Detection of olfactory receptor I7 self assembled multilayer formation and immobilization using a Quartz Crystal Microbalance

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Abstract

A self-assembled multilayer based on a mixed MHDA-Biotinyl PE self-assembled monolayer followed by the addition of a biotin-avidin system was built up on a the gold electrode of a quartz crystal microbalance which was used to monitor the deposition. With the view to producing an odorant sensing device, an olfactory receptor (OR), ORI7, was immobilized on the self-assembled multilayer. The ORI7 OR originates from a large group of proteins belonging to the I subfamily of G protein coupled receptors that binds odorant ligands.

All steps formation were followed with the QCM-D. Valuable information was obtained regarding the composition of each layer, providing evidence of the high dissipation effect of nanosomes adhesion. Also, based on the results, an explanation for multilayer formation and binding relations between components is proposed.

Keywords: G_{olf} , olfactory receptor, QCM, quartz crystal microbalance, biosensor, self-assembled multilayer.

1. Introduction

First reports of quartz crystal microbalance (QCM) use for very small mass measurements date from 1959. At that time, Sauerbrey described a piezoelectric crystal based oscillation system for accurate mass deposition measurements of thin metal layers in vacuum [Sauerbrey, 1959]. Since then, use of the microbalance technique has been reported in several different experiments in vacuum and air media. However, it became a widespread technique for biological measurements when the piezoelectric crystal was successfully made to reliably oscillate in liquid media. This resulted in the application of the QCM technique to many biological problems [Nomura et al., 1982].

Kanazawa et al. demonstrated a theoretical formula for accurate prediction of frequency variations of a quartz crystal (QC) in liquid media from its oscillations in air and its association with solution properties, i.e. density and viscosity [Kanazawa et al., 1985]. Later, models for a QC oscillating with mass and liquid loading were developed in order to allow accurate calculations of mass deposition on the QC surface oscillating in liquid media [Martin et al., 1991; Thompson et al., 1991; Kipling et al., 1990]. It was demonstrated for QCs oscillating in solution that Sauerbrey's equation is not always valid as it stands. No direct conversion, unless special cases where certain experimental conditions are supposed, can be made between frequency variations and mass deposited on the QC surface [Martin et al., 1991; Rickert et al., 1997; Janshoff et al., 2001].

At present it is proposed that there is trapping of water molecules between deposited layers on the QC surface as well as between the molecules of each layer. These act as solid mass attached to the QC surface, causing the frequency variations to be associated

with phenomena other than just the layer mass increase [Janshoff et al., 2001; Rickert et al., 1997]. This trapping is dependent on the type of molecules or substances that constitute the layer and has been reported to contribute to as much as 70 percent of the frequency variation of the QC in some cases [Rickert et al., 1997]. Indeed, frequency variations are also caused by acoustic energy dissipation due to changes in the viscosity of the layer, as well as changes in the solution properties (density and viscosity) which may arise from the addition of other solutions [Martin et al., 1991; Ghafouri et al., 2000]. As it stands, understanding QC behaviour in liquid media is a complex phenomenon, and it requires more information than just the frequency variation to be understood.

Nowadays there are quite different approaches for the accurate conversion of frequency variation to mass deposition. One of the proposed methods accomplishes this conversion by a complex calibration of the oscillating QC in the working solution to compensate for unwanted effects, i.e. interfacial liquid properties, thin film viscoelasticity, electrode morphology, and the mechanism of acoustic coupling [Matsuno et al., 2001, Stengel et al., 2005]. Another method, more widely accepted, is acoustic coupling. It proposes the network analysis of the resonance curve obtained from a QC oscillating in liquid media [Thompson et al., 1991, Martin et al., 1991] and uses the fit of the resonance curve to a Butterworth-Van Dyke (BVD) equivalent circuit. The motional resistance is associated with deposited layer viscoelastic properties and the parallel capacitance with electrode electrical coupling, and hence electrical double layer modifications [Thompson et al., 1991, Liu et al., 2005]. There is one final approach, based on a registered technique, namely the QCM-D, which stands for quartz crystal microbalance with dissipation measurements. In this technique, both frequency

