedlar bags by a liquid organic gas blender and were injected into the detection chamber by a syringe pump. All the experiments were performed under controlled environmental conditions with 10 % of humidity at 25 °C. The obtained olfactory biosensor showed a good sensitivity and selectivity to diacetyl with a dose-dependent response in the concentration range between 10^{-13} and 10^{-7} M.

In their second study [99], they intended to improve the immobilization efficiency for ORs to improve the performance of the olfactory biosensor. Here, ORs in membrane fractions were immobilized on the biosensor surface via covalent bond but without precise control over the orientation of ORs, see Fig. 1.5c. For this, initially, the gold surface was functionalized by the formation of SAMs of 16-mercaptohexadecanoic acid thanks to the strong affinity between gold and thiols. Then, terminal carboxylic groups on SAMs were activated by treatment with

1-ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride/ sulfo-N-hydroxysuccinimide (EDC/NHS). As a result, SAMs with active esters were obtained, which allowed for attaching primary amines of ODR-10 via the amide bonds. Similar to their previous study, SAW was used as transduction technique. Three odorants having similar structures, including diacetyl, butanone and 2,3-pentanedione, were used to test the performance of the obtained olfactory biosensor. Odorants were prepared in DMSO at 0.5 M. Saturated vapor of odorants were used as stimuli after they were diluted to the desire concentrations with nitrogen by a liquid organic gas blender. The olfactory biosensor showed good sensitivity and selectivity to the specific analyte (diacetyl) with a linear response in the same concentration range as obtained in their first study. In addition, compared to the olfactory biosensor developed in their first study, the sensitivity of the system was two times higher and a much lower detection limit was obtained. The stability was also greatly improved from 2 days to 7 days (stored at 4 °C).

In their third study [100], to further improve the performance of the olfactory biosensors, they developed an original aptamer-assisted specific immobilization technique with better control over the orientation of ORs (c.f. Fig. 1.5d). Here, ODR-10 was expressed heterologously in HEK-293 cells with a 6×His-tag on the N terminus. Western blot and RT-PCR confirmed the expression of His_6-tagged ODR-10 at the protein level and mRNA level, respectively. For the immobilization, initially, the gold surface was functionalized with mixed SAMs composed of thiol-modified anti-His_6 aptamers and 11-mercaptoundecanoic acid. The second thiol had dual functions of blocking free sites on the gold surface and reducing steric hindrance. Then, the membrane fractions carrying the His_6-tagged ODR-10 were added. As a result, ODR-10 was captured specifically by the anti-His_6 aptamers with precise control over its orientation. QCM was used as the transduction technique. Moreover, in this study, physical adsorption method was also used to illustrate the influences of immobilization methods on the performances of OR-based biosensors. To test the sensitivity and selectivity of the obtained olfactory biosensor, various odorants were analyzed, including specific diacetyl and some other non-specific odorants such as isoamyl acetate, anisole, lavender, butanone, and 2,3-pentanedione. The odorants were freshly prepared in Tedlar bags by a liquid organic gas blender and then injected into the analysis chamber at a constant flow rate using a syringe pump. The olfactory biosensor prepared by aptamer-assisted specific

immobilization showed improved sensitivity and selectivity, compared to that prepared by physical adsorption. Its detection limit was as low as 1.5 ppm (v/v). Moreover, it had good stability for 7 days, in contrast to 2 days for the biosensor prepared by physical adsorption.

In Table 1.1, we list all the presented olfactory biosensors based on membrane fractions carrying ORs for comparison.

1.3.2.2. Nanovesicles

Another strategy developed to provide a natural lipidic environment to maintain the stability and biological function of ORs consists in using nanovesicles (NVs). They are spherical structures that encompass a lipid bilayer displaying membrane proteins and encapsulating cytoplasmic compounds. NVs are usually secreted by a cell via a process called exocytosis. Thanks to their ability to entrap molecules, nanovesicles are very attractive candidates for drug delivery and nanomedicine
[108, 109]. Another potential application involves using such structures as a miniature cell-like prototype to study cellular signaling process [110]. Through which, Pick *et al.* [110] showed that nanovesicles are able to perform the same signaling process as the "mother" cell. Furthermore, nanovesicles are also very attractive as biomaterials for the development of olfactory biosensors and bioelectronic noses [84, 111-113]. Firstly, they retain the original, natural environment of ORs. Secondly, they can be prepared in large quantities and can be frozen and stored for many weeks without the loss of biological activity. Thirdly, they can serve as miniaturized artificial cells to replace natural ones since their nanometric size are more suitable for their incorporation into nanoscale olfactory biosensors.

Table 1.1. Summary of the olfactory biosensors based on membrane fractions carrying ORs.

Reference	**Hou *et al.* [92]**	**Vidic *et al.* [96]**	**Sung *et al.* [97]**	**Wu *et al.* [98]**	**Wu *et al.* [99]**	**Du *et al.* [100]**
Olfactory receptor	Rat OR I7	Human OR1740	C. elegans ODR-10	C. elegans ODR-10	C. elegans ODR-10	C. elegans ODR-10
Expression system	Saccharomyces cerevisiae yeast cell	Saccharomyces cerevisiae yeast cell	E. coli	Human breast cancer cells MCF-7	Human breast cancer cells MCF-7	Human embryonic kidney cells HEK-293
Structure stabilization	Membrane fractions	Nanosome	Membrane fractions	Membrane fractions	Membrane fractions	Membrane fractions
Immobiliza-tion	Antibody-directed specific immobilization	Antibody-directed specific immobilization	Physical adsorption	Physical adsorption	Covalent binding	Aptamer-assisted specific immobilization
Transduc-tion technique	EIS	SPR	QCM	SAW	SAW	QCM
Odorants tested	Specific: Octanal Heptanal Control: Helional	Specific: Helional Control: Octanal	Specific: Diacetyl Control: Hexanal Heptanal Octanal Decanal	Specific: Diacetyl Control: Ethanol Butanol Pentane-dione Hexanal Isoamyl acetate	Butanone 2,3-pentane-dione	Specific: Diacetyl Control: Butanone Pentane-dione Isoamyl acetate Anisole Lavender
Analysis milieu	Aqueous solution	Aqueous solution	Vapor	Vapor	Vapor	Vapor
Detection limit	10^{-12} M	–	10^{-12} M	10^{-13} M	1.2 10^{-14} M	1.5 ppm
Long term stability	7 days	–	–	2 days	7 days	7 days

Finally, they can be used for signal amplification thanks to the conserved cell signaling pathways. Below we present some interesting examples of nanovesicle-based olfactory biosensors.

