

PHYSIOLOGICAL AND ARTIFICIAL SYSTEMS FOR ODOUR RECOGNITION

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ABSTRACT: The design of an artificial nose requires two types of contributions: transducers, to convert chemical information carried by the odorant molecules into an electric signal, and information from the biological olfactory system on how chemical structures are associated to odours. The current availability of different types of gas sensors, fulfilling the required properties, and the increasing information on the biochemistry of olfactory transduction make such project feasible. Transducers based on conducting polymers are currently being employed in artificial devices for gas discrimination and recognition, currently applied to food headspace analysis, environmental monitoring and clinical diagnostic. The use of odorant-binding proteins of the olfactory system in the fabrication of biosensors for odours could in the future improve the performance of electronic noses.

Keywords: olfaction, structure-odour relationships, odorant-binding proteins, conducting polymers.

INTRODUCTION

The pressing need for objective and reliable evaluation of flavour and the increasing knowledge in the physiology and biochemistry of the olfactory system have recently prompted several research projects aimed at the design of an artificial system for measuring odours, a sort of "electronic nose".

Although at present we are still far from reproducing the multitask performance of our nose with an electronic device, several instruments, that have been already on the market for a couple years, are currently being employed in food quality control and environmental monitoring.

Applications in the clinical and other fields are the object of current research.

These artificial systems utilise inorganic (such as metal oxides) and organic (such as conducting polymers) gas sensors, but no attempt has been made to apply biosensors to this field.

The present contribution reviews the main recent findings in the biochemistry of odour sensing, with particular regard to odourant-binding proteins (OBPs), describes the artificial odour detector based on conducting polymers, and explores the possibility of using proteins of the biological olfactory system in the fabrication of biosensors for odours.

Before going into detailed descriptions of the results obtained, however, it is worth analysing a question that is fundamental for any project aimed at the design of an artificial nose: what is an odour? In other words, which are the molecular parameters most relevant to the sensation that we call odour? These same parameters have to be measured by an artificial system, whatever the approach used. We can answer the first question only with reference to the biological system (odour is the molecular property measured by the nose), therefore, a certain knowledge on how our nose discriminates odours is essential for identifying the type of analysis that artificial sensors have to perform on volatile compounds.

Therefore, an artificial nose requires two types of contribution:

- ?? gas sensors, capable of interacting with chemical in the vapour phase in a reversible, specific and sensitive fashion;
- ?? information on the algorithm used by our biological nose for discriminating different odours.

THE BIOLOGICAL OLFACTORY SYSTEM

General overview

Most of the current information on the biochemistry of odour perception is the result of very recent research, started in the early 1980's, but experiencing a tremendous expansion at the beginning of the 1990's, after the discovery of the olfactory receptor proteins [1-4].

For several decades, however, generations of chemists have studied the odour of natural and synthetic compounds and tried to correlate olfactory properties with structural parameters of the molecules. The enormous amount of information collected have enabled the definition of some general criteria and guidelines for relating olfactory sensation and chemical data. We can thus summarise these results by stating that odour is related to stereochemical parameters of the odorant molecule, such as size, shape and position of functional group. There are many excellent books and reviews illustrating these concepts with abundant examples taken from all the areas of organic chemistry and relative to all odour classes [5-7].

A simple classical example is reported in Fig.1.

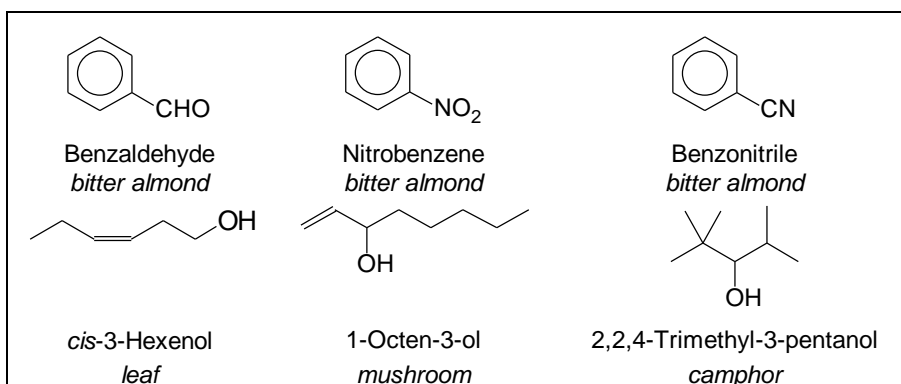


Figure 1. Influence of functional groups on the odour of the molecule.

