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Advances in the production, immobilization, and electrical characterization of olfactory receptors for olfactory nanobiosensor development

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Abstract

The animal olfactory system represents the gold standard of olfactory biosensors with its capability to identify and discriminate thousands of odorant compounds. In order to mimic the performances of natural olfactory sensors it is necessary to develop methods and techniques for the production, immobilization and electrical characterization of olfactory receptors. We review in this paper some of the advances we obtained in these fields.

Keywords: Electronic nose; Biosensors; Nanotechnology; Olfactory receptors

1. Introduction

The mammalian nose is the gold standard of odour detection, displaying both an unmatched chemical space (estimates vary from tens of thousands up to one million of compounds) and the highest sensitivity (a dog being capable of detecting traces of fatty acids [1]). These performances are due to both the primary olfactory message, which is elaborated at the level of the olfactory receptors expressed by the olfactory sensory neurons, and its subsequent neuronal processing. In vertebrates, each of the about 10^7 neurons of the olfactory epithelium mainly expresses 1 of the 1000 genes of olfactory receptors [2]. This is the largest genome subfamily in mammals (approximately 1–4% of the whole genome), which emphasizes the evolutionary importance of chemical detection in animals. Each individual receptor can bind multiple odorants with distinct affinities and specificities [3] but some olfactory receptors can display a relatively restricted odour specificity to a set of few chemically related molecules [4,5].

In vivo, olfactory receptors perform odour detection in a physiological medium: they are inserted in the plasma membrane of the olfactory ciliae of sensory neurons, which are located at the olfactory epithelium surface. They function in association with small hydrosoluble proteins at the outside of the cell (the olfactory binding proteins (OBPs)) thought to play in particular a role in the transport of odour molecules toward the receptors. At the inside of the cells, the olfactory receptors interact with the α subunit of a trimeric G protein (Gαolf) [6].

The olfactory receptors pertain to the rhodopsin subfamily of G-protein coupled receptors (GPCR), also named "serpen-

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tine" receptors or 7TM (for seven transmembrane segments) because their polypeptide chain spans the plasma membrane seven times. The transmembrane segments delimit a binding pocket for the ligand. Most of the knowledge on GPCRs, and on olfactory receptors, has been acquired from studies on a few archetypal molecules of the family, namely rhodopsin and the β-adrenergic receptor. Up to now, rhodopsin is the only GPCR whose three-dimensional structure is known [7,8], however models for olfactory receptors based on this structure have already proven valuable [9].

Given the fantastic odour space detected by the olfactory receptors, it is tempting to harness them to some generic electronic device that could be endowed with some of the most prominent properties of animal olfaction: discrimination, specificity and sensitivity. The olfactory receptors display some natural characteristics that make them appropriate for an electronic olfactory nanobiosensor. Among others we emphasise that:

- The binding of the odorant ligand elicits a conformational change of the receptor protein which, by itself, might generate a change of the electrical properties of the protein.
- This conformational change may even be enhanced by the interaction of the receptor with the Gα subunit, since Gα dissociates from the receptor upon odorant stimulation and docking of the ligand to the receptor. This molecular event has already been detected by opto-electronic devices for rhodopsin [10,11].
- As other proteins, olfactory receptors may be endowed, by genetic engineering, with specific tags, in order to enable their detection, grafting and orientation in the biosensor device.

Based on the low expression yield of recombinant olfactory receptors (see below) a platform integrating nanoelectrodes (or nanoelectromechanical systems NEMS, in general) and a single or few olfactory receptors seem to be the best suited for the development of these new olfactory biosensors. The advantages of such a platform are several fold: it relies on nanotechnologies that supply cheap unitary nanodevices, it can be fabricated with different configurations, it can be manufactured with different conducting materials (silver, gold or platinum) that can be previously functionalised in order to provide a favourable platform for immobilising receptors; it is able to function in aqueous solution (e.g. physiological medium) and it can be arranged in nanobiosensor arrays to enhance its sensitivity and specificity.