T. H. Park's group has greatly contributed to the development of this type of olfactory biosensors. In the first example [112], they expressed the human olfactory receptor 2AG1 (hOR2AG1) fused with a *flag tag* and a *rho-tag* import sequence in HEK-293 (Fig. 1.6a). After transfecting the cells with the expression vector, the production of nanovesicles containing hOR2AG1 was induced by a cytochalasin B treatment under agitation (Fig. 1.6b). The nanovesicles were separated from the cells and collected via multiple centrifugations. The expression of hOR2AG1 in both cells and nanovesicles was confirmed by Western blot. According to the analysis by field emission scanning electron microscope (FE-SEM), the obtained nanovesicles had a well-defined sphere-like shape with a uniform diameter of ~200 nm. To assess the activity of the nanovesicles carrying hOR2AG1, a Ca^{2+} signaling assay was carried out using the fluorescent Ca^{2+} indicator. It was found that such nanovesicles retained most of the activities for cellular signal transduction (Fig. 1.6c). Then, they were combined with a single-walled carbon nanotube-based field effect transistor (swCNT-FET), see Fig. 1.6d. For their immobilization, nanovesicles were attached to the surface of carbon nanotube covered with poly-D-lysine by electrostatic interactions. To evaluate the performance of the obtained olfactory biosensor, specific odorant amyl butyrate and some non-specific odorants with similar structures such as propyl butyrate, pentyl valerate, and butyl butyrate were used in aqueous solution. Thanks to the advantages of nanovesicles with cell signaling pathways for signal amplification, the obtained system exhibited a human-like selectivity with single-carbon resolution and a very high sensitivity with a detection limit of 1 fM. However, unlike the olfactory neurons, nanovesicles lack the restoration functionality. Indeed, in live cells Ca^{2+} pumps enable the restoration of the intracellular ion level at the end of the interaction. Therefore, this absence of restoration could highly affect the reusability of the system and make it less suitable for high throughput measurements.

In the second example, this group explored the application of this kind of system for medical diagnostics [84]. The idea was to make lung cancer diagnosis based on the detection of a VOC biomarker such as heptanal. For this study, an important effort was made on the selection

of ORs that have a high sensitivity and specificity to this VOC molecule by building a library of HEK-293 cells expressing 30 types of human ORs. Among the tested ORs, hOR1J2, having not only good sensitivity but also very good selectivity for heptanal, was selected and used for olfactory biosensor development. The nanovesicles carrying hOR1J2 were produced by transfecting HEK-293 cells using cytochalasin B.

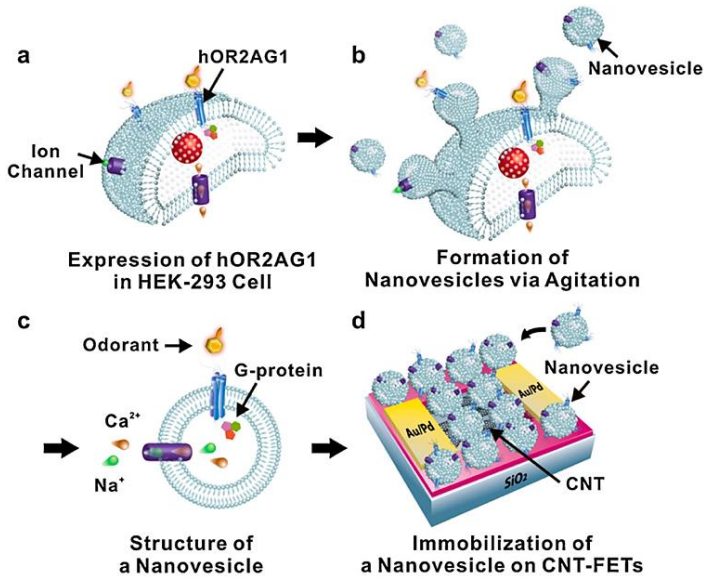


Fig. 1.6. Schematic illustration for the preparation of nanovesicles carrying ORs and their combination with a carbon nanotube-based FET transducer to build an olfactory biosensor [112].

To increase the expression of ORs, the receptor-transporting protein 1S (RTP1S) was also expressed in the cells since it was known to facilitate the traffic of the ORs in mammalian cells [114]. Moreover, the cells were also transfected with the $G\alpha_{olf}$ gene to enhance the intracellular signaling process. Afterwards, nanovesicles were immobilized on swCNT-FET covered with poly-D-lysine by electrostatic interactions. Initially, the efficiency of the obtained olfactory biosensor for heptanal detection was validated in standard buffer solution. Then, to verify its capability for practical diagnosis, the detection of heptanal contained in human blood plasma was examined. It was proven that the system was

able to selectively detect heptanal at a concentration as low as 1×10^{-14} M, a sufficient level to distinguish the blood of a lung cancer patient from the blood of a healthy person.

Besides nanovesicles with natural lipid bilayer, Khadka *et al.* [113] reconstituted insect odorant receptors into artificial lipid bilayers such as liposomes for the development of an ultrasensitive olfactory biosensor. In their previous works [115], the production of such liposomes was optimized. Therein, three odorant receptors OR10a, OR22a, and OR71a from fruit fly *Drosophila melanogaster* were recombinantly expressed, purified and integrated into liposomes (100-200 nm). Then, the liposomes were covalently attached to SAMs-modified gold surface, as illustrated in Fig. 1.7. Both quartz crystal microbalance with dissipation (QCM-D) and EIS were used as transduction system. To evaluate the performances of the obtained olfactory biosensors, various odorants were tested, including methyl salicylate (specific odorant of OR10a), methyl hexanoate (specific odorant of OR22a), 4-ethyl guaiacol (specific odorant of OR71a), and E2-hexanal as negative control. Odorant solutions were prepared in phosphate buffer solution (PBS) containing 1 % DMSO for better solubility. The authors showed that the olfactory biosensors based on EIS were much more sensitive. These biosensors were able to sensitively and selectively detect their corresponding specific odorant at femtomolar concentrations. Nevertheless, liposomes are large, unstable, and difficult to prepare with precisely controlled size and stoichiometry. To overcome it, novel technology based on nanodiscs has emerged.