The three aromatic molecule, benzaldehyde, nitrobenzene and benzonitrile share the same typical olfactory note of “bitter almond”, despite their different functional groups. On the other hand, the three alcohols, cis-3-hexenol, 1-octen-3-ol and 2,2,4-trimethyl-3-pentanol are endowed with markedly different olfactory characters. Thus, shape is more important than the nature of functional groups. The position of the functional group, instead, may be of some relevance in determining the “oriented profile” of the molecule, that is the shape presented to the olfactory receptor protein.

After the understanding of the main biochemical elements of olfactory transduction, these concepts became obvious, and the interactions of odorants with their specific receptor proteins was regarded as a particular case of the general way in which small organic molecules are recognised by proteins. Examples include the interactions of neurotransmitters and hormones with their receptors, as well as the specificity of enzymes towards their substrates.

It must be added that olfaction is probably more complex and may involve other types of interactions, where, for instance, the type of functional group plays a major role. This is suggested by the observation that some chemical classes, such as lower amines, thiols and esters, can be associated with typical odour descriptors.

All this information on how chemical structure is analysed by the nose to produce olfactory sensations represents the reference point when designing gas sensors for discriminating odours. Going back to the examples of Fig. 1, sensors capable of discriminating between an aldehyde and a nitro group would rate the odours of benzaldehyde and nitrobenzene as very different from each other, while these compounds

smell similar to the human nose. Also, sensors recognising the hydroxyl group as the main structural element of the three alcohols in Fig.1, would fail to discriminate their different olfactory notes.

Biochemical research has recently discovered a large family of transmembrane receptors, unique to the olfactory system [2]. Specific interactions of these proteins with odorant molecules triggers an enzymatic cascade, involving production of cyclic AMP or IP3, as second messengers, and leading eventually to the opening of ion channels and depolarisation of the neuron. Thus, chemical information, carried by volatile molecules in terms of stereochemical parameters, is translated into electric signals. Our knowledge on the structure of olfactory receptor proteins, in particular regarding their interactions with odorants, is still too poor and fragmentary to be of any use in the design of an artificial system. However, there is another class of proteins in the olfactory system, capable of reversibly binding molecules of odorants. These are soluble proteins, found in the nasal mucus and called OBPs (odorant-binding proteins) on the basis of their binding properties.

Structure and function of odorant-binding proteins

Odorant-binding proteins were among the first biochemical elements of the olfactory system to be identified [1]. They were discovered in the attempt to find olfactory receptors, using a ligand-binding approach. The experiment involved fractionation of a protein extract from nasal mucosa and testing of individual fraction for binding capacity toward a very potent odorant, 2-isobutyl-3-methoxypyrazine, in its tritiated form. This molecule, that is the natural compound responsible for the typical odour of bell peppers, was found to selectively bind, among all the proteins of the crude extract, a polypeptide of about 20 kDa with a

dissociation constant in the micromolar range.

The first OBP was purified from the cow and structurally characterised [8,9]. As all the other OBPs, later described, it belongs to a large superfamily of carrier proteins, called lipocalins and generally involved in the transport of hydrophobic ligands in aqueous media [3,4,10]. This assignment was based on sequence similarity and later confirmed by the determination of its three-dimensional structure by X-ray crystallography [11,12]. The structure of the bovine OBP (which is present in solution as a homodimer) presents the typical motif of β -barrel, common to all lipocalins so far studied. It consists of eight β -strands tightly packed in the shape of a cup. A segment of β -helix and a ninth short β -strand complete the structure of the monomeric unit. Figure 2 reports the amino acid sequence of bovine OBP and a cartoon showing its three-dimensional folding.



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1    AQEEEEAEQNLSSELSGPWRTVYIGSTNPEKIQENGPFRTYF
41   RELVFDDEKGTVDYFYSVKRDGKWKNVHVKATKQDDGTYV
81   ADYEGQNVFKIVSLSRTHLVAHNINVDKHGQKTELTGLFV
121  KLNVEDEDLEKFWKLTEDKGIDKKNVVFLENEDHPHPE

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Figure 2. Amino acid sequence and three-dimensional folding of homodimeric bovine OBP.