There are three main challenging aspects in the fabrication of these new olfactory nanobiosensors, namely: (i) to produce the desired olfactory receptors, (ii) to reach resolution levels in the fabrication of the nanodevices comparable to the single olfactory protein size (plus functionalisation layers), and (iii) to anchor the olfactory receptors on the nanoelectrodes.

Concerning the olfactory receptor production one must keep in mind that GPCRs, and ORs in particular, suffer from a number of biochemical properties which make them difficult to handle (rhodopsin is a notable exception): they are usually refractory to high-yield expression (reasonably 10^3 to 10^4, exceptionally 10^6 copies per cell), and they have to remain in a lipidic bilayer to retain their structure and function (in counterpart immobilization onto surfaces seems to stabilize them [12]) Yet a number of tricks may be used to overcome these difficulties. Among them, protein engineering allows the grafting of tags, so that specific anti-tag antibodies then allow "fishing-out" the receptors from a membrane fraction preparation for protein detection, specific binding and orientation. Purification of olfactory receptors has already proved not to be required, since olfactory epithelium membrane preparations retain their odour detection capacity once coated on electronic sensor device [13].

Concerning the resolution levels for nanoelectrode fabrication, resolution levels around 20 nm and below have been reported by using electron beam lithography (E-Beam), nanoimprint lithography (NIL) and focused ion beam (FIB). These resolution levels may be sufficient for nanotransducer development purposes.

Finally, concerning the anchoring of single olfactory receptors in-between the nanoelectrodes, a variety of approaches to immobilize proteins according to precisely defined patterns on solid supports have been recently demonstrated [10], which can be applicable to the development of the nanobiosensor. Among others we cite:

- The fabrication of GPCR protein monolayers by means of the Langmuir–Blodgett technique [12] or by transferring engineered GPCRs from natural plasma membrane [10]. It is worth noting that the thermal stability of rhodopsin is increased (150 °C versus 65 °C) in Langmuir–Blodgett films [12] deposited on electrodes, which may be a favourable factor for preparing a batch of identical electrodes.
- The immobilization of rhodopsin, as the representative of the olfactory receptors GPCRs subfamily, via specific biotinylated antibodies. Biotinylated thiols are inserted in an ω-hydroxy-undecanethiol SAM layer, and a monolayer of neutravidin provides the link between the thiols and the specific biotinylated antibodies [14,15].

These two techniques for the transfer of protein monolayers onto substrates are well adapted to conductive planar surfaces and can be perfectly applicable to the development of the nanobiosensor.

Additional aspects relevant for the development of the olfactory receptor nanobiosensor refer to its theoretical modelling, signal detection and nanoscale characterisation.

Up to date, the modelling of the properties of single molecular nanoelectronic devices constitutes a rather difficult issue, in particular, on what concerns devices based on biomolecules. The difficulties in the modelling are related to both the nature of the molecules themselves and the relevant role played by contacts in these systems. Currently this field is in fast development and named also as molecular electronics, bio-electronics, molecular junctions, molecular wires, etc. As a general trend, from top to bottom the above theoretical approaches are valid for as simple molecules as benzene-1,4-dithiolate self-assembled monolayers or gold nanowires, as well as for more complex molecules, like DNA or receptor proteins.

Concerning electrical signal detection, very low noise wide bandwidth pre-amplifiers are required. Expected requirements

for the instrumentation are DC electrical currents resolution levels below nA with measurement times of a few μs, and capacitance resolution levels of a few aF. Commercial electronic instrumentation does not satisfy all these requirements, thus making necessary the development of custom-made preamplifiers.

Finally, concerning nanoscale structural and electrical characterisation of the nanobiosensor, different scanning probe techniques can be used (STM, AFM, SNOM, etc.). Among them, the two best adapted to the nature of the biosensor seem to be AFM and SNOM. The former provides spatial resolution levels below 1 nm, and is able to work in a physiological environment and to perform electrical measurements [16]. The latter is able to resolve fluorescent targets at a scale below 50 nm, and hence able to detect the presence of single receptors in the nanobiosensor.