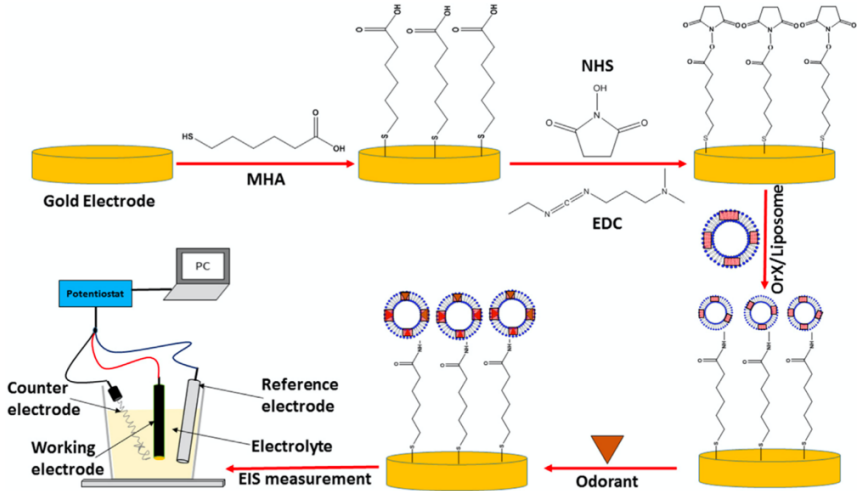


Fig. 1.7. Schematic description of covalent immobilization of liposomes onto gold electrodes [113].

In Table 1.2, we list all the presented olfactory biosensors based on nanovesicles carrying ORs for comparison.

Table 1.2. Summary of the olfactory biosensors based on nanovesicles carrying ORs.

Reference	**Jin et al. [112]**	**Lim et al. [84]**	**Khadka et al. [113]**
Olfactory receptor	Human OR2AG1	Human OR1J2	OR10a, OR22a and OR71a from Drosophila melanogaster
Expression system	Human embryonic kidney cells HEK-293	Human embryonic kidney cells HEK-293	Sf9 insect cells
Structure stabilization	Nanovesicles	Nanovesicles	Liposomes
Immobilization	Electrostatic interaction	Electrostatic interaction	Covalent binding
Transduction technique	CNT-FET	CNT-FET	QCM-D
			EIS

Odorants tested	Specific: Amyl butyrate Control: Propyl butyrate Pentyl valerate Butyl butyrate	Specific: Heptanal Control: Hexanal Octanal Nonanal	Specific: Methyl salicylate (OR10a) Methyl hexanoate (OR22a) 4-ethylguaiacol (OR71a) Control: Methyl salicylate (OR22a) Methyl hexanoate (OR10a) E2 hexenal (OR71a)
Analysis milieu	Aqueous solution containing Ca^{2+}	Aqueous solution containing Ca^{2+}	Aqueous solution
Detection limit	10^{-15} M	10^{-14} M	10^{-12} M (OR10a) 10^{-15} M (OR22a) 10^{-16} M (OR71a)

1.3.2.3. Nanodiscs

Nanodiscs are soluble artificial self-assembling phospholipid bilayer containing transmembrane proteins. They are able to maintain the native membrane environment of olfactory receptors. Recently, this technology has become an important tool to functionally reconstitute membrane proteins. To do so, the membrane protein is solubilized transiently with a detergent in the presence of phospholipids and an encircling amphipathic helical protein belt such as a membrane scaffold protein (MSP). Upon removal of the detergent, typically via adsorption to hydrophobic beads, the target membrane protein simultaneously assembles with phospholipids into a discoidal bilayer. Nanodiscs have several advantages. Firstly, ORs embedded in nanodiscs are stable since they are in a native membrane environment. Secondly, the encircling MSP belt renders the entire assembly soluble. Finally, it is possible to control the size of nanodiscs depending on MSP length. For example, the diameter of bare nanodiscs can be tuned between 9.5 to 17 nm. Therefore, nanodiscs are very promising for the development of olfactory biosensors and bioelectronic noses, [116-119] as illustrated by three examples in the following part.

In a first example, A. T. C. Johnson's group combined ORs-embedded nanodiscs with CNT-FET to develop olfactory biosensors [116]. In this study, three recombinant mouse olfactory receptors mOR174-9, mOR203-1 and mOR256-17 were expressed in Sf9 insect cells with an

N-terminal His-tag to simplify the purification and to ensure their oriented immobilization on the Ni-coated CNTs. Moreover, the ORs were prepared in two distinct nanoscale constructs: digitonin micelles and nanodiscs for comparison. Then, they were immobilized via a polyhistidine tag onto Ni-coated CNTs as described previously, which was efficient in controlling the orientation of the ORs (Fig. 1.8a). To test the performances of the obtained olfactory biosensors, eight odorants were used: eugenol, 2-heptanone, heptanal, acetophenone, dinitrotoluene, n-amyl acetate, methyl benzoate, and cyclohexanone. For odorant sampling, a system with three flows (humidity, sampling, background) was used, allowing the control of humidity and odorant concentrations in vapor. The results showed that nanodiscs carrying ORs were extremely stable in solution. In contrast, the digitonin micelles aggregated rapidly and thus needed to be used immediately after production. Moreover, after fabrication, nanodiscs based devices were much more stable with reproducible responses for longer than one month, while micelle-based devices remained active with stable responses for only ~5 days. Finally, the obtained olfactory biosensors had good sensitivity and selectivity with rapid responses and full recovery to baseline on seconds time scales.

