

# Chapter 10

## Biosensors Based on Odorant Binding Proteins

Krishna C. Persaud and Elena Tuccori

**Abstract** Both the sensillary lymph of insects and the nasal mucus of vertebrates contain large amounts of small soluble proteins, odorant-binding proteins that specifically and reversibly bind odors and pheromones. Proteins from different sources have affinities toward a wide range of compounds with different sizes and shapes. They can be easily expressed in heterologous systems, they show high thermal stability and it is possible to modify their binding sites by site-directed mutagenesis. We describe the development of an odor sensing biosensor array based on immobilization of odorant binding proteins on to suitable transducers. Using a quartz crystal microbalance platform as a transduction element, it is possible to detect and measure quantitatively concentrations of volatile analytes at parts per million concentrations in air.

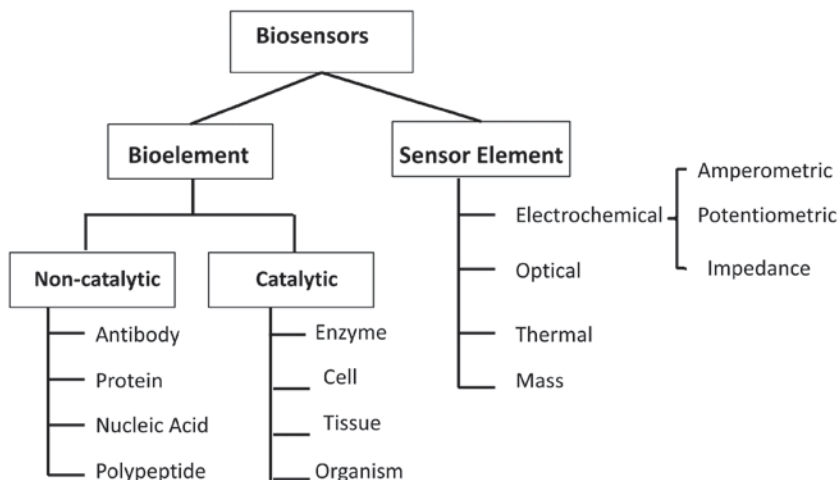
### 10.1 Introduction

A biosensor can be defined as an analytical device that converts a biological interaction into a measurable electrical signal [1]. Biosensors are commonly composed of a recognition element (receptor), a signal conversion unit (transducer) and an output interface. It is possible to classify them on the basis of commonly used receptor and transducer elements as summarized in Fig. 10.1. This involves a combination of two steps: a recognition step and a transduction step. The recognition step involves a biological sensing element or receptor that can recognize biological or chemical analytes in solution or in the ambient atmosphere. The receptor may be proteins, such as antibodies or enzymes, DNA, peptide sequences or whole cells. The receptor elements are in close contact with a transducer that converts the analyte-receptor interaction into a quantitative electrical or optical signal [2].

The detection of chemical signals in the environment, which provides information on food, mates, danger, predators and pathogens, is essential for the survival of most mammals and insects. Animals have developed a highly sophisticated olfactory system, able to distinguish between thousands of diverse volatile compounds.

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**Fig. 10.1** Biosensor transduction methods

Humans, for example, can detect many thousands of different molecules [3]. There is much interest in development of olfactory biosensors that may mimic some aspects of performance of the olfactory system and these activities have been reviewed by Persaud (2012) [4] and Du (2013) [5].

Vertebrates and insects detect volatile compounds from the environment and integrate these signals with other sensory stimuli such as sight and hearing in order to obtain a complete map of the immediate environment. In vertebrates and in insects, the olfactory neurons are separated from the air by a protective layer made up of the nasal mucus and sensillary lymph, respectively. The odorants, and pheromones which are hydrophobic and volatile molecules, have to cross this hydrophilic barrier to reach the olfactory receptor neurons. Both the sensillary lymph of insects and the nasal mucus of vertebrates contain large amounts of small soluble proteins, odorant-binding proteins that specifically and reversibly bind odors and pheromones [6]. The very high concentrations of these proteins in their respective fluids, as well as their rapid turnover, suggest functions important in the perception of olfactory stimuli. They are involved in the binding of numerous hydrophobic ligands with affinity in the micromolar range. The high conformational stability of these proteins as well as the wide variety of OBPs isolated in mammals and insects make them interesting in constructing biosensors that can also function as chemical sensors for volatiles [7]. In this chapter we describe the development of an odor sensing biosensor array based on odorant binding proteins.

### **10.1.1 Biosensor Recognition Elements**

The recognition element is the biological component of the biosensor that produces the signal. There are various types of recognition elements, ranging from whole

cells to specific molecules. Recognition elements can be divided into two general categories: non-catalytic elements and catalytic elements [8, 9].

Non-catalytic elements, such as antibodies, protein receptors, polypeptides, DNA, are often used for direct detection biosensors, in which the interaction with the target compound is monitored in real time. Antibodies are the most commonly used non-catalytic recognition elements, because they are highly specific, versatile and have high affinities towards the target molecules.

For the catalytic elements, the recognition elements can be enzymes, organelles, whole cells or organisms. They are used primarily in indirect detection biosensors, in which the interaction between the biological component and the target analyte releases a detectable second molecule.

Enzymes are the most widely used catalytic detectors, because they have high level of amplification in biorecognition processes and good selectivity. The most important enzymes from an analytical point of view are the oxidoreductases, which use oxygen or nicotinamide adenine dinucleotide (NAD) to catalyze the oxidation of substrates, or hydrolases, which catalyze the hydrolysis of compounds. For example, glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid, which forms the basis of glucose monitoring for diabetics [1].

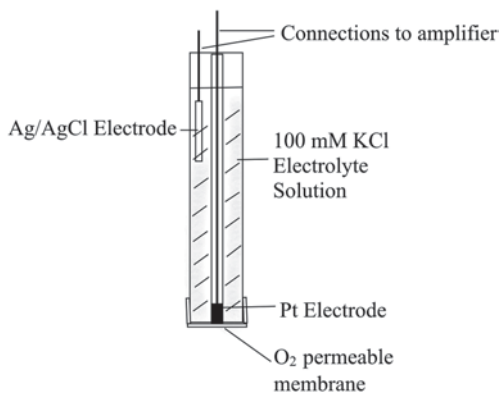
### 10.1.2 Transducers

The transducer converts the bio-recognition event into a measurable signal. Transducers can be clustered in four main classes on the basis of the method used [10]:

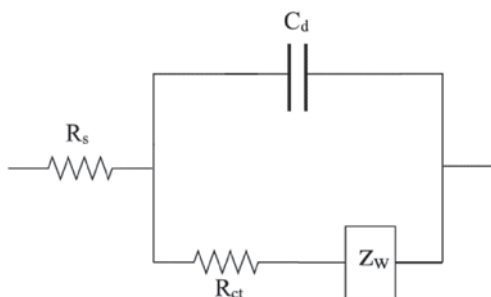
1. Electrochemical detection methods;
2. Optical detection methods;
3. Acoustic (mass detection) methods;
4. Thermal detection method (not described here).

Amperometric and potentiometric systems typify the most commonly used electrochemical transducers. The detection of analytes by the biological elements of biosensor often generates chemical species that can be measured by electrochemical methods. The principle of operation of amperometric biosensors is defined by a constant potential applied between a working and a reference electrode. The imposed potential promotes a redox reaction at the electrode surface, which produces a current. The magnitude of this current is proportional to the concentration of electro active species present in solution [11–13]. The simplest amperometric biosensor in common usage is the Clark oxygen electrode (Fig. 10.2). This consists of a platinum cathode at which oxygen is reduced and a silver/silver chloride reference electrode. When a potential is applied to the platinum cathode, a current proportional to the oxygen concentration is produced. In potentiometry, a glass membrane or a polymeric membrane electrode is used for measuring the membrane potential resulting from the difference in the concentrations of  $H^+$  or other positive ions across the membrane. Amperometric or potentiometric transducers can be employed in the

**Fig. 10.2** Clark oxygen electrode. The reaction chamber is separated from the electrodes by a semipermeable membrane, which permits oxygen to diffuse from the reaction buffer into the potassium chloride solution that bathes the electrodes: a platinum cathode and a silver anode. A voltage is applied between the electrodes and the resulting current (in the  $\mu\text{A}$  range) is proportional to the concentration of oxygen



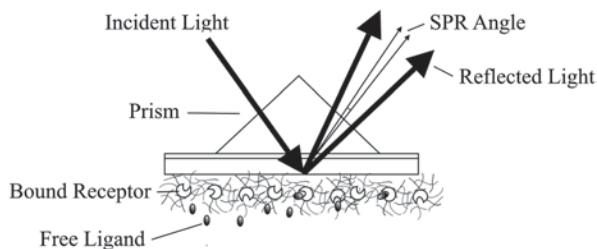
**Fig. 10.3** Randles equivalent circuit. Impedance measurements are often fitted to the Randles equivalent circuit, where  $R_{ct}$  is the charge-transfer resistance,  $C_d$  is the differential capacitance,  $R_s$  is the solution-phase resistance and  $Z_w$  is the Warburg diffusion element



case of catalytic receptor elements, mainly enzymes that convert the target analyte into a detectable product.

Biosensors based on impedance, can be also classified as electrochemical transducers. Impedance measurements involve application of a small sinusoidal alternate current (AC) voltage probe and determination of the current response. Impedance measurements are often fitted to the Randles equivalent circuit shown in Fig. 10.3, where  $R_{ct}$  is the charge-transfer resistance,  $C_d$  is the differential capacitance,  $R_s$  is the solution-phase resistance and  $Z_w$  is the Warburg diffusion element [14]. The Randles circuit describes the Faradaic impedance. In this case, it is necessary to have a redox species in solution where it is possible to monitor the charge transfer resistance. Impedance sensors detect a change in one of these equivalent circuit parameters due to the direct interactions of the target analyte with the probe.

Optical sensors rely on the optical transduction of the signal and comprise ultraviolet, visible and infrared spectrophotometry in transmission or reflectance modes. The relationship between the incident light intensity and the transmitted radiation is given by the Beer–Lambert law. Optical methods have been used classically to determine analyte concentrations. Properties like absorption, refractive indices, fluorescence, phosphorescence, chemiluminescence, etc., can be used in order to

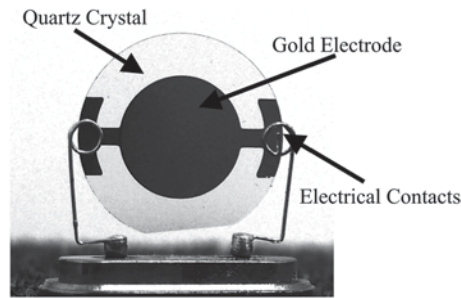


**Fig. 10.4** Surface plasmon resonance sensor. SPR measures minute changes in refractive index at and near the surface of the sensing element. SPR measurement is based on the detection of the attenuated total reflection of light in a prism with one side coated with a metal. When a p-polarized incident light passes through the prism and strikes the metal at an adequate angle, it induces a resonant charge wave at the metal/dielectric interface that propagates a few microns. The total reflection is measured with a photodetector, as a function of the incident angle. For example, when an ligand binds to a recognition element (protein, antibody or other) that is immobilized on the exposed surface of the metal the measured reflectivity increases. This increase in reflectivity can then be correlated to the concentration of the ligand

monitor the biological recognition in biosensors. The devices can be miniaturized by using optical fibers, which act as light guides. The detectors are often semiconductor photodiodes. These devices are often used for remote analysis as the light signal is resistant to electrical noise. Optical fiber biosensors can be used in combination with different types of spectroscopic techniques, e.g. absorption, fluorescence, phosphorescence, surface plasmon resonance, etc [15].

Surface Plasmon Resonance (SPR) is a real-time, label-free, optical detection method for studying the interaction of soluble analytes with immobilized ligands or receptors [16–18]. SPR measures minute changes in refractive index at and near the surface of the sensing element. SPR measurement is based on the detection of the attenuated total reflection of light in a prism with one side coated with a metal (Fig. 10.4). When a p-polarized incident light passes through the prism and strikes the metal at an adequate angle, it induces a resonant charge wave at the metal/dielectric interface that propagates a few microns. The total reflection is measured with a photo detector, as a function of the incident angle. For example, when an antigen binds to an antibody that is immobilized on the exposed surface of the metal the measured reflectivity increases. This increase in reflectivity can then be correlated to the concentration of the antigen [19].

Mass transducers measure small changes in mass due to the interaction between the analyte and the biological component of the biosensor. The piezoelectric silica crystals called quartz crystal microbalances (QCM) are the most common mass transducers (Fig. 10.5) and is used to measure very small mass changes in the order of picograms [20–22]. The principle is based on the piezo-electric properties of quartz crystals. Indeed, if an electrical field is applied through quartz, the inner dipoles are re-orientated and a crystalline mechanical strain is observed. When the



**Fig. 10.5** Quartz crystal microbalance. A typical quartz crystal microbalance (QCM) used for mass measurements. If a potential difference is applied between the electrodes of the QCM, the physical orientation of the crystal lattice is distorted, resulting in a mechanical oscillation of a standing shear wave across the bulk of the quartz disk at a characteristic vibrational frequency (i.e. the crystal's natural resonant frequency). The direction of the oscillation depends on the exact geometry of the cut of the quartz crystal

crystal is included in an appropriate electronic circuit, the oscillation frequency measured is closed to the resonant frequency and the generated wave amplitude reaches a maximum value. Modification of a physical characteristic of the resonator, for example changes in global mass or thickness, lead to a variation of the resonant frequency. The frequency change is directly proportional to the mass of the crystal [23]. For biosensors, mass changes occurring when the modified transducer surface interacts with the detected species are common, and this change can be easily measured.

### 10.1.3 Immobilization

One key factor in the construction of a biosensor is to develop an immobilization method for binding biomolecules on the surface of the transducer. The immobilization technique should not alter the activity, structure and function of the biological component and should assure long-term stability of the active layer of the biosensor. Biological films need to be immobilized using reproducible methods, and once formed these should adapt to different environments, maintaining their stability and activity during changes in temperature, pH, ionic strength and chemical composition [19, 24–26].

The principal methods of immobilization are:

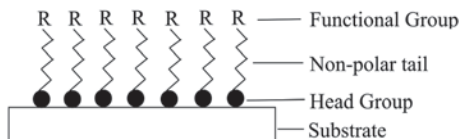
1. Physical or chemical adsorption;
2. Covalent binding;
3. Entrapment within a membrane, surfactant matrix, polymer or Microcapsule;
4. Cross-linking between molecules.

The immobilization method employed depends on many factors, but in general the method needs to be compatible with the biomolecules to be immobilized, the sensor surface or matrix and ultimately the end use of the sensor.

Adsorption of biomolecules from solution onto solid surfaces can proceed via either physical or chemical interactions. Physical adsorption involves Van der Waals forces, ionic binding or hydrophobic forces, whereas in chemical adsorption there is a sharing or transfer of electrons to form a chemical bond. The main advantage associated with direct adsorption onto solid surfaces is that is a simple method which can be performed under mild conditions. In general, however, biomolecules that are adsorbed on to a surface exhibit some degree of reversibility, and with few exceptions, the forces involved in the binding are not very strong. Moreover, irregular distributions of randomly oriented proteins are commonly observed on the surface [27, 28]. Since such protein molecules exhibit multiple-binding sites, they can bind to solid surfaces through various groups in a disorganized manner. Despite these problems, simple adsorption remains the major method used in clinical assays.

An alternative approach to attach biomolecules on sensor surfaces is via covalent binding. Biomolecules are immobilized on solid surfaces through the formation of defined linkages. Covalent binding of biomolecules to the surface is a procedure resulting in minimal loss of biomolecules activity. This method has been employed to improve uniformity, density and distribution of the bound proteins, as well as the reproducibility of the surfaces. Problems associated with instability, diffusion, aggregation or inactivation of proteins can also be overcome by using covalent immobilization. Biomolecules such as enzymes and proteins expose many functional groups on their surface that can be used for covalent immobilization on the transducer; these include amino-acid side chains (e.g. amino groups of lysine), carboxyl groups (aspartic acid and glutamic acid), sulfhydryl groups (cysteine), etc. It is important that functional groups involved in the immobilization reaction of biomolecules on the surface do not result in a loss of activity. Suitable functional groups which are available for covalent attachment are also present on some transducer materials (hydroxyl groups on silica). Metal surfaces such as gold and silver can be modified by reaction with hydroxyalkanethiols to generate hydroxyl, carboxyl or amino groups which may react with enzymes or proteins. This technique includes the Self Assembled Monolayer's (SAMs) method. SAMs are well organized two- or three-dimensional supramolecular structures formed by the adsorption of an active surfactant on a solid surface [29–31] (Fig. 10.6). The spontaneous self assembly is driven by specific interactions between the head functional groups of the “self-assembling molecules” and the surface, followed by a self-organization of a monomolecular film on the surface, which is stabilized by non-covalent interactions among the same molecules. This process is widely used to modify solid surfaces.

The class of monolayer, commonly used in biosensor applications is based on the strong adsorption of disulfides (R–S–S–R), sulfides (R–S–R) and thiols (R–SH) on a metal surface, gold, platinum, silver [32]. Gold is the substrate more commonly used for growing SAMs, since it is possible to obtain thin gold films by thermal evaporation; it is inert to the gases present in the atmosphere and is easy to clean.



**Fig. 10.6** Self assembled monolayers. The spontaneous self assembly is driven by specific interactions between the head functional groups of the “self-assembling molecules” and the surface, followed by a self-organization of a monomolecular film on the surface, which is stabilized by non-covalent interactions among the same molecules

Thiols are chemisorbed on gold through the oxidation of the S-H bond, followed by a reductive elimination of hydrogen and formation of thiolate ions, while the adsorption of disulfides is due to a simple oxidative addition of the S-S bond on the gold surface. The formation of a self assembly monolayer of alkylthiols on gold surfaces has a biphasic kinetic behavior. After an initial fast step which is led by the reaction of the sulphur group on the gold surface, a slow second phase starts the formation of a crystallized surface, where alkyl chains relax from the disordered state and organize into unit cells, thus forming a two-dimensional crystal [33].

SAMs of thiols on gold seems to be the most promising technique for making protein biosensors. Alkylthiols used in the SAMs often carrying carboxylic functional groups that may be easily activated by carbodiimide, forming stable peptide bonds with the proteins' amino groups.

The procedure of entrapping biological components in polymer gels, membranes or surfactant matrices has been used with success in the past and it is still widely employed [34]. The immobilization of an enzyme in a polymeric gel or behind a membrane is a relatively straightforward process. A number of polymers have been used for the inclusion of enzymes, cells and organelles. These include polyvinyl alcohol, polyvinyl chloride, polycarbonate, polyacrylamide and cellulose acetate. Methods used for the entrapment of biological components, however, suffer from the disadvantage of leakage of the biological species during the use, resulting in a loss of activity [35].

Cross-linking of biological components by multifunctional reagents affords good stability to the adsorbed enzymes or proteins. Glutaraldehyde, which couples with the lysine amino groups of enzymes, is by far the most common cross-linking agent in biosensor applications. Biosensors, where the enzymes were immobilized in a glutaraldehyde matrix or in combination with natural polymers as chitosan, were often used [36]. However, there are a number of disadvantages associated with this method: the reaction is difficult to control, the protein layer formed is usually gelatinous and not rigid and large amounts of biological material are required. Cross-linking can result in the formation of multilayer's of protein or enzyme resulting in low activity of the immobilized layers and large diffusion barriers to the transport of the biological species may result, leading to slow interactions [35].

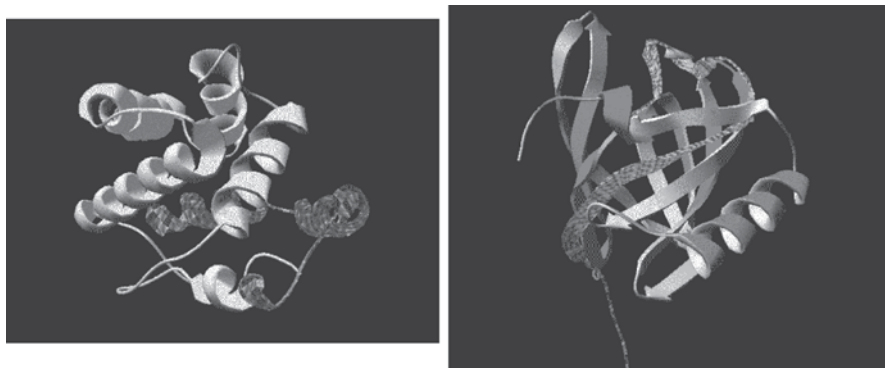


## 10.2 Odorant Binding Proteins

Discovered almost simultaneously by Pelosi and coworkers in vertebrates [37] and by Vogt and Riddiford in insects [38], it was shown that the nasal mucus of vertebrates and the chemosensillar lymph of insect antennae contain large amounts of small soluble proteins, odorant-binding proteins, that specifically and reversibly bind odor molecules and pheromones [6, 39, 40]. The very high concentrations of these proteins in their respective fluids, as well as their rapid turnover, suggest functions that are important for the individual or for the species. They are involved in the binding of numerous hydrophobic ligands with affinity in the micromolar range. Some similar proteins such as pheromone-binding proteins (PBPs) [41], major urinary proteins (MUPs) in rodents [42–46] or salivary lipocalin (SAL) in the boar [47, 48] are clearly involved in vertebrate chemical communication, binding with high specificity sex pheromone components. On the contrary, the sub-class of odorant binding proteins (OBPs) has the ability to bind a wide range of ligands, without displaying high specificity [6, 39, 40].

When OBPs were first isolated in vertebrates, it was hypothesized that they could be responsible for recognizing olfactory stimuli acting as carriers or scavengers for the hydrophobic molecules of odorants, but their exact role in olfaction is still uncertain. It is largely agreed that OBPs function as solubilizers for odorants [49–52]. Generally, odorants are small hydrophobic molecules that are unable to easily cross the air-liquid interface of the olfactory epithelium in vertebrates or the sensillary lymph in insects. Upon binding to the hydrophilic water soluble OBPs, odorants become solubilized and can thus be transported across perireceptor space, to odorant receptors (ORs). This serves to protect the ligands against degradation by enzymes, such as UDP-glycosyltransferases and sensillary esterases [53]. Opposed to the view that OBPs function as general odorant carriers, the numerous OBPs discovered and the selectivity of each of these for odorant subclasses, in addition to interactions with specific subclasses of olfactory sensory neurons, suggest that OBPs serve a much wider function [54, 55].

It is now proposed that OBPs are involved in the peripheral processing of olfaction. This is demonstrated by the generation of LUSH mutant *Drosophila* flies, which fail to display normal behavioral responses to odorants. Kim et al. (1998) [56] developed mutant flies for LUSH, an OBP expressed in the third antennal segment of *Drosophila*, from a 3 kb genetic deletion, which included a transcription unit for LUSH, thereby preventing its expression. These LUSH mutants did not display the normal aversive behavior to high concentrations of short-chain alcohols, observed in control flies. Interestingly, the reintroduction of the LUSH transgene allowed recovery of the avoidance response. Xu et al (2005) [57, 58] further demonstrated that LUSH mutants do not exhibit social aggregation in response to the pheromone 11-cis vaccenyl acetate. Together, these findings highlight OBPs as essential for olfaction, and necessary for processing of specific odorants. It may suggest that OBPs are selectively binding to distinct odor subgroups, and thus, in combination with the selectivity of ORs, contribute to the responsiveness and sensitivity of OSNs, and ultimately, evoked behavioral responses.



**Fig. 10.7** Structure of OBPs from insects and vertebrates. The figure shows OBPs from the wasp *Polistes dominula* (left), and pig *Sus scrofa* (right)

### 10.2.1 Structure of Odorant Binding Proteins

The secondary structures of OBPs are widely different between vertebrates and insects. The insect's OBPs are mainly organized in  $\alpha$ -helical domains, in contrast to the  $\beta$ -barrel motif found in vertebrates (Fig. 10.7).

The three dimensional structure of an insect OBP is very compact and stabilized by three interlocked disulphide bridges connecting six cysteines, which is the fingerprint of this protein family. The first OBP of insects was discovered at the beginning of the 1980's in the giant moth *Antheraea polyphemus* [38] using a tritium-labelled specific pheromone (E, Z)-6,11-hexadecadienyl acetate as a probe. A great number of proteins similar in their amino acid sequences to the OBP were later identified in many Lepidopteran species, *Lymantria dispar* [59], *Manduca sexta* [60], *Bombyx mori* [61] and many others. According to their length and the number of cysteines, insect OBPs can be grouped into: classic OBPs (having the typical six-cysteine signature), tandem OBPs (constituted by two classic OBPs linked by few amino acids), C-plus OBPs (containing more cysteines in addition to the six of the conserved motif), C-minus OBPs (presenting only four of the six conserved cysteines), and atypical OBPs (having a variable number of additional cysteines and generally a longer C-terminus) [62–67]. Beside odorant binding proteins, a second class of polypeptides has been identified in the lymph of chemosensilla: chemosensory proteins (CSPs). OBPs are believed to be an evolved form of CSPs which are mainly present in primitive arthropods [68].

Vertebrate odorant-binding proteins were discovered almost simultaneously with insect OBPs using a ligand-binding approach. Pelosi et al. [37] detected in the nasal olfactory mucosa of bovines and other mammals soluble proteins able of binding 2-isobutyl-3-methoxypyrazine, an odorant with a low detection thresholds. Several members of this family were later purified from different mammals: pigs, rabbits [69], mice [70], porcupine [71] and humans [72]. OBPs expressed in mammals are dimers or monomers of acidic polypeptides of about 17–20 kDa, belonging to the

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DSDI AVK KYL HAVPEPVLAK CLKESGLEAD KDKLLSDEST VDQ GKFSCLI  
ACTLKDNGALVNGELKYDVL SELLSKLLTN KEDKLQERLE KACIPEGA  
NAKNDCEYIG KIMQCKLSKA KEMGL
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**Fig. 10.8** Amino acid sequence of OBP1 from the wasp *Polistes dominulus*

superfamily of lipocalins [6]. They are synthesized in several glands located in the respiratory region of the nasal tissue, such as the lateral nasal glands and the glands of the septum [73–75]. Vertebrate OBPs are mainly organized in a  $\beta$ -barrel structure, with a calyx-shaped cavity, made up of eight antiparallel  $\beta$ -sheets with a short segment of  $\alpha$ -helix at the C-terminus [76, 77]. The ligand binding site is located in the core of the  $\beta$ -barrel, as was demonstrated by the use of a selenium-containing ligand, 2-amino-4-butyl-5-propylselenazole [78]. Figure 10.7 compares the structure of vertebrate and insect OBPs.

OBPs from different sources display specific affinities toward a wide range of compounds with different sizes and shapes. They can be easily expressed in heterologous systems, they show high thermal stability and it is possible to modify their binding sites by site-directed mutagenesis. These factors make them attractive for the design of biosensors that can be used for detection of ligands in the liquid phase or in vapor phase.

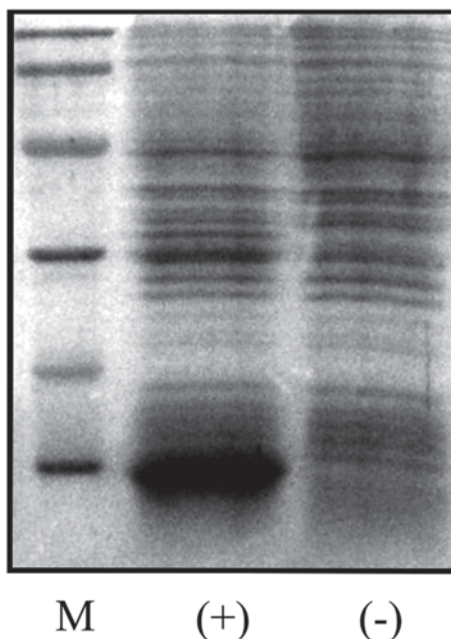
## 10.2.2 Construction of an OBP Biosensor

### 10.2.2.1 Protein Expression

Because the amino-acid sequences of many OBPs are available, it is now a fairly straightforward procedure to create plasmids containing the appropriate gene sequence coding for a particular protein using either vertebrate or invertebrate OBPs. For example, the amino acid sequence for OBP1 from the wasp *Polistes dominulus* is shown in Fig. 10.8. The nucleotide sequence of the OBPs can be easily insert inside a plasmid, such as pET (Promega). The obtained expression vector was used to transform *E. coli* BL21(D3) cells (Agilent technology). The construct included the nucleotide sequence coding for the protein, an ATG codon at the 5' end and a stop codon at the 5' end. These two extra codons were inserted in the sequence of primers in the forward and reverse direction respectively, to allow the correct expression of the protein.

Bacteria colonies were grown overnight in 10 ml Luria-Bertani/Miller broth containing 100 mg l<sup>-1</sup> of ampicillin. The culture was diluted 1:100 with fresh medium and grown at 37 °C until the absorbance at 600 nm reached 0.7 AU. At this stage, 0.4 mM isopropyl thio- D-galactoside was added to the culture to induce expression. After 2 h at 37 °C, the cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 7.4 and lysed by sonication. The recombinant OBP was present at this stage in the supernatant and was expressed with yields of about 15 mg l<sup>-1</sup>

**Fig. 10.9** Electrophoretic analysis in denaturing conditions (14% SDS-PAGE) of bacterial cultures expressing OBP, sampled before (-) and after (+) induction with isopropyl-beta-D-thiogalactopyranoside



of culture. The proteins were purified by two chromatographic steps on anion-exchange resin DE-52 (Whatman), using a gradient of 0.5 M NaCl in Tris buffer, followed by gel filtration on Superose-12. Fractions were analysed by SDS-PAGE and by UV spectroscopy to evaluate the amount of DNA co-eluted with the protein. At the end of the purification procedure, the proteins were more than 95% pure, as judged by SDS-PAGE, and virtually free from DNA.

The protein was expressed in good yields (around 15 mg of OBP per litre of bacterial culture) and in soluble form.

Figure 10.9 shows the electrophoretic analysis in denaturing conditions (14% SDS-PAGE) of crude bacterial cultures expressing OBP, sampled before (-) and after (+) induction with isopropyl-beta-D-thiogalactopyranoside. The expressed protein had a molecular weight of about 15 kDa.

### 10.2.2.2 Quartz Crystals

Piezoelectric AT- cut quartz crystals, with a resonance frequency of 20 MHz and 7.95 mm of diameter were used as the transduction elements for an OBP biosensor. The crystals were coated on both sides with a layer of gold (Au geometric surface of 4.9 mm diameter). The gold was deposited on the quartz surface with an adhesion layer of titanium.

### 10.2.2.3 Protein Immobilization

Proteins were immobilized on the gold surfaces of the quartz crystals using two different methodologies:

- direct covalent immobilization
- Covalent immobilization—Self assembled monolayer (SAM)

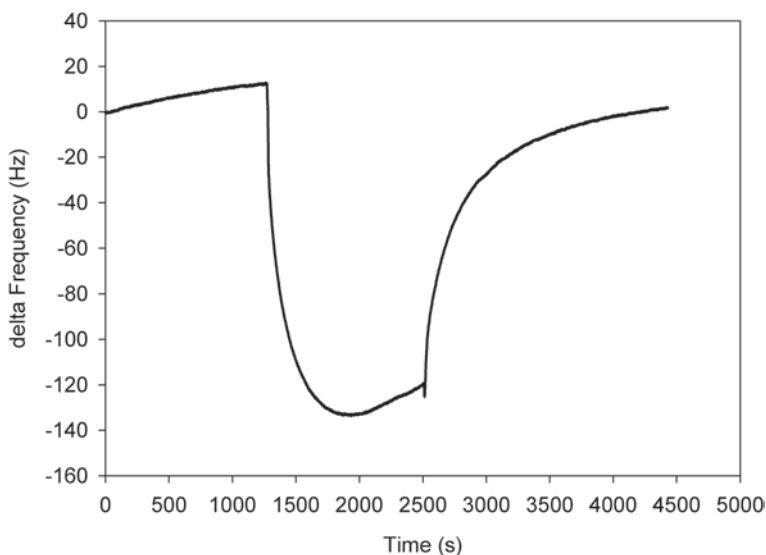
Before immobilization of proteins, the gold surface was rinsed with absolute ethanol and double distilled H<sub>2</sub>O in case of new crystals. If quartz crystals were to be reused, they were cleaned by dipping the crystal in Piranha solution (1:3=30% H<sub>2</sub>O<sub>2</sub>: H<sub>2</sub>SO<sub>4</sub>) for few minutes to remove any organic residues from the surface.

### 10.2.2.4 Direct covalent immobilisation

For direct covalent immobilization, we modified the OBP by adding a cysteine residue at the N-terminal end of the protein—allowing a stable bond to be formed between the thiol group of cysteine and the gold surface. The immobilisation was carried out by spreading 5 µl of proteins (2.3 mg ml<sup>-1</sup>) on the gold surface. The protein was added about every 2 hours for at least four times. After that, the surface was gently rinsed with sterile double distilled H<sub>2</sub>O and was left to dry. The same procedure was applied for the gold electrode on the other side of the QCM.

### 10.2.2.5 Covalent Immobilization

OBPs were immobilised on the gold surface through covalent immobilization via a Self assembled monolayer (SAM). Thiocetic acid (TA) ((R)-5-(1,2-dithiolan-3-yl) pentanoic acid). The sulphur atoms of the TA forms a strong bond with the gold, while the other end of the molecule is free to bind to the proteins. TA (100 mM in absolute ethanol) was dropped on the gold surface of the crystal, repeatedly every 20 min, for at least 2 hours. The same procedure was used for the gold electrode on other side of the quartz crystal. The SAM procedure was carried out in a controlled environment under nitrogen. The quartz crystal was then rinsed with an excess of absolute ethanol and was left to dry at air. In order to activate the carboxylic acid groups of the SAM, 20 µl of a solution consisting of a mixture of ethyl(dimethylaminopropyl)carbodiimide (30 mM) and N-hydroxysuccinimide (60 mM) was placed on the gold surface for 2 hours. The solution was then rinsed with distilled water and was dried at air. These procedures for the SAM activation were applied to both sides of the gold surfaces for all the quartz crystals. The immobilization of proteins on the activated SAM layer was carried out by pipetting the OBP solution onto the gold surface and leaving it for 1 hour before gently rinsing with distilled water and drying in air. The amount of protein deposited on to the gold surface corresponded to about 10 µg of OBP.



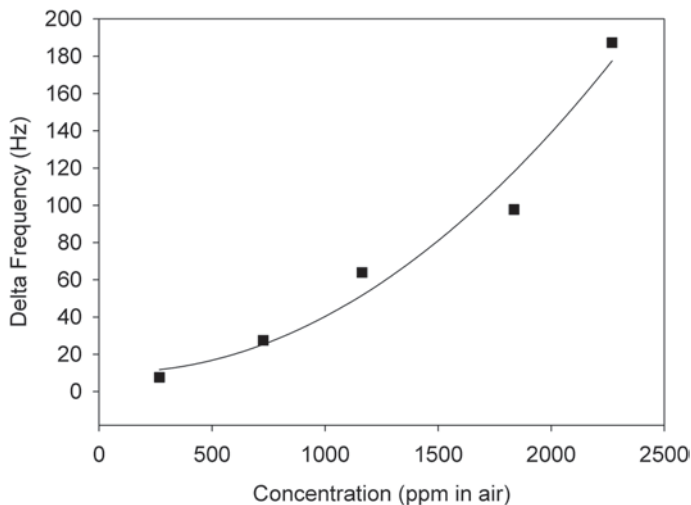
**Fig. 10.10** Response of a QMB coated with *P. dominulus* OBP to a pulse 350 ppm of 2-isobutyl-3-methoxypyrazine in air

The best performance were obtained using covalent immobilization by using self assembled monolayers rather than direct covalent binding of the proteins.

### 10.3 Sensor Responses

Although the binding affinities of odorant binding proteins have been characterized to a range of analytes in solutions [49, 79, 80], there is little data available about how they interact with ligands in the vapour phase. Here we report our first experiments in this area.

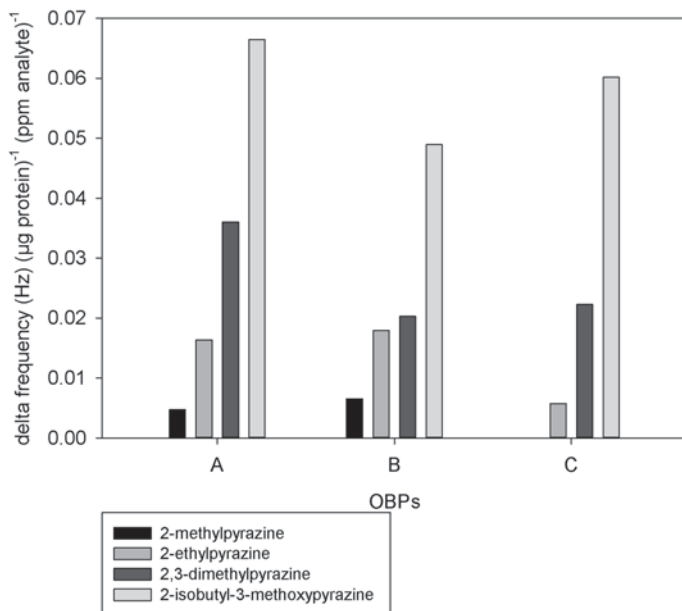
The raw response signal from a QCM with immobilized odorant binding proteins from the *Polistes dominulus* to 350 ppm of 2-isobutyl-3-methoxypyrazine in air are shown in Fig. 10.10. The decrease of the quartz crystals basic oscillator frequency due to the changes of mass over time is recorded as the sensing signal. Sensors were stabilized under a constant flow of air before the introduction of target gas in order to obtain a reference frequency and eliminate the effect of flow dynamics. The baseline frequency was then re-established by a flow of clean air in order to remove bound analytes. The sensor showed good reversibility as well as stability over time, although the baseline frequency measured was prone to long term drift. Control experiments with gold surfaces on bare crystals indicated that responses specific to the presence of OBPs were being detected.



**Fig. 10.11** Concentration-response curve for 2,3-dimethylpyrazine—QMB coated with pig OBPM2. A quadratic polynomial  $f=y_0+ax+bx^2$  was fitted with coefficients  $y_0=10.57$ ,  $a=-0.005$ ,  $b=3.45E-5$  with  $R^2=0.97$

Intriguingly, pyrazines are a group of chemicals to which good responses are normally found from proteins over a range of species from ligand binding experiments carried out in solution [80]. In the vapour phase, for all species of proteins immobilized on quartz crystal microbalances we observed a concentration-dependence in the responses to these compounds. The change in frequency of the QMB is proportional to concentration of analyte in the vapour phase as illustrated in Fig. 10.11, which can be fitted with a quadratic polynomial. In this case the concentration-response curve of pig OBP to 2,3-dimethylpyrazine is shown, and similar response curves with different initial slopes are seen with other types of OBPs, or with other analytes for the same OBP. Mass transduction mechanisms in QMBs have been reviewed by Mecea [81] who indicates that the QMB is not only a sensitive mass sensor but it is also an actuator generating a mega gravity field on the surface of the quartz resonator. It is claimed that the very high mass sensitivity of the QCM is explained by the very high acceleration acting on the deposited film.

We have investigated the selectivity of proteins from different sources to a range of different pyrazines. This indicated that the binding sites are sensitive to the size and shapes of the ligands. There are some intriguing similarities and differences in the trends found. Both the protein from the pig and the protein from the wasp gave similar trends in terms of response—2-isobutyl-3-methoxypyrazine always giving the best response, although the protein from the wasp was less sensitive than the pig protein as shown in Fig. 10.12.



**Fig. 10.12** Normalised responses of *A*—pig *Sus scrofa* OBP, *B*—wasp *P. dominula*, *C*—locust *L. migratoria* OBP immobilized on quartz microbalances to pyrazine derivatives, 2-methyl pyrazine, 2-ethyl pyrazine, 2,3-dimethylpyrazine, 2-isobutyl-3-methoxy pyrazine

## 10.4 Conclusions

The exceptional stability of OBPs to thermal denaturation and proteolytic degradation makes these proteins interesting for incorporation into sensing devices. There is now a large body of knowledge available on the protein sequences, ligand binding specificities and affinities, as well as crystal structures for key members of the family, so it is possible to tailor proteins in the future for selected target analytes. Few papers so far describe the potentialities of OBPs as biosensors. We show here that it is possible to express different OBPs from many sources using conventional molecular genetics methods. These proteins can be immobilized and are able to capture volatile analytes from the vapour phase. Using a quartz crystal microbalance platform as a transduction element, it is possible to detect and measure quantitatively concentrations of volatile analytes at ppm concentrations in air. Further work is necessary to improve the versatility of these new sensors in matching the complexity and variety of environmental odours.

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