To our knowledge no nanobiosensor with the properties outlined above has been reported in the current literature, in spite of the efforts devoted by different groups worldwide. In what follows we review significant advances we have achieved recently in the different aspects relevant for the development of the olfactory nanobiosensors integrating olfactory receptors.

2. Olfactory receptor production

The rat I7 olfactory receptor has been expressed heterologously in yeast (*Saccharomyces cerevisiae*) [17]. Since antibodies to the receptor itself are available, tags are not included in the constructs for detection, quantification, and grafting. Localization of I7 olfactory receptor expressed in yeast has been performed by immunodetection and confocal microscopy, revealing the presence of the receptor at the plasma membrane. Ultrastructural localization of the I7 OR has been refined at the molecular level by immunogold labeling and electron microscopy, revealing the location of the olfactory receptors at their functional sites in the yeast membrane (Fig. 1). Gold grains are present on the plasma membrane (arrows) and vesicles near the plasma membrane.

Immunoblot analysis and glycosylation studies show that the I7 receptor is present as a mannose-glycosylated monomer [17]. The level of expression of the I7 receptor has been estimated to be around 1.5×10^5 receptors/yeast cell. Even if this expression level may seem quite low, it is among the highest obtained for GPCRs in this system and it appears to be sufficient for the development of nanobiosensors with a few or single receptors.

A high-level of functional expression has been demonstrated through a luciferase reporter [17]. Receptor expression was induced with 2% galactose at 15 °C in yeast transformed to co-express the I7 OR and $G_{olf,}$ and its functional activity was evaluated by the luciferase reporter placed under an inducible promoter, triggered by docking the odorant ligand to its related olfactory receptor. Bioluminescence measurements were thus performed in response to odorant stimulation, and the dose–response curves plotted as a difference to controls obtained by replacing odorants with water (Fig. 2).

Samples with olfactory receptors have been prepared in the form of membrane fraction, by cell disruption followed by centrifugation to eliminate cell walls, unbroken cells and cytoplasmic content, and subsequent ultrasonication [12]. The resulting membrane fraction is composed of circularized nanosomes with sizes ranging from 500 to 40 nm (Fig. 3). Given the low density of the I7 olfactory receptors on the yeast membrane the number of receptors in the smallest nanosomes is expected to reach the level of a single receptor.

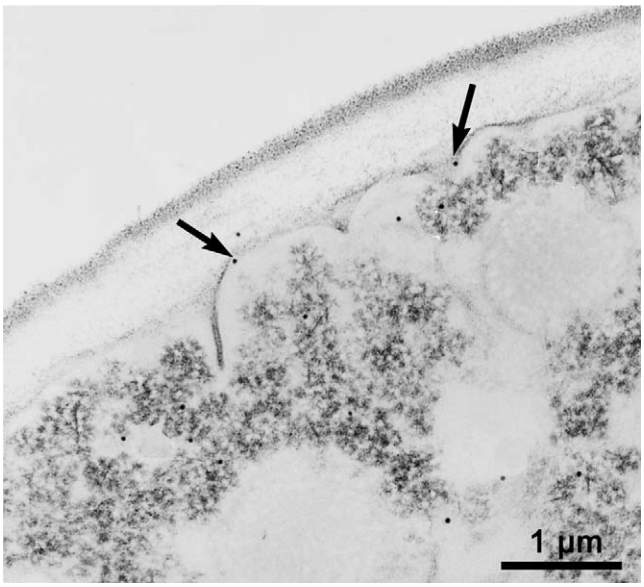

Fig. 1. Ultrastructural localization of the I7 OR in *Saccharomyces cerevisiae*. Transmission electron microscopy image of immunogold labelling of a yeast cell expressing the I7 olfactory receptor.