Murugathas *et al.* developed olfactory biosensors based on insect ORs-embedded nanodiscs and CNT-FET with very high sensitivity [117]. In their study, four olfactory receptors from *Drosophila melanogaster*: OR10a, OR22a, OR35a, and OR71a were recombinantly expressed using a baculovirus-mediated Sf9 insect cells. Then, they were incorporated into nanodiscs. For their immobilization on CNTs, first, the 1-pyrenebutanoic acid succinimidyl ester (PBASE) molecules were attached noncovalently onto the CNT sidewalls via π–π interaction. Then, the amine groups on ORs were anchored onto the NHS ester group on the PBASE molecule via a nucleophilic substitution reaction, see Fig. 1.8b. It is important to note that this immobilization strategy cannot control the orientation of the ORs. To test the performances of these olfactory biosensors, four odorants including methyl salicylate, methyl hexanoate, trans-2-hexen-1-al and 4-ethylguaiacol were used. They were dissolved in PBS with 1 % DMSO to obtain concentrations ranging between 1 fM to 10 pM. It was demonstrated that the four olfactory biosensors selectively responded to their respective positive odorant with limits of detection in the low femtomolar range.

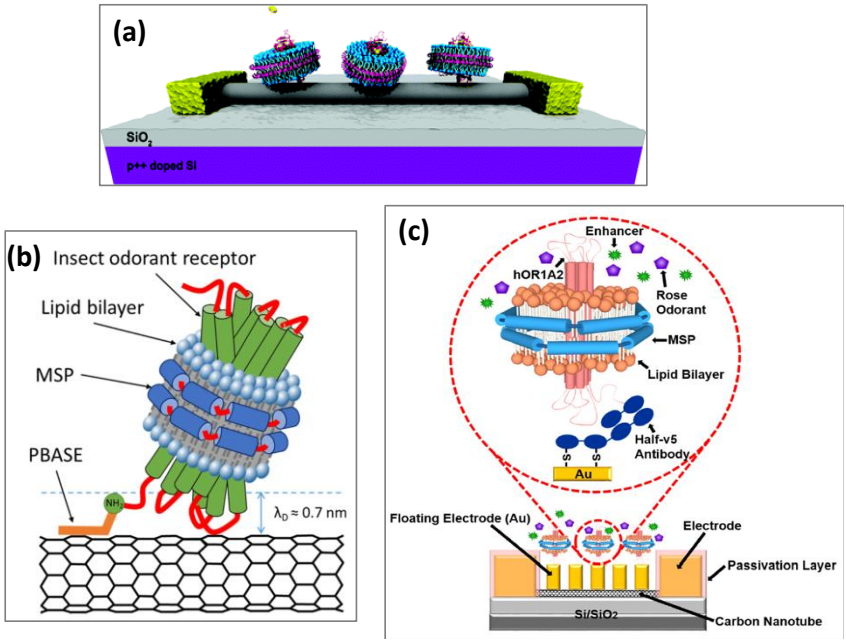


Fig. 1.8. Schematic illustration of nanodiscs carrying ORs immobilized on CNT [116-118].

T. H. Park's group developed an olfactory biosensor capable of quantitatively assessing rose scent components in real samples such as rose oil [118]. Here, human olfactory receptor 1A2 (hOR1A2) was overexpressed in *E. Coli* and purified via affinity chromatography. Then, the purified hOR1A2 were embedded into nanodiscs (15-20 nm) and immobilized on a floating gold electrode-based CNT-FET. For the immobilization, first, a cysteine monolayer was formed on the gold electrode surface using N-acetyl-L-cysteine, on which half-v5 antibody (Ab) fragments were anchored via disulfide bonding. Subsequently, the hOR1A2 was incorporated into the system which specifically bound to the antibody, as shown in Fig. 1.8c. To assess the performance of the olfactory biosensor, specific odorants geraniol and citronellol and non-specific odorants trimethylamine and amyl butyrate were used, additionally, with benzyl salicylate used as an enhancer. These odorants and rose oil solutions were prepared in HEPES buffer solution. The obtained system was able to quantitatively recognize geraniol and citronellol down to 1 fM and 10 fM, respectively, and with very good selectivity. Interestingly, for the first time, this work demonstrated that

the sensitivity of olfactory biosensors can be greatly improved in the presence of an enhancer like benzyl salicylate. Where, ORs in the presence of 1 nM benzyl salicylate responded to a rose scent with ~10^3 times lower concentrations. Finally, it was also proven that the olfactory biosensor could recognize geraniol in complex environments such as rose oil.

Furthermore, based on the same principle, T. H. Park's group also developed olfactory biosensors by using trace-amine-associated receptors (TAARs) [119, 120]. TAARs belong to the G-protein coupled receptor family which were identified in many animal species (mouse, rat, humans) and were attributed to different functions associated with binding trace amines. Since amines such as diamines (putrescine and cadaverine) are often responsible for bad odors i.e. coming from bacterial decomposition of proteins, olfactory biosensors and bioelectronic noses incorporating TAARs may be promising for assessing food quality and olfactory pollution, etc.

Finally, with all these progresses made on olfactory biosensors, it becomes possible to design multiplexed system for the development of bioelectronic noses. For instance, T. H. Park's group initiated studies in this direction [102, 121]. Moving even further, they reported a portable and multiplexed bioelectronic sensor that combines human olfactory and taste receptor with a multichannel CNT-FET for food freshness assessment (Fig. 1.9) [121].

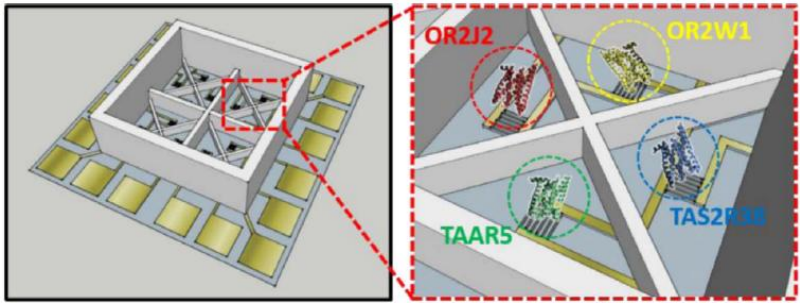


Fig. 1.9. Schematic illustration of the bioelectronic sensor that combined human olfactory and taste receptor with a multichannel carbon nanotube FET [121].