The ligand sits inside the hydrophobic cavity delimited by the eight β -strands, as was clearly shown in the X-ray map with the use of a selenium-containing odorant [11,13]. The amino acids lining the binding pocket contain hydrophobic residues, with a high abundance of aromatic groups. These residues confer a rather "smooth" surface to the binding site, that explains the poor specificity of OBP towards different odorants.

In fact, several ligand-binding studies, mainly performed with the bovine and the porcine proteins, have shown that volatile compounds, different in chemical structure and in olfactory properties, present similar affinities to OBPs. However, other odorants fail to bind to OBPs, even at high concentrations [14-16]. Fig. 3 reports some examples of good ligands and non-ligands, together with their odour description.

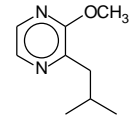
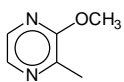
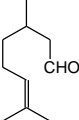
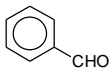
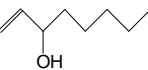
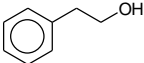
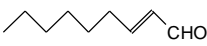
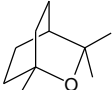
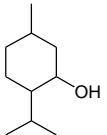
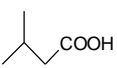
<p>Good ligands ($K_D : 1-10 \mu M$)</p>	<p>Non-ligands ($K_D >100 \mu M$)</p>
<p>2-Isobutyl-3-methoxypyrazine <i>bell peppers</i></p> 	<p>2-Methyl-3-methoxypyrazine <i>Nutty</i></p> 
<p>Citronellal <i>citrus</i></p> 	<p>Benzaldehyde <i>bitter almond</i></p> 
<p>1-Octen-3-ol <i>mushroom</i></p> 	<p>2-Phenylethanol <i>floral</i></p> 
<p>2-Nonenal <i>cucumber</i></p> 	<p>Cineole <i>eucalyptus</i></p> 
<p>Menthol <i>minty</i></p> 	<p>Isovaleric acid <i>sweaty</i></p> 

Figure 3. Examples of ligands and non-ligands for the bovine OBP.

The conclusion, that can be drawn from these studies, is that OBPs present a certain ability to discriminate odorants, but far from the fine tuned capability of the olfactory system.

But a discriminating system, whether biological or artificial, requires the use of several sensors, each tuned to a certain range of the spectrum to be analysed. Thus, the colour visual system utilises three pigments each sensitive to a broad region of the electromagnetic spectrum. This is actually a good example of a system based on poor selectivity sensors and still capable of fine discrimination. In principle, OBPs could perform a first discriminating function towards odorants, before they reach the membrane-bound receptors. The experimental data so far available do not allow us to draw such conclusion, nor to support any of the different hypotheses on the physiological role of OBPs. However, the availability of different types of these proteins, exhibiting different specificities to chemical structures, indicates that OBPs could be utilised in the production of biosensors for odour discrimination.

In fact, since the discovery of the first OBP, several members of this class have been purified and characterised. In some cases, OBPs with complementary binding affinities to odorants have been described in the same animal species [17]. At present, three to five sub-classes of odorant-binding proteins have been identified in several species, but their number is increasing as finer techniques are being applied to this research.

ARTIFICIAL SYSTEMS FOR ODOUR MEASUREMENT

From the preceding section describing the characteristics of binding proteins found in biological olfactory systems, we can make use of

certain concepts in designing artificial sensory systems. The sensors need to be able to respond rapidly and reversibly to adsorbed chemical species, but they do not need to be highly specific to a particular chemical. Nature makes use of many binding proteins that have broad affinities to a range of chemical species, but information processing in the olfactory bulb and higher parts of the brain allows us to distinguish between many complex mixtures of chemicals on the basis of the relative responses from each type of odour receptor [1]. In general, it is difficult to design sensors that are chemically specific and yet applicable to a broad range of measurements. An approach that has proved successful in recent years has been the development of broad-specificity arrays of sensors of a variety of technologies [18,19] that allow patterns of signals to be extracted to characterise a complex gas mixture or odour on the basis of response patterns that can be used as fingerprints. Instruments based on this principle have been designed for laboratory use to analyse samples under highly controlled conditions. The core of the system developed at UMIST, Manchester, UK, and University of Pisa, Italy, comprises an array of conducting polymer sensors mounted on a ceramic substrate together with associated electronics. The sensors used consist of electrically conducting polymers made up of derivatised pyrroles, thiophenes and other heterocyclic compounds. The sensor arrays were designed so that each sensor element was broadly tuned in terms of chemical selectivity but was different in sensitivity and selectivity from other elements in the array. Each sensor behaves as a chemiresistor, and after exposure to an odour, the percent change in resistance from a baseline measured in clean air is recorded. This approach has the advantage that the array can respond to many thousands of chemical species due to the broad selectivity of the adsorbent surfaces.