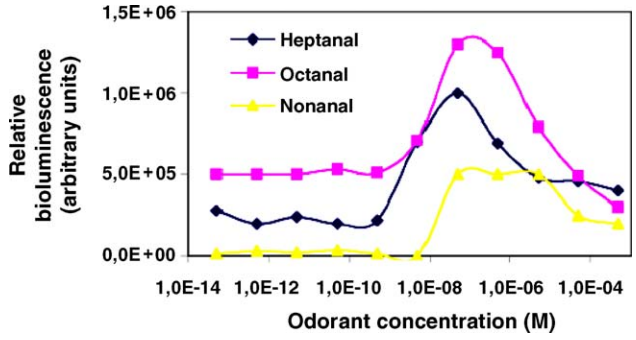

Fig. 2. Differential bioluminescence dose–response upon stimulation of yeast-expressed I7 olfactory receptor with octanal, heptanal or nonanal.

3. Olfactory receptor immobilization on conducting substrates

A new method to specifically immobilize the olfactory receptors onto conducting substrates (gold) has been developed, consistent with that developed for rhodopsin immobilization [14,15]. Being a method based on immunoreactions it constitutes a method for on-site purification of the biomaterial. The new method starts with the formation of a mixed self-assembled monolayer on a gold substrate followed by blockage with unrelated antibody, fixation of neutravidin, immobilisation of the

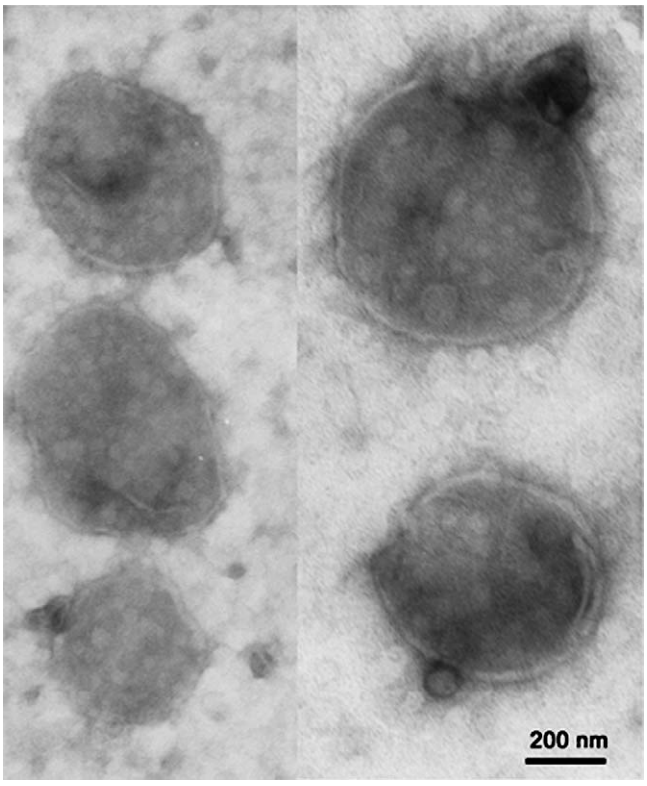

Fig. 3. Transmission electron microscopy image after negative staining of the membrane fraction sample of yeast expressing an olfactory receptor showing the presence of nanosomes with sizes ranging from 40 to 500 nm.

biotinylated antibody specific to the receptor, and finally immobilization of the I7 olfactory receptor within its membrane fraction (Fig. 4).

Control of the immobilization procedure can be performed at each step by means of electrochemical impedance spectroscopy as detailed in Ref. [15]. Validation of the procedure has been performed on a large-scale gold substrate (0.1 cm^2) with bovine rhodopsin as immobilized receptor [14,15], as well as with I7 olfactory receptor [18].