For this, three olfactory receptors (human OR2J2, OR2W1 and trace amine-associated receptor TAAR5) and one taste receptor (TAS2R38) were produced in *Escherichia coli*. A 6×His-tag was fused to the C-terminus of the receptor proteins for purification and oriented immobilization on the Ni-coated CNTs. Notably, these receptor proteins were reconstituted using a detergent micelle method to maintain their original structure. To immobilize the receptor proteins, initially, CNTs were incubated in 4-carboxybenzene diazonium tetrafluoroborate solution. Subsequently, the carboxylic acid of the 4-carboxybenzene was activated by EDC/sulfo-NHS in an appropriate buffer. Then, CNTs were immersed in Nα,Nα-bis(carboxymethyl)-L-lysine hydrate (NTA-NH_2) solution. Afterwards, they were washed with deionized water and immersed in a solution of $NiCl_2$ for coating CNTs with Ni^{2+}. Finally, thanks to the 6×His-tag fused to the C-terminus, olfactory receptors and taste receptor were immobilized on the Ni-coated CNTs with controlled orientation via the formation of coordination bonds. In this study, a customized portable and multiplexed bioelectronic sensor was constructed for the analysis of odorant and taste molecules, including octanol (specific ligand of OR2J2 and OR2W1), hexanal (specific ligand of OR2W1), trimethylamine (specific ligand of TAAR5), and goitrin (specific ligand of TAS2R38). They are known indicators of food contamination. It was demonstrated that the obtained device selectively distinguished mixtures of these molecules as well as individual odor and taste molecules with high sensitivity. Thus, such a system was suitable for efficient monitoring of food freshness as well as for other applications requiring on-site analysis.

In Table 1.3, we list all the presented olfactory biosensors based on nanodiscs carrying ORs for comparison.

Table 1.3. Summary of the olfactory biosensors based on nanodiscs carrying ORs.

Reference	**Goldsmith *et al.* [116]**	**Murugathas *et al.* [117]**	**Lee *et al.* [118]**	**Son *et al.* [121]**
Olfactory receptor	Mouse OR174-9, OR203-1 and OR256-17	OR10a, OR22a, OR35a, and OR71a from *Drosophila melanogaster*	human olfactory receptor 1A2	(human OR2J2, OR2W1 and trace amine-associated receptor TAAR5 TAS2R38

Expression system	Sf9 insect cells	Sf9 insect cells	*E. coli*	*E. coli*
Structure stabilization	Digitonin micelles and Nanodiscs	Nanodiscs	Nanodiscs	Micelle
Immobiliza-tion	Covalent binding	Non covalent interactions using PBASE	Antibody-directed specific immobilization	Covalent binding
Transduction technique	CNT-FET	CNT-FET	CNT-FET	CNT-FET
Odorants tested	Eugenol 2-heptanone Heptanal Acetophenone Dinitrotoluene N-amyl acetate Methyl benzoate Cyclohexanone	Methyl salicylate Methyl hexanoate Trans-2-hexen-1-al 4-ethylguaiacol	Geraniol Citronellol Trimethylamine Amyl butyrate Rose oil	Octanol Hexanal Trimethylami ne Goitrin
Analysis milieu	Vapor	Aqueous solution	Aqueous solution	Aqueous solution
Detection limit	~7 ppb for dinitrotoluene	1 fM	1 fM	pM range
Long term stability	> 1 month (in humid environment)	Not mentioned	Not mentioned	~7 days

To summarize, all these examples have proven that ORs are very promising sensing materials for the development of olfactory biosensors and bioelectronic noses. In particular, their use makes it possible to attain a high sensitivity and selectivity, even at the concentration level occurring in the human nose. Today, their production in large quantities are possible based on various expression methods. By keeping ORs in a lipidic environment using membrane fractions, nanovesicles or nanodics, their biological activities are conserved with good stability even in suboptimal environmental conditions after being incorporated into bioelectronic noses. With ingenious design of immobilization technique to control the OR orientation on the biosensor surface, the performances of the olfactory biosensors in terms of sensitivity and stability can be highly improved. Nevertheless, the wide application of this type of device remains limited by their short lifespan and their poor repeatability.

1.3.3. Odorant Binding Proteins Based Olfactory Biosensors and Bioelectronic Noses

Other attractive biomimetic receptors for olfactory biosensors are the odorant binding proteins. These relatively small proteins are suitable elements for several reasons. Firstly, they present a remarkable stability to high temperature and pH variations, and have low susceptibility to proteolytic degradation [5, 58]. Secondly, as mentioned previously, OBPs have broad specificity and can reversibly bind a large spectrum of odorants with a micromolar range dissociation constant. Thirdly, their hydrophilic nature considerably facilitates their expression, purification and thus allows a large-scale production in bacterial systems. Fourthly, site-directed mutagenesis enables to tune the binding properties of OBPs and the introduction of special tags to control their immobilization on biosensors and bioelectronic noses [122, 123]. Finally, it is possible to couple these proteins to an electrical, optical or acoustic transduction technique to build olfactory biosensors and bioelectronic noses. In the last decade, different groups from around the world have contributed to their development [122-136]. Nevertheless, despite the great stability of OBPs, the most challenging task is to maintain their three-dimensional structure and biological activity over time when immobilized onto the biosensor and exposed to odorants. Indeed, being naturally present in an aqueous environment (the olfactory mucus), the humidity level of the working milieu is a very important factor. Therefore, many OBP-based biosensors were designed to analyze and detect odorants in the liquid phase and were proven to be highly sensitive. Moreover, several studies showed that OBP-based biosensors were also efficient in detecting odorants in the gas phase. In this part, we will present the development of diverse olfactory biosensors and bioelectronic noses based on OBPs for the analysis of odorants in both aqueous and gaseous phase.

1.3.3.1. Odorant Analysis in the Liquid Phase

Thanks to the good solubility and stability of OBPs, they can be used directly without the lipidic environment. Q. Liu's group developed several OBP-based olfactory biosensors using different transduction systems such as EIS [125, 126] and localized surface plasmon resonance (LSPR) [127]. In one of these studies [126], they used a human odorant binding protein to develop an olfactory biosensor with potential applications for medical diagnosis. Recombinant hOBP was

expressed with a polyhistidine tag at the C-terminus in the host human cells. Then, the cell extracts were analyzed and the expression of the proteins was confirmed by SDS-PAGE. The obtained hOBPs were immobilized on aluminum oxide surface of nanopores, as schematically illustrated in Fig. 1.10.