Extremely selective information for discrimination between adsorbed chemical species or mixtures can be obtained by analysis of the cross-sensitivities between sensor elements. The relative responses between sensor elements produce patterns that may be unique 'fingerprints' that may be used as odour descriptors. This strategy has been successful in the design of chemical sensors that are capable of detecting some volatile chemicals that are difficult to detect by other methods. The modulation of electrical conductivity of conducting polymers by external physical and chemical interactions make them attractive for use in chemical sensing. They are currently used extensively for quality control of raw materials and finished products in the food industry, detection of off odours in packaging, control of contaminants in plastic materials, malodours in agriculture and in the chemical industry.

Conducting Polymer Sensor Arrays

Conducting polymers based on aromatic or heteroaromatic compounds such as polypyrrole and polythiophene are sensitive to many odorants and a reversible change in electrical conductance is observed. Several characteristics of these materials make them attractive for use as odour sensors. There are few problems due to poisoning, rapid reversibility, use at room temperature, rapid response absorption/desorption within seconds to most volatile chemicals, and a long sensor lifetime of several years, depending on the type of usage. On the other hand, many of the materials also respond to water vapour and the competition for binding on the surface causes poor sensitivity to odour molecules at high humidity levels. Understanding of how volatile chemicals interact with conducting polymers is still poor, but it is believed that reversible

conformational changes and/or charge transfer take place between volatile odour chemical and polymer. Of the heterocyclic molecules available, polypyrrole, polythiophene, and polyaniline represent some of the most stable of the organic conducting polymers and have been the focus for most of the research. To develop conducting polymers that are useful for sensing volatile chemicals, a good understanding of the physical, chemical and electrical mechanisms is required. The problems of repeatable and reproducible manufacture are not trivial. The base unit of polypyrrole is pyrrole, a five-member heteroaromatic ring containing nitrogen. Polypyrrole is synthesised from the pyrrole monomer by mild oxidation, using either chemical or electrochemical methods. The polymer formed is usually a black solid, the exact form depending on the nature of preparation, the counterions are usually incorporated in the conducting polymer during oxidation of the monomers. The stoichiometric ratio of monomer units to counterions is generally 3:1 - 4:1, decreasing slightly with molecular weight and negative charge of the anion.

The microstructure of the polypyrrole layer can be significantly altered by the introduction of counterions. Ions with more than one charge will tend to associate with the same number of charges on the polymer chain, causing distortion of an anisotropic (ordered) chain as the distance between the anion and the associated chain charges is minimised. As the polymer structure becomes more isotropic this effect will diminish, as the disordered structure will generally possess have more positive charges in the vicinity of the counterion.

In a semiconductor the band gap is narrow, allowing electrons to be energetically excited from one band to another, thereby enabling current to flow. The thermal excitation of an electron results in a 'hole' in the top of the valence band. The resulting positive charge is

delocalised over the entire material, no local lattice distortion occurring within the crystalline material. Remaining electrons in the valence band are able to jump to this 'hole', leading to the appearance of metallic character. The addition of impurity (dopant) increases the number of charge carriers, which coupled with the high mobilities of the charge carriers in the crystalline lattice, leads to high conductivity's for doped semiconductors. Conducting polymers behave as if they are semiconductors, but they display many small band gaps and the mechanism of conduction may involve three dimensional charge hopping between and along polymer chains.

We have created sensor arrays of up to thirty-two elements consisting of substituted conducting polymers that have unique specificities to different chemical species. Fig. 4 illustrates the way the sensors operate.