and dissipation measurements are accomplished. The functioning of the device is based on removing stimulation of the QC after an excitation impulse, allowing the QC to oscillate freely in medium, showing a typical exponential decaying frequency curve. The excitation, removal of stimulation and frequency measurement of the QC is cyclically repeated throughout the experiment. Then, the acoustic energy dissipation in QCM experiments can be obtained by measuring the dissipation factor (D) which is inversely proportional to the decay time constant of the QC free oscillation frequency curve. It has been demonstrated that dissipation provides helpful information for elucidating the phenomenon associated with viscoelastic effects of the deposited layer [Hook et al., 2002, Voinova et al., 1999].

An interesting possibility for QCM sensors in biological applications is the development of odorant sensors using a variety of methods [Lu et al., 2001, Ko et al., 2005, Wu, 1999]. Here we explain the first steps toward the production of such an odorant sensor based on grafted olfactory receptors with a QCM transducer. Olfactory receptors (ORs) are a large group of proteins belonging to the subfamily I of G protein coupled receptors (GPCRs) that bind odorant ligands. These receptors are predicted to contain seven transmembrane helices that change their relative orientation upon odorant stimulation, resulting in the conformational change of the receptor and productive interaction of its intracellular loops with G_{olf} the α subunit of the heteromeric G protein. Each OR recognizes multiple odorants and most odorants are recognized by several ORs [Minic et al., 2005 (A)].

For the construction of an odorant recognition biosensor, I7 OR has been immobilized on a self-assembled multilayer composed of a mixed MHDA-Biotinyl PE self-

assembled monolayer and a biotin-avidin system which allows binding of biotinylated antibodies [Minic et al., 2005 (B)]. In this case biotinylated antibodies specific to I7 OR (Biot-Anti-I7) were bound to the surface. The whole self assembled multilayer was build on the surface of a quartz crystal, an each step formation was followed using the QCM-D technique.

2. Materials and methods

Construction of the multilayer has been achieved via consecutive exposure of the QCM to solutions of MHDA-Biotin PE solution, neutravidin solution, a solution of the Biot-Anti-I7 and to solution containing the I7 OR itself. Both frequency and dissipation variations are recorded for each of the assembly steps of the multilayer.

Here, we propose the use of nanosomes incorporating the I7 ORs. We use this approach as it is important for this ORI7 transmembrane protein to remain in the membrane fraction to retain its conformational structure. These nanosomes have been produced with sizes ranging from 50 nm to 500 nm, and each one is thought to contain one or two I7 olfactory receptors. It has been shown that the nanosome size can be tuned depending on preparation conditions [Minic et al., 2005 (C)].

The final aim of the I7 OR layer formation is the development of an odorant biosensor. However, only a small mass increase due to odorant recognition is expected. Therefore the response will be amplified by the addition of guanosine triphosphate (GTP). The GTP molecule has been shown to activate a mechanism which favors the release of the G protein associated to a ligand-binding process, and therefore can be used to illicit a

much larger mass decrease (Fig.1), which should be more easily detected with the QCM [Minic et al., 2005 (C)].

2.1 Biomaterials and chemicals

Dipalmitoyl-sn-Glycero-3-phosphoethanolamine-N-(biotinyl) (biotinyl-PE) and 16-meraptohexadecanoic acid, 90% were obtained from Avanti Polars and Sigma-Aldrich respectively. Neutravidin was purchased from Pierce. The Biot-Anti-I7 and the nanosomes incorporating the I7OR were obtained from I.N.R.A- Neurobiologie de l'Olfaction et de la prise Alimentaire [Minic et al., 2005 (C)]. The buffer solution used was phosphate buffer saline (PBS) from Sigma-Aldrich containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4.