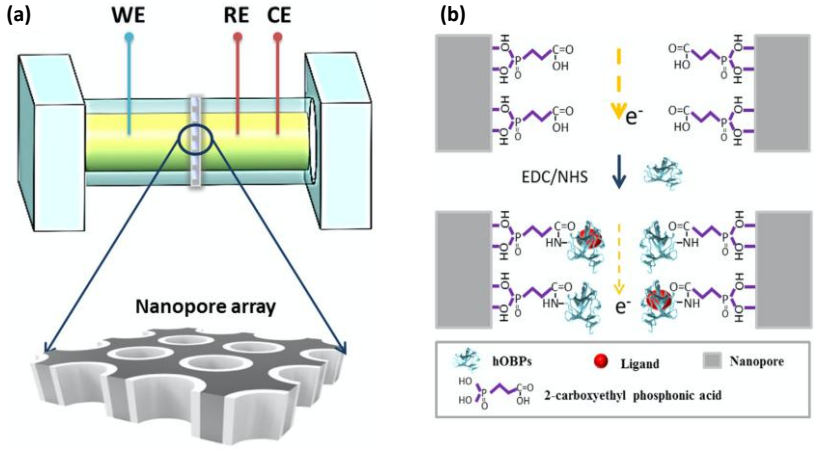


Fig. 1.10. Olfactory biosensor based on nanopore array functionalized with hOBP [126] (a) EIS experimental setup (WE: working electrode; RE: reference electrode; CE: counter electrode) (b) hOBP immobilization process.

First, the nanopore surface was functionalized with 2-carboxyethyl phosphonic acid by the formation of SAMs. Then, carboxylic groups were activated by EDC/NHS, which generated active esters that allowed for the anchoring of the primary amines of hOBPs via the formation of amide bonds. In this study, EIS was used as the transduction technique. Benzaldehyde and two fatty acids: lauric acid and docosahexaenoic acid, considered as potential biomarkers for cancers and other serious diseases, were chosen as targets to test the efficiency of the device. The three compounds were dissolved in methanol at different concentrations ranging between 10^{-8} and 10^{-4} mg/mL. The obtained system showed dose-response for the three odorants with good sensitivity.

For the incorporation of OBPs into the biosensors, an efficient immobilization is crucial and can highly affect the sensitivity of the

device. To highlight this, Manai *et al.* [124] compared the performances of OBP-based olfactory biosensors functionalized by two different chemical grafting strategies. In this study, a wild type OBP from pig (wtOBPpig) and its mutant with 6×His-tag at the N-terminus (m6hisOBPpig) were used as sensing material. They were attached to the surface of poly-crystalline diamond micro-cantilevers used as signal transducers. In the first immobilization method (Fig. 1.11a), hexanoic acid was covalently attached to hydrogen-terminated diamond surfaces then the carboxylic groups were activated by EDC/NHS, to allow the anchoring of wtOBPspig via amide bonds. In the second method (Fig. 1.11b), the hydrogenated diamond surface was first functionalized with Nα,Nα-Bis-(carboxy- methyl)-L-lysine hydrate (NTA), followed by the addition of nickel(II) chloride hexahydrate, which led to the formation of Ni-NTA complex. This complex showed a high affinity with 6×His-tag at the N-terminus of the mutant m6hisOBPpig. The first immobilization strategy resulted in random orientation of the proteins on the sensor surface whereas the latter approach enabled control over the orientation of the proteins. The sensing performances of the obtained olfactory biosensors based on diamond microelectromechanical systems (MEMS) were evaluated and compared for the analysis of 2,4-dinitrotoluene (2,4-DNT) and 2-isobutyl-3-methoxypyrazine (IBMP). The odorants were prepared in aqueous buffer solution. The olfactory biosensor based on the second immobilization method showed 20 % higher sensitivity for the two odorants. This could be explained by a greater immobilization yield and better odorants accessibility to the proteins binding sites. Furthermore, a better repeatability of the sensor response was also observed. As previously mentioned, with the first immobilization method, OBPs were randomly oriented on the sensor surface. Thereby, resulting in the number of available active sites varying from one biosensor to another. In contrast, with the second immobilization method, all the receptors were well oriented, thus, providing a similar number of binding sites for a more reproducible sensors response.

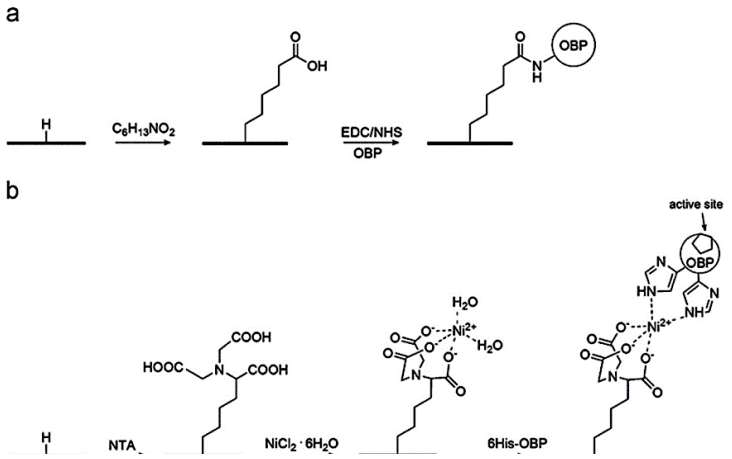


Fig. 1.11. Two strategies for OBP immobilization on diamond surface (a) with random orientation (b) with controlled orientation [124].

As mentioned before, it is possible to tune binding properties of OBPs based on site-directed mutagenesis. In such a way, a set of OBPs with differential affinity to target odorants can be designed and produced, which paves the way for the development of multiplexed bioelectronic noses [122, 123]. Recently, our team carried out a preliminary proof-of-concept study on the development of an OBP-based bioelectronic nose [122]. Three derivatives of the third rat odorant binding protein (OBP3) were used as sensing material, including a "wild type" protein (OBP3-w), and two modified proteins named OBP3-a and OBP3-c. The binding properties of these proteins were customized by modifying the amino acid sequence of their binding pockets. A lysyl residue was introduced into the OBP3-a binding pocket to improve the affinity of the protein for aldehydes. For OBP3-c derivative, bulky amino acids were introduced in its binding pocket. Consequently, resulting in the binding site being cluttered and rendering the receptor incapable of binding with any odorants. Thus, it was used as the negative control. The three recombinant His-tagged proteins were expressed in *Escherichia coli* then purified using immobilized metal affinity chromatography (IMAC). Moreover, a cysteine was added at the N-terminus of the OBPs to enable their immobilization by direct self-assembly on the gold surface of a prism. Importantly, the N-terminus was located on the opposite side of the binding pocket entrance, ensuring the promotion of correct protein orientation by anchoring at this point.