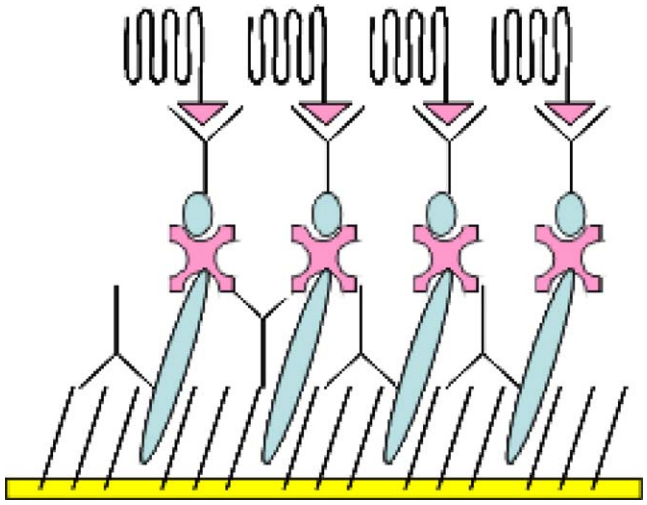

Fig. 4. Schematic representation of the immobilization procedure developed. Layers from bottom to top: mixed self-assembled monolayer, blockage layer with antibodies, neutravidin, biotinylated specific antibody and I7 olfactory receptor within its membrane fraction.

4. Micro/nanotransducer design and fabrication

In view of the success of impedance spectroscopy in the monitoring of the olfactory receptor anchoring process, a good candidate for a micro/nanotransducer for the olfactory biosensor consists of a scaling down of an electrochemical cell. Micro- and nanoelectrochemical cells have been designed and fabricated for this purpose. Fabrication has been based on a combination of standard microfabrication processes and focused ion beam milling. The size of the working electrode of the microelectrochemical cells fabricated range from 80 μm down to 40 nm in diameter (Fig. 5) [19], allowing the microdevices to probe the response from thousands of receptors down to a single receptor.

5. Electronic instrumentation

We have designed and fabricated a new low noise and wide-bandwidth transimpedance preamplifier, specially suited for low

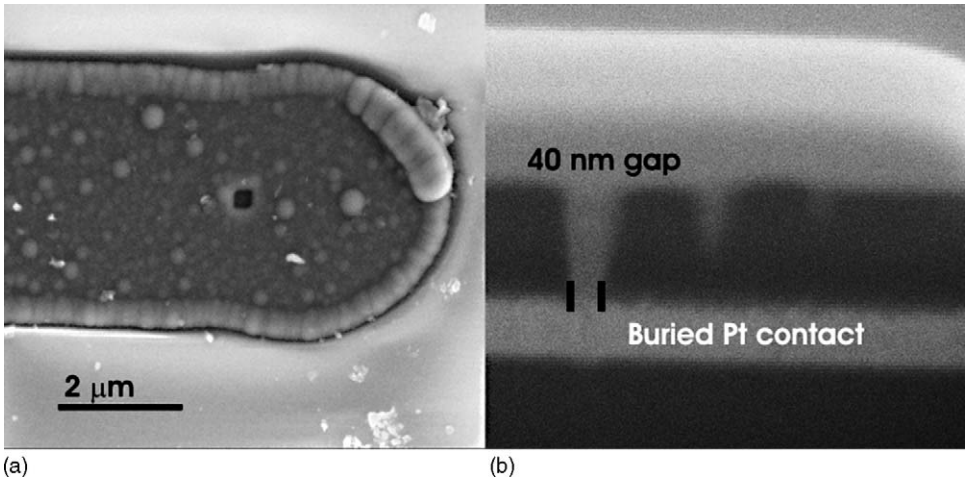

Fig. 5. (a) SEM image of a working nanoelectrode fabricated by focused ion beam milling showing an aperture of 40 nm. (b) SEM image of the cross section of the nanoelectrode showing the 40 nm aperture of the nanoelectrode.