2.2 Equipment

QCM-D measurements were performed using the Q-sense D300 system (from Q-sense AB, Västra Frölunda, Sweden). An axial flow chamber (model QAFC 302, from Q-sense AB, Västra Frölunda, Sweden) was used for crystal mounting and solution circulation. The volume of the sensor cell is 100 μ l and the whole volume of the temperature loop used to circulate solution with controlled temperature is 800 μ l. Polished AT-cut quartz crystals with fundamental frequencies of 5 MHZ were used to complete the experiment (from Q-sense AB, Västra Frölunda, Sweden). The diameter of the active Au electrode is 14 mm. All measurements were carried out at a constant temperature of 24 °C, controlled by the Q-sense system. Changes in frequency (ΔF) and dissipation (ΔD) were recorded at natural oscillation frequency (5 MHZ) and three harmonics (14.9 MHZ, 24.7 MHZ and 34.6 MHZ).

2.3 Preparation of mixed self-assembled monolayer

Gold electrodes for the QCM were functionalized by immersion in a mixed solution, composed of 1mM MHDA and 0.1 mM Biotin-PE in ethanol, over a period of 21 hours. After this period of time it was assumed that an optimal functionalization had been achieved on both faces of the crystal. The crystal was rinsed several times with pure ethanol solution and then dried under an argon gas flow.

2.4 Mass deposition experiments

Continuous QCM-D measurements were initially taken in pure PBS solution. This allowed the system to stabilise and ensured that a stable baseline was achieved. Neutravidin at 5×10^{-7} M concentration in PBS was then added to the system for 88 min before a PBS solution was again applied to remove unbound neutravidin. Biot-Anti-I7 at 1×10^{-6} M concentration in PBS was then added for 82 min before again washing with PBS solution. Finally, nanosomes, with attached I7 OR, was added at a concentration of 30 $\mu\text{g/ml}$ (10 μl of the concentrated I7 OR solution diluted in 1 ml PBS) for 60 min, before a last washing step with PBS. The final structure of the multilayer formed is as depicted in Fig. 2.

3. Results and discussion

3.1 Study of self assembled multilayer formation

Figure 3 shows the effect of the in-situ functionalisation of the quartz crystal. Initially the signal baseline is stable as the quartz crystal is subjected to the PBS solution. Each of the steps in the production of the multilayer system causes an increase in the mass on

the surface of the quartz crystal. This damps the oscillation of the crystal, causing the resonant frequency to decrease and causing the dissipation to increase.

The addition of neutravidin yields a $\Delta F = -80$ Hz and $\Delta D = 0.66$ (A in Fig. 3) as it binds to the thiol SAM on the electrode surface. Biot-Anti-I7 addition then causes further changes of $\Delta F = -90$ Hz and $\Delta D = 1.2$ respectively (B in Fig. 3). The final step, the addition of I7 OR coated nanosomes, causes changes of $\Delta F = -70$ Hz and $\Delta D = 4.8$ (C in Fig. 3).

The molecular mass of the first two components of the multilayer are, Neutravidin = 60 kDa, Biot-Anti-I7 = 150 kDa. The molecular mass of the nanosomes is not known. They have an approximate diameter of 100 nm, after 2 minutes sonication at 0 degrees, and are described as being spherical lipid bilayers containing one or two I7 OR proteins, each one having a molecular weight of 39 kDa [Minic et al., 2005 (C)]. Accordingly, the total mass is not expected to be much higher than 80 kDa, but a high dissipation effect is expected due to the water trapped inside nanosomes. From the nanosomes description, we conclude that its average density is much lower than densities of neutravidin and Biot-Anti-I7. The nanosomes are much larger than the Biot-Anti-I7, which is bigger than Neutravidin.

If we assume the theoretical 1 to 2 relation for neutravidin and Biot-Anti-I7 deposition as shown in figure 2, ΔF should increase in relation. Based on the molecular weight proposed, ΔF for the nanosomes is expected to be similar that for the neutravidin deposition, assuming that each nanosome occupies an area for 2 Biot-Anti-I7 (1 to 2 relation). An even higher ΔF is expected from the nanosomes adhesion due to

dissipation effects. However, the change in ΔF will also depend on the packing density of each of the components.