In this study, great effort was made to optimize the density and orientation of OBPs grafted on the microarray since it may influence the binding properties of the proteins as well as the signal intensity of the obtained bioelectronic nose. Indeed, in an ideal case, the proteins would form a well-organized monolayer with their binding pockets pointing upwards to facilitate the access of odorants. If the OBP layer on the biosensor was not dense enough to ensure steric and electrostatic repulsion between the proteins, this will result in a random orientation of the proteins with less accessible binding sites. On the contrary, if the density of OBPs was too high, the formation of additional disordered layers could be favored. Resulting in the proteins on the top layer to have random orientation which may block access to the binding sites of the proteins in the underlayer. Moreover, it would be difficult to prepare reproducible biosensors under such conditions.

Surface plasmon resonance imaging (SPRi) was used to detect odorant binding. SPRi is a real-time, highly sensitive, label-free, and noninvasive optical transduction technique. It is particularly interesting for the development of bioelectronic noses. Thanks to the imaging mode, the interactions between the analytes and multiplexed biosensors can be simultaneously monitored with the access to real-time kinetic information. The performance of the obtained bioelectronic nose was investigated by the analysis of three odorant molecules: β-ionone, hexanal, and hexanoic acid. β-ionone can bind to both OBP-w and OBP-a with a higher affinity for OBP-w. Hexanal strongly binds to OBP-a. Hexanoic acid was used as a negative control. The odorant samples were prepared by diluting pure products in filtered running buffer. The obtained bioelectronic nose exhibited a detection limit of 200 pM for β-ionone that is among the lowest values reported in literature. Moreover, very remarkably, the system was able to detect odorants having a molecular weight of 100 g/mol (hexanal). This value is lower than the limit of detection in mass commonly admitted for commercial SPRi systems (200 g/mol) without amplification. This result suggested that the SPR signal was not solely due to the binding of odorants but was further amplified by the potential conformational change of the protein which resulted from the binding event. Moreover, the distinct binding properties of the OBP derivatives enabled very high selectivity particularly at low concentrations of odorants. Finally, with appropriate regeneration procedures, the biosensor showed a good measurement-to-measurement and chip-to-chip repeatability, as well as good stability with a lifespan of up to nearly two months.

1.3.3.2. Odorant Analysis in the Gas Phase

Some other groups have challenged the development of OBP-based olfactory biosensors and bioelectronic nose for the analysis of odorants in gas phase [128-134]. Di Pietrantonio and collaborators [128-130] published several studies on such bioelectronic noses based on SAW biosensor array. In one of these studies [128], they used three different OBPs: wild-type OBP from cow (wtbOBP), a double mutant of the cow OBP (dmbOBP) and a wild-type pig OBP (wtpOBP). The DNA sequence of cow OBPs was combined with a 6xHis-tag, expressed in *E. coli*, then purified using Ni-NTA coated agarose beads and followed by a second chromatography step on fast performance liquid chromatography (FPLC). Pig OBPs were extracted and purified from fresh nasal tissues of the animal. To immobilize the sensing materials, the electrodes of the sensor were coated with a thin gold film to allow the formation of self-assembled monolayer thanks to the high affinity between the gold and the thiol group of the proteins. In this study, each of the three OBPs was deposited on a resonator by a simple droplet deposition method. The response of the bioelectronic nose was monitored upon the injection of R-(–)-1-Octen-3-ol (13-61 ppm) and R-(–)-carvone (9-80 ppm) at different concentrations carried by N_2 flow. It was demonstrated that the three OBPs preserved their full functionality when exposed to the air environment. The obtained bioelectronic nose showed good selectivity and sensitivity with the capacity of detecting low concentrations of octenol and carvone. Despite being fast and simple, the conventional droplet deposition technique did not allow a good reproducibility of surface densities of the OBP coating. Therefore, it is not suitable for large-scale sensor production. For this purpose, Di Pietrantonio and collaborators investigated more sophisticated but precise deposition methods such as matrix assisted pulsed laser evaporation and laser-induced forward transfer [129, 130]. They showed that these new deposition processes did not affect the activity of OBPs and the fabricated olfactory biosensors showed similar performances.

In a second example, Gao *et al.* [132] developed a bioelectronic nose for efficient human body odor detection to meet the needs of many related fields (disease diagnosis, cosmetic, forensic science, etc.). For this, OBPs from the anthropophilic mosquito, *Anopheles gambiae* (AgOBPs) were used. A poly-histidine tag was fused to the N-terminal of three different AgOBPs: AgOBP5, AgOBP6 and AgOBP7 and the receptors were expressed in *E. coli*. After purification, AgOBPs were

immobilized on the surface of CMOS-compatible silicon nanowires (SiNW) array using 3-Aminopropyltriethoxysilane (APTES) and cross-linker glutaraldehyde. As presented in Fig. 1.12, the SiNW surface, possessing hydroxyl groups, was first functionalized with SAMs of APTES possessing primary amino groups. Then, the cross-linker glutaraldehyde was used to covalently anchor AgOBPs onto the surface of SiNW. This functionalization technique is commonly used for protein immobilization in biosensor development [137]. However, it does not allow to control the protein orientation.