level *I–V*, capacitance and impedance spectroscopy measurements, to be directly connected to the micro/nanotransducers [20]. The fabricated preamplifier displays a DC gain of 1 GΩ on a bandwidth of either 0–1 Hz or 0–100 Hz, and an AC gain of 80 MΩ on either an AC bandwidth of 1 Hz to 1 MHz or 100 Hz to 1 MHz. Capacitance sensitivity up to a few aF and current sensitivity of a few fA have been demonstrated under experimental conditions [21]. The performance of the preamplifiers matches the expected changes in the electric signals due to the conformational changes of the olfactory receptors at the level of a single or a few olfactory receptors.

6. Theoretical modeling

In order to interpret experimental measurements we have developed an equivalent circuit modelling of an olfactory receptor (OR) [22]. By taking the rhodopsin as an appropriate prototype of an OR we have considered the spatial structure of the rhodopsin molecule. By associating elemental RC impedances to each link between two neighbour aminoacids distant apart for an interaction radius *R*, we have constructed the corresponding impedance network which mimics the electrical response of the rhodopsin in its natural (dark) and activated state (light). The network impedance depends on the radius of interaction *R*, which is chosen as the independent variable. Then the network has been analyzed in terms of its graph properties and the equivalent network circuit solved to determine its global impedance. By taking the first and last aminoacids of the rhodopsin primary structure as ohmic contacts, the relative change in the modulus of the impedance between the natural and activated state can be computed as a function of the interaction radius defined above. Simulation results are given in Fig. 6. As can be seen the model predicts a detectable change of the global impedance (up to about 30%) associated with the conformational change due to the docking of the odorant ligand, i.e. to the very sensing action of the receptor.

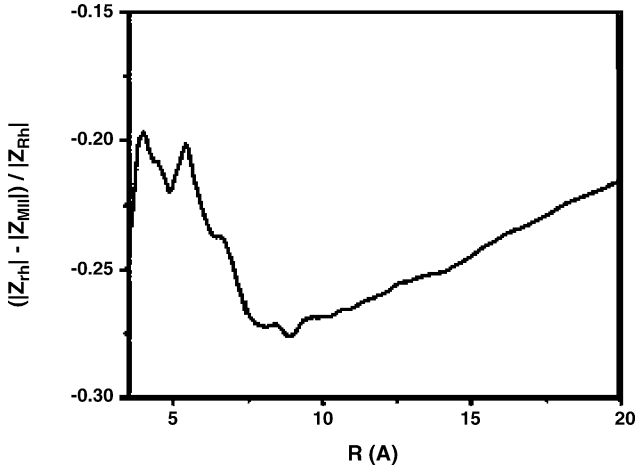

Fig. 6. Difference between the absolute value of the network impedance which mimics rhodopsin in dark $|Z_{Rh}|$ and metarhodopsin-II in light $|Z_{MII}|$, normalized to $|Z_{Rh}|$ as a function of the interaction radius *R*.

7. Nanoscale structural and electrical characterization

In order to assist in the structural and electrical characterisation of the micro/nanodevices fabricated, a custom atomic force microscopy set up for simultaneous structural and electrical nanoscale characterization of the samples and devices has been developed. The set up consists of a commercial atomic force microscope (AFM) coupled to the custom transimpedance preamplifier described in Section 5. This set up allows performing simultaneous measurements of the topography and of both the DC current and the AC small signal impedance of a sample [23], as well as of the electronic noise [24]. This can be useful for the proper characterization of the nanobiosensors at the nanoscale.

8. Conclusions

In the present paper we have reviewed recent advances we performed in the development of methods and techniques for the production, immobilization and electrical characterization of olfactory receptors for the development of artificial olfactory biosensors integrating olfactory receptors and nanoelectrodes. In view of the advances achieved, it is concluded that at present no fundamental difficulty is foreseen in the development of such nanobiosensors, what can make them to come to reality sooner than expected.

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