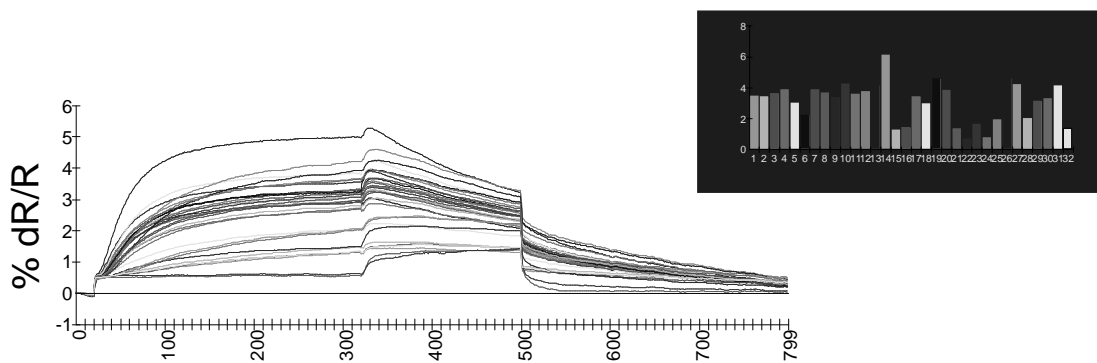


Figure 4. Raw response of a 32 element conducting polymer array to an odour pulse, and extraction of a normalised pattern from the steady state portion of the response.

When an odour is presented, all of the sensors respond with a reversible change of resistance. The intensity of the response is dependent on the affinity of chemical species for individual sensors, and is proportional to the concentration present. If the steady-state response of each sensor to the absolute sum of the response of the entire array is taken, then the raw data between the two cursors can be transformed into a pattern, that is unique to that particular chemical species, or mixture, and can be used as a 'fingerprint' to identify it. The response to a single chemical is proportional to its concentration (Fig. 5).

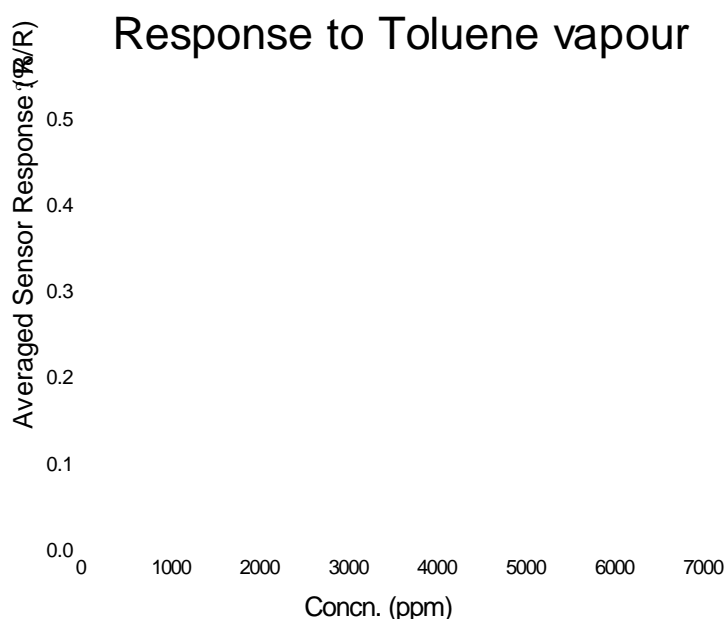


Figure 5. Concentration-response curve to toluene vapour

Adsorption Mechanisms and Sensor Design

The exact nature of the interactions between the sample molecules and the organic conducting polymer surface remain largely unknown. Identification of the interactions undergone at the sensor-vapour interface is an important factor in the design and selection of specific

sensor materials. For adsorbates other than n-alkanes, the overall interaction between the adsorbent and adsorbate contains a contribution due to specific interactions. The magnitude of this specific component can be evaluated by use of a dispersion interaction reference state, based on measurements of the free energy of adsorption of n-alkanes [20]. A number of parameters have been used as the basis for the reference state, such as the decadic log of sample vapour pressure, molar polarisability and the dispersion contribution to the enthalpy of vapourisation ($\Delta H_{\text{VAP}}^{\text{D}}$). We have applied solvation equations using sample, or solute, parameters as descriptors to the correlation and prediction of adsorption phenomena. These phenomena may include specific retention volume values, $\log(V_g)$, or gas-liquid partition coefficients on various adsorbents. The equations used have been variations on the general solvation equation

$$\log(SP) = c + rR_2 + s\chi_2^H + a\chi_2^H + b\chi_2^H + l\log(L^{16}) \quad (1)$$

where SP is the experimental data; R_2 the solute excess molar refraction, a measure of the specific interactions involving π and n electrons pairs; χ_2^H the solute dipolarity/polarisability, a measure of the dipole-dipole or dipole-induced dipole interactions; χ_2^{H} and χ_2^{B} the effective hydrogen bond acidity and basicity of the solute; and L^{16} the solute gas-liquid partition coefficient of hexadecane at 298° K, a measure of the dispersion interactions. Other parameters may also be used, such as solute boiling point or solute vapour pressure. The constants c, r, s, a, b and l are found by multiple linear regression analysis. These types of investigations allow us to specify the

selectivity and sensitivity of a given polymer to an analyte of interest, and also give us a tool for the design of new materials.

Data Processing

The patterns produced by such sensor arrays consist of multidimensional data that is difficult for the human observer to visualise. Often the requirement is to determine whether an incoming odour is similar or different to previously seen samples. A preference is to be able to see directly where a sample odour sits in relation to a number of other known substances. A problem arises when the human observer wishes to examine the multidimensional data produced by odour sensor arrays. To devise a simple means of data inspection from the responses of sensor arrays, Sammon's nonlinear mapping algorithm or principal component analysis (PCA) as a linear projection algorithm, can be applied to the pattern generated by odour sensing system (see Fig. 6).

Figure 6. Cluster analysis using principal components analysis of response

patterns for 1,8-cineole (CINEOLE.DBA), citral (CITRAL.DBA), citronellol (CITRON.DBA), and isoamyl acetate (ISOAMYL.DBA). Each point corresponds to a response pattern reduced from multidimensions to two dimensions

Any application of conducting polymer sensor arrays (or other types of sensor technology) will rely heavily on the data processing and pattern recognition software associated with it. The response to a volatile odour of each sensor is proportional to the concentration, and is unique for each type of single or complex odour. With each sensor in an array having a certain response character, an array of sensors with broad but different chemical specificities provides a measurement pattern of broad overlapping selectivity. These responses, or signals, are processed to produce a set of descriptors for the input, which can be identified as a "fingerprint" for an odour, and then saved into a database for further manipulation within statistical pattern recognition methods, cluster analysis, and artificial neural networks. The input data into these methods is a normalised pattern of responses of each sensor relative to the whole array. For pure solvents the normalised patterns produced are almost concentration-independent. However, in general, when monitoring a complex odour or chemical mixture the patterns are non-linearly concentration dependent. This creates difficulties for information processing.

It is desirable to discriminate between odours, compare one odour sample with another, and get an estimate of intensity or concentration of the odour. Ideally, it is required to map these properties to the human perception using descriptors. The use of neural networks within artificial sensory analysis has been growing in momentum in recent years. The ability to recognise pattern characteristics from relatively small pieces of information has led to growing interest in the possible

applications and development within sensory recognition. A variety of pattern recognition techniques, including neural networks, may be applied to the classification of different odours, quantitative prediction and recognition of unknown gases and odours. Backpropagation, used for multilayer perceptron networks, is probably the most widely used neural network paradigm. This algorithm has been used with good results with a wide variety of odour recognition problems. In this case an exemplar set of data is measured experimentally, and patterns for each odour class of interest are collected. The neural network is then used to associate these patterns with outputs that are descriptors. Once trained, such a system is able to generalise, and is able to recognise even noisy patterns [21].

PERSPECTIVES USE OF BIOSENSORS IN ODOUR RECOGNITION

The chemical characteristics of OBPs, as briefly summarised in one of the previous sections, fulfill most of the requirements for being used as biosensors in a sort of artificial nose. The main advantage of OBPs are:

- ?? they bind odorants in a reversible fashion; both the binding and the dissociation processes are fast enough to produce responses in real time;
- ?? their binding spectrum is rather broad: this characteristic allows the use of only few proteins in the analysis of the complex diversity of odorants;
- ?? OBPs of different and complementary binding specificities are described and their structures are known;
- ?? they can be obtained in large quantities and in their active conformation by expression in heterologous systems, such as bacteria and yeasts;

?? they can be covalently immobilised without losing their binding properties;

?? they are exceptionally stable to thermal denaturation, withstanding temperatures above 80° C; this property makes them particularly suitable to be used in biosensors for environmental monitoring

The main open question is related to how to convert the binding event into an electrical or optical signal, that could be easily measured. While several alternative approaches are being considered at present, a closer study of the three-dimensional structure of OBPs and of their conformational modifications following interactions with odorants, will certainly indicate a strategy to solve this problem.

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