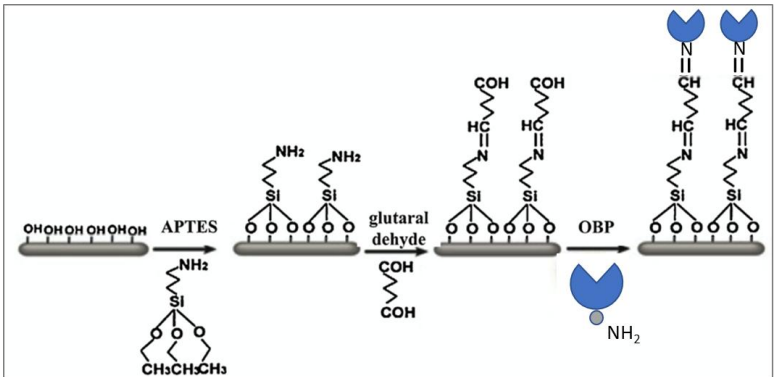


Fig. 1.12. The immobilization of OBPs on silicon nanowire array [132].

The performances of the obtained bioelectronic nose were evaluated using odorants related to human body odor. For evaluating the sensitivity and particularly the selectivity of the obtained system, two sets of odorants were used. The first panel of odorants had the same functional group but different chain lengths, including hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid and dodecanoic acid. The second panel of odorants had a similar single chain scaffold and distinct functional groups, including nonanoic acid, hexanoic acid, geranylacetone, linalool, methyl dodecanoate. Very satisfyingly, the bioelectronic nose had high sensitivity to some of these odorants down to several ppb, and outstanding size and functional group selectivity. Moreover, the responses of devices from different batches were highly reproducible. Finally, when stored at -20 °C, the device conserved a stable activity for 30 days with a decrease in the signal speed and intensity only observed after the first day of storage.

In Table 1.4, we list all the examples of OBP-based olfactory biosensors given above. To summarize, all these examples showed clearly that OBPs are very promising for the development of olfactory biosensors and bioelectronic noses, in particular, thanks to their good stability and tunable biological properties. When coupled to an appropriate transduction system, the obtained sensors can not only be used to analyze odorants in the liquid phase but also in the gas phase. Therefore, these tools have promising applications in diverse fields.

1.4. Conclusions and Perspectives

This chapter has presented the basic mechanism of olfaction and the important biological elements in the olfactory system. It has given an overview of significant progress made for the development of biomimetic olfactory biosensors and bioelectronic noses based on sensing materials from the olfactory system (i.e. olfactory cells and tissues, ORs, OBPs). In particular, ingenious strategies are highlighted, which have been developed to solve some key technical issues for building robust, sensitive and selective devices. Such olfactory biosensors and bioelectronic noses have improved performances compared to classical eNs. They present great potential for applications in diverse fields, including environmental monitoring, food safety, disease diagnosis, as well as quality control for food, beverages, perfume and cosmetics.

Nevertheless, for these applications, in most cases, the analysis of odorants in the gas phase is required. Unfortunately, olfactory biosensors and bioelectronic noses are limited for gas sensing by their short lifespan and their poor repeatability. For this, the new tendency is to design novel sensing materials that are analogues of proteins but with much better stability, such as peptides. Indeed, peptides are particularly attractive thanks to their chemical robustness, diverse physicochemical properties, and easy synthesis and production. Moreover, most importantly, they can reach similar affinity to that of olfactory proteins either by rational computational design (molecular modeling and molecular docking, virtual screening, and molecular dynamics simulation, etc.) or by high-throughput selection such as phage display.

Table 1.4. Summary of the OBP-based olfactory biosensors.

Reference	**Lu *et al.* [126]**	**Manai *et al.* [124]**	**Hurot *et al.* [122]**	**Di Pietrantonio *et al.* [128]**	**Gao *et al.* [132]**
Odorant binding protein	Human OBP	Pig wtOBP + mutant m6hisOBPpig	OBP3 from rat + 2 mutants	wtbOBP from cow + double mutant (dmbOBP) and wtpOBP from pig	OBP5, OBP6 and OBP7 from *Anopheles gambiae* mosquito
Expression system	Human host cell	*E. coli*	*E. coli*	*E. coli* and pig nasal tissues	*E. coli*
Immobilization	Covalent binding	Covalent binding Random using EDC/NHS Oriented using Ni-NTA	Au-S bond	Au-S bond	Cross-linking
Transduction technique	EIS	MEMS	SPRi	SAW	Silicon nanowire array (SiNW)
Odorants tested	Benzaldehyde Lauric acid Docosahexaenoic acid	2,4-dinitrotoluene (2,4-DNT) 2-isobutyl-3-methoxypyrazine (IBMP)	Specific: β-ionone Hexanal Control: Hexanoic acid	Octenol Carvone	Hexanoic → dodecanoic acid Geranylacetone Methyl dodecanoate Linalool
Analysis milieu	Aqueous solution	Aqueous solution	Aqueous solution	Vapor	Vapor
Detection limit	10^{-9},10^{-12},10^{-8} mg/mL respectively	–	200 pM for β-ionone	0.39-2.8 ppm for octenol 1.4-1.8 ppm for carvone	2 ppb for nonanoic acid
Long term stability	–	2 months (in air at room temperature)	2 months at 4 °C	–	30 days at -20 °C

In order to get closer to the performance of the human nose, many other elements must be taken into account for the development of bioelectronic noses. Firstly, concerning the sensing materials, their diversity must be greatly increased. The human nose possesses about 400 different types of ORs, while so far all reported bioelectronic noses based on ORs and OBPs contain less than five sensing probes. The utilization of peptides can provide an efficient solution. Secondly, the human nose uses the combination of two types of recognition principles. Indeed, some ORs are "narrowly tuned" and thus specific to few odorants while some others are "broadly tuned" and cross-reactive to many odorants. It is such a combination that gives the human nose the extraordinary capacity of discriminating over 10,000 of different odors. Therefore, it is important to take inspiration from this and combine cross-reactive and specific sensing materials on the same multiplexed sensor system for better discrimination capacity. Thirdly, a hydrated sensing environment will be important to conserve the function and stability of sensing materials. In the human nose, mucus provides a hydrated environment thanks to the presence of large glycoproteins (mucins). In bioelectronic nose, the integration of its analogue will be very beneficial for considerably improving the lifespan and repeatability of the device. Fourthly, the transduction system should enable multiplexed format and temporal response with supplementary odorant recognition information. Fifthly, appropriate data processing with the rise of artificial intelligence and by taking inspiration from the unique functioning of olfactory neurons will also help in improving the performances of the bioelectronic noses. Finally, for their wide industrial applications, there is an urgent need to standardize the bioelectronic noses for odor analysis.

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