Based on the values of ΔF taken from fig. 3, and according to the previous description of each of the layer components, we can propose that neutravidin is well distributed on the surface. The concept chosen for the packing of the layer arises from comparison of ΔF and ΔD values relative to each one of the layers. Where ΔF is high (high mass deposition or high viscoelastic effects due to layer formation) and ΔD is small (small contribution of viscoelastic effects), it is assumed that a compact layer has been formed. This assumption relies on the fact that mass deposition is accomplished in a more ordered manner, not allowing big empty spaces to be formed and or filled by water (this would imply a higher ΔD). The reason for the good distribution of neutravidin compared to the other two components is assumed to be due to the small size of this molecule and its affinity with the SAM underlayer. The Biot-Anti-I7 deposition has a larger ΔD than the neutravidin deposition (although it is still much lower than the change for nanosomes), albeit with a similar ΔF . Consequently, the Biot-Anti-I7 layer is not as well packed as the neutravidin layer.

While ΔF due to Biot-Anti-I7 deposition is similar to the neutravidin, ΔD is approximately double. This suggests that the mass of Biot-Anti-I7 deposited is not as high as the mass of the neutravidin deposited. The molecular weight of the former is double of the latter, meaning that if we assume a 1:1 binding ratio, a ΔF around double should be expected for Biot-Anti-I7 deposition. An even higher ΔF should be expected if there is also a greater increase in ΔD . However, this does not agree with the general binding model proposed in fig. 2 where a 2:1 Biot-Anti-I7 neutravidin relation is

proposed based on the free binding sites of neutravidin. The explanation for this is that the antibody biotinylation process is not selectively targeted to the best binding site, so antibodies are quite randomly grafted to the neutravidin layer. This can mean that the Biot-Anti-I7 does not necessarily bind to all the binding sites on the neutravidin surface and even that its orientation, together with its bigger size, can ‘block’ neighbouring vacant binding sites.

When it comes to the nanosome layer formation, ΔD is very high compared to the previous layer depositions, while ΔF is lower. This means that the effective nanosome-antibody binding ratio is even lower than that found for neutravidin - Biot-Anti-I7. The increase in ΔD is explained by the volume of water present, both trapped between adjacent nanosomes and inside the bilipidic spheres containing I7 OR. However, the water is not so tightly trapped as to produce big ΔF variations,. Added to this, the dissipation increased because of the increased viscoelasticity of the multilayer as a whole. The low binding ratio is assumed to be caused by the random orientation of the I7 OR present on the nanosomes surface, meaning that the I7 OR is not always optimally positioned for binding, and also due to poor orientation in some of the grafted antibodies, causing similar problems.

Finally, the repeatability of the whole layer formation was successfully verified, with mean values and standard deviation as indicated in table 1.

4. Conclusion

A self-assembled multilayer with I7 OR as the top layer was successfully constructed on the surface of a gold quartz crystal. The formation of the multilayer was monitored with

the QCM-D. Valuable information which regards mass and packing density was obtained for each layer. Evidence for the high dissipation effect of the addition of nanosomes adhesion was found. In the present work, the binding ratio for neutravidin - Biot-Anti-I7 and Biot-Anti-I7 - nanosome was found to be lower than 1:1, however successful formation of the multilayer has been proved. Such functionalized quartz crystal response to the addition of different odorants, and how the response is affected in presence of GTP, will be explored.

Modelling will be the object of future work, using modelling software QTools (provided by the QCM-D manufacturer, Q-sense) for conversion of the four ΔF curves and the four ΔD curves (natural oscillation frequency and overtones recorded) to variations in mass deposition, viscosity and elasticity attributed to each of the layers formed. It is worth noting that for this modelling to be adequately accomplished, model parameters associated for each of the components of the layers should be optimised.

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Figure captions

Fig. 1: Proposed mechanism for odorant detection.

Fig. 2: Self-assembled multilayer formation.

Fig. 3: ΔF (top) and Dissipation (bottom) curves in response to self assembled multilayer formation.

Table 1: Repeatability of the multilayer formation: mean values and standard deviation for each layer deposition.

Figure 1

Figure 2

Figure 3

Table 1

		ΔF mean value	ΔF standard deviation	ΔD mean value	ΔD standard deviation
Layer formation	Neutravidin	-83.34	3.40	0.85	0.39
	Biot-Anti-I7	-96.67	17	0.92	0.22
	Nanosomes	-71.67	2.36	4.93